Marker development and gene identification for blackleg (*Leptosphaeria maculans*) disease resistance in canola (*Brassica napus*)

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By

Zining Wang

In Partial Fulfillment of the

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Marker development and gene identification for blackleg (*Leptosphaeria maculans*) disease resistance in canola (*Brassica napus*)

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

DOCTOR OF PHILOSOPHY

FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. Manuscripts follow the style recommended by the Theoretical and Applied Genetics journal. The thesis begins with a general introduction and literature review. Three manuscripts each contain an abstract, an introduction, materials and methods, results and discussion. The thesis ends with a general discussion, references and appendixes.

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Abstract

Wang, ZN., Ph.D., the University of Manitoba, June, 2007. Marker development and gene identification for blackleg disease (*Leptosphaeria maculans*) resistance in canola (*Brassica napus*)

Major Professors: Drs. Genyi Li and Peter BE McVetty

A consensus ultradense genetic recombinant map was constructed using sequence related amplified polymorphism (SRAP) markers and used to tag a blackleg disease resistance gene *LepR3* in 'Surpass 400'. Marker development was also performed using comparative genomic sequencing to find single nucleotide polymorphism (SNP) markers for the other two disease resistance genes *Rlm1* and *Rlm3* in 'Quinta' and 'Glacier', respectively. The objective of gene mapping is to use molecular markers linked closely to disease resistance genes for marker assisted selection (MAS) in cultivar development and for pyramiding different resistance genes into a cultivar to improve the degree of resistance. Furthermore, cloning of these disease resistance genes will be essential to fully understand the underlying mechanism of disease resistance and to manipulate disease resistance genes for managing the disease effectively.

An ultradense genetic recombinant map was developed with a 58 DH line population of 'Westar × Zhongyou 821' and in total 13,551 SRAP markers were integrated on 19 linkage groups that corresponded to the 19 chromosomes in *B. napus*. All SRAP markers were generated with a total of 1,634 primer combinations

including 12 fluorescently labeled primers and 442 unlabeled ones. These 13,551 markers were put into 1,055 bins, resulting in a map length of 1,604.8 cM. Furthermore, all 19 linkage groups were assigned to the previously reported N1–N19 linkage groups of *B. napus* by integrating 55 microsatellite or simple sequence repeat (SSR) markers that had been used to construct previous maps in this species.

The dominant resistance gene *LepR3* in 'Surpass 400', introduced from *B. rapa* subsp. *sylvestris* through gene introgression, was targeted for marker development and gene cloning. To use the ultradense genetic recombination map, the same primer combinations for the map construction were used to find the common SRAP markers that were linked to the disease resistance gene *LepR3* in the segregating DH line population of 'Westar' and 'Surpass 400'. The integration of these SRAP markers allowed the use of SRAP markers on the map to find SRAP markers linked more closely to *LepR3*. With 384 primer combinations, two SRAP markers, R269 and G278 linked to *LepR3* were developed and R269 was found to correspond to SRAP marker 1217Ar269 on the N10 linkage group, which contained 508 markers.

The SRAP markers on the N10 linkage group were selected to screen the gene tagging population and three markers, 210Ay442, 0127Fr382 and 1128BG275, were found to co-segregate with the gene *LepR3*. After analysis of these SRAP markers with the population of 3,900 plants, 52 recombinant plants between SRAP markers 1217Ar269 and 0127Fr382 were selected for further analysis and the region containing the gene *LepR3* was identified. Moreover, SRAP marker 0127Fr382 was

found to be the closest marker to the resistance gene *LepR3* with a genetic distance of 0.3 cM.

Map-based identification of a candidate gene for the resistance gene *LepR3* to blackleg in 'Surpass 400' was then pursued. After the SRAP markers linked to *LepR3* were sequenced, the sequences were used for BLASTn analysis and these markers landed on two syntenic regions on the 5th chromosome of Arabidopsis. Four SNPs located in four *Brassica* homologs of At5g57035, At5g57345, At5g57670 and At5g57830 were developed and used to analyze the 52 recombinant plants selected previously. The genetic map for these four SNPs showed the same gene order in *B. napus* as in Arabidopsis. These SNP markers also showed similar genetic distance to the *LepR3* as that of SRAP markers 1217Ar269 and G278. It was confirmed that the *LepR3* gene did not fall in this syntenic region. SNP markers including SNP13280, SNP13530, SNP13930, and SNP14950 in *B. napus* were also developed with Arabidopsis genes At5g13280, At5g13530, At5g13930, and At5g14950. Further analysis of these SNPs with these 52 recombinant plants showed that SNP13930 co-segregated completely with the resistance gene. A bacterial artificial chromosome (BAC) clone A48M23 anchoring a *B. rapa* homolog of Arabidopsis At5g13930 was identified with the primers designed based on the sequence of At5g13930. BAC end sequences and gene sequences obtained through PCR with the BAC DNA demonstrated that the genes on this BAC clone showed the same gene order as in Arabidopsis. In this small region, four SNP markers SNP13930, SNP14060, SNP14210 and SNP14220 were developed with Arabidopsis genes At5g13930,

At5g14060, At5g14210 and At5g14220, respectively. These four SNPs showed no recombination among these 52 recombinant plants, indicating that the resistance gene *LepR3* was in this region and one of these genes should be the candidate for the *LepR3* locus. After searching the flanking genes in Arabidopsis covering about 1,060 kb (At5g13290-At5g16000), At5g14210 was found to be the only gene sequence coding for a *R*-gene-like protein. It codes for a leucine-rich repeat transmembrane protein kinase, which has been demonstrated to be one kind of the resistance gene such as *Xa21* and *Xa26* found in rice. Therefore the *Brassica* orthologous gene of At5g14210 is a good candidate for the blackleg resistance gene *LepR3* in 'Surpass 400'.

The resistance genes *Rlm1* in 'Quinta' and *Rlm3* in 'Glacier' have been mapped on the N7 linkage groups by other researchers. In order to pyramid the N7 genes with the *LepR3* gene in 'Surpass 400', SRAP markers and SNP markers were developed for the N7 disease resistance gene loci. The genetic inheritance of these two resistance genes were confirmed by screening the F_2 and F_3 populations of 'Westar' × 'Quinta' and 'Westar' × 'Glacier' with blackleg isolates. The SRAP markers were screened for *Rlm1* by using 256 pairs of primers. Marker B342 was the closest marker for *Rlm1* with a genetic distance of 1.5 cM. This marker was also linked to *Rlm3* with a genetic distance of 6.8 cM. SNP80870, developed from the N7 region, was the closest marker to *Rlm3* with a genetic distance of 3 cM. There was a genetic distance of 11.3 cM between SRAP B342 and SNP80870. These markers could be used for MAS and gene pyramiding. Production of improved and possibly durable resistance to blackleg by

marker assisted gene pyramiding is the ultimate goal of this study. The development of molecular markers for several blackleg resistance genes offers the opportunity for gene pyramiding via MAS in cultivar development. The marker SNP14210 was developed from 'Surpass 400' targeting the resistance gene to the PG2 isolate from wild *B. rapa*.

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CHAPTER 1

GENERAL INTRODUCTION

Blackleg, caused by *Leptosphaeria maculans*, is a serious fungal disease that can cause major yield losses in canola and other cruciferous oilseed crops (West et al. 2001). As a global disease of canola, blackleg causes annual yield losses of 5 to 7% (Brumby 2006). Since the virulent strain of blackleg in canola was first detected in 1975 in Western Canada (Gugel and Petrie 1992), blackleg pathogen has widely spread in the canola growing areas in Canada. Research on avirulence genes from the pathogen, resistance genes from the plant and pathogen-host interactions have been conducted for more than twenty years. Breeding for disease resistance is the most effective disease control method.

Genetic mapping is an important tool to identify molecular markers linked closely to disease resistance genes. These molecular markers are useful for marker assisted selection in breeding and for gene cloning. Sixteen blackleg disease resistance genes have been mapped from different sources: *B. napus, B. rapa, B. juncea* and Arabidopsis. These mapped resistance genes come from different cultivars and the gene mapping has been performed using different marker systems which makes it difficult to compare the mapped genes from different studies. Additionally, several genes were mapped onto the same linkage groups, making gene identification more difficult. For example, five blackleg resistance genes *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* were mapped on linkage group N7 (Rimmer 2006). Two other resistance genes, *LmR1* and *CLmR1* from 'Shiralee' and 'Cresor' were also mapped on the same linkage group. These latter two resistance genes were believed to be the same resistance gene (Mayerhofer et al. 2005). These maps

are not universal and are difficult to be used by other research groups. Further, some of the linked molecular markers on these maps are not close enough for marker assisted selection. As two old cultivars from Europe, 'Quinta' and 'Glacier' have Mendelian resistance genes to blackleg that are still used in many cultivars today. 'Surpass 400' has a resistance gene introduced from *B. rapa* ssp. *sylvestris* (Li and Cowling 2003) and this gene is believed to be different from those genes in 'Quinta' and 'Glacier' (Yu et al. 2004). In order to find closely linked molecular markers for these important genes including *Rlm1* from 'Quinta', *Rlm3* from 'Glacier' and *LepR3* from 'Surpass 400' for marker assisted selection, sequence related amplified polymorphism (SRAP) was used in this study to map these important blackleg resistance genes.

Marker assisted gene pyramiding for blackleg resistance may be an efficient way to utilize disease resistance genes. This approach combines a few target genes identified by genetic markers into a single genotype (Servin et al. 2004). The advantages of this strategy are to save time and simultaneously target multiple genes that could not be followed by traditional breeding methods. Through marker assisted selection (MAS), several disease resistance genes in rice including *Bt*, *Xa21* and *Xa7* were pyramided into a restoring line 'Minghui 63' and *Pi1, Pi2, Qbp1* and *Qbp2* were pyramided in a maintaining line 'Zhenshan 97'. These lines greatly improved the resistance of a hybrid rice cultivar 'Shanyou 63' to bacterial blight, blast, stem borer and brown plant hopper (He et al. 2004b). Singh et al. (2004) pyramided three bacterial blight resistance genes (x*a5, xa13* and *Xa21*) in one material via MAS.

Single resistance genes may be easily overcome by pathogen recombinants and mutations, such as the resistance genes that have been overcome in France and Australia

(Li et al. 2003, Rouxel et al. 2003a, Sprague et al. 2006). Breeding of cultivars with durable resistance is very important to control disease. The canola cultivar 'Surpass 400' was reported to have one dominant resistance gene *LepR3* to PG2; 'Glacier' two dominant resistance genes *Rlm2* and *Rlm3* to PG2; 'Quinta' one dominant resistance gene *Rlm1* to PG3 and one resistance gene *Rlm4* to PG4 respectively. These dominant Mendelian genes controlling vertical resistance may be easily overcome by the pathogen mutants. However, these specific resistance genes can be pyramided into one cultivar and may increase its resistance to most of the isolates from all four PGs. The pyramided resistance might be more durable than the lines with single resistance genes. The cultivars with multiple resistance genes may last longer by reducing the chance that the pathogen recombinants or mutants may overcome all resistance genes simultaneously. So the first step for gene pyramiding is to identify closely linked genetic markers for the important blackleg resistance genes mentioned previously.

Disease resistance gene cloning is the foundation for the study of *R* gene structure, function and interaction with avirulence genes. Since the gene for gene hypothesis was described (Flor 1942), a large number of disease resistance genes that have been identified follow this concept. More than 54 disease resistance genes have been cloned from different plant species. The commonly used methods for cloning these genes with limited information are through map-based cloning or insertional mutagenesis (Bechtold et al. 2000). Most of the resistance genes identified encode different types of proteins with conserved domains, such as kinase domain, transmembrane domiain, a nucleotide binding site (NB) and leucine rich repeats (LRR) (Dangl and Jones, 2001). LRR domain may participate in protein to protein interactions. An NB domain may activate defense

responses and play an important role in signaling etc. These features of resistance genes may be very helpful to the identification of blackleg resistance genes in *Brassica* species.

Although 16 genes have been mapped in *Brassicas* (Rimmer 2006), none of the resistance genes has been cloned, possibly because of the complicated nature of *Brassica* genomes which contain highly duplicated regions and rearrangements (Mayerhofer et al. 2005). Although a cDNA sequence from *B. nigra* (*Lm1*) was identified with degeneracy primers designed based on conserved sequences of *R* genes (Wretblad et al. 2003), more work needs to be done to confirm if it is an *in vivo* resistance gene.

'Surpass 400' is one of the cultivars containing *B. rapa* ssp. *sylvestris* resistance released by the Pacific Seed Company of Australia in 2000. The ancestry of 'Surpass 400' includes an accession of *B. napus* resynthesized from wild *B. rapa* ssp. *sylvestris* and *B. oleracea* ssp. *alboglabra* (Crouch et al. 1994, Anon. 2001), indicating that the single dominant blackleg resistance gene in 'Surpass 400' may be different from resistance genes in other cultivars of *B. napus* (Li et al. 2003). *LepR3* in 'Surpass 400' controls seedling resistance and was mapped on N10 (Yu et al. 2004). Cloning of *LepR3* from 'Surpass 400' will initiate the functional analysis of blackleg resistance genes and the effective use of these genes in breeding.

Chapter 2

Literature Review

2.1. Triangle of U

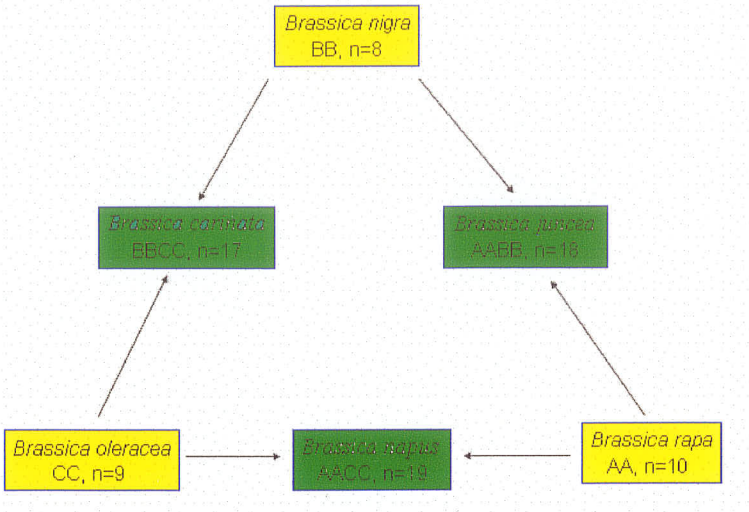


Fig.2.1. Genetic relationships among *Brassica* species in U Triangle. Diploid species are *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC). Between each pair of the diploid species are three allotetraploid species including *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC).

The triangle of U (Fig.2.1) is a theory about the origin and relationships among the members of *Brassica* genus. It was named after Woo Jang-choon (Nagaharu U in Japanese), a Korean botanist working with synthetic hybrids between diploid and tetraploid species (U. 1935).

There are three ancestral diploid genomes, *B. rapa* (AA, n=10), *B. nigra* (BB, n=8) and *B. oleracea* (CC, n=9). In particular, there are several subspecies in *B. rapa* and *B. oleracea* that belong to important vegetables and oilseeds, such as cabbage, Chinese cabbage, broccoli and cauliflower. Three allotetraploid species including *B. juncea* (AABB, n=18), *B. napus* (AACC, n=19) and *B. carinata* (BBCC, n=17) evolved

naturally through interspecific hybridization between these three diploid species. Canola and rapeseed are two main crops in *B. napus*.

2.2 Blackleg disease

Blackleg, caused by *L. maculans*, is a serious fungal disease in Canada, Australia and Europe that causes annual yield losses of 5 to 7% in canola production (Brumby 2006). Since the virulent strain of blackleg of canola was first detected in 1975 in Western Canada (Gugel and Petrie 1992), it has widely infected the canola growing areas in Canada. Several methods such as crop rotation, weed control, seed treatment, foliar fungicide, and crop residual treatment were used to control the disease in the fields (Arthur 1996); however, use of resistant cultivars is the most effective means to control this disease.

2.3 Blackleg Disease Cycle

The blackleg disease has two stages (Fig.2.2). The blackleg fungus lives on canola stubble and infects seeds with dormant mycelium over the winter. The fungus develops pseudothecia (perithesia) on the stubble during winter. The long lived airborne ascospores are released from the pseudothecia and travel long distances with wind to infect canola plants. Ascospores can cause leaf lesions at the seedling stage. The earlier the infection occurs, the more likely stem cankers develop, eventually resulting in severe yield loss. During the growing season, the fungus produces pycnidia that exude asexual pycnidiospores (conidia). The spores can spread short distances by rain splash and cause secondary infection within a crop. Seedling infection may come from infected seed or airborne spores. Once leaves are infected, the whole plant can be infected through the

plant vascular system.

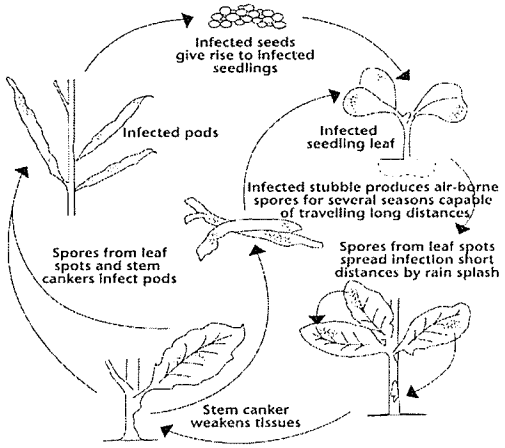


Fig 2.2. Blackleg disease cycle (available at www.canola-council.org/blacklegcycle.aspx): Ascospores of blackleg develop on the stubble over the winter and can travel long distance with wind in the spring to infect new plants, causing primary infection. Pycnidiospores from infected plants can spread to other plant parts by rain splash and cause secondary infection. Infected seeds can also give rise to infected seedlings.

2.4 Pathogenicity groups

Table 2.1. Differential *B. napus* cultivars used to classify interaction with isolates of *L. maculans**

		B. napus differentials			
Pathogenicity group		Westar and Liradon (no *R* gene)	Glacier (*Rlm2*/*Rlm3*)	Quinta and Columbus (*Rlm1*/*Rlm4*)	Jet Neuf and Maluka (*Rlm4*)
PG2	A3	+	-	-	-
	A4	+	-	-	+
PG3	A6	+	+	-	-
	A2	+	+	-	+
PG4	A5	+	+	+	-
	A1	+	+	+	+
PGT	-	+	-	+	+

* References for this table: Koch et al. 1991, Kuswinanti et al. 1995, Balesdent et al. 2002, Rouxel et al. 2003b, Rimmer 2006

There are two types of the blackleg isolates, A (virulent) and B (non aggressive) types, defined on the basis of symptoms on *B. napus* (Johnson and Lewis 1994). The blackleg isolates in the A type group have been subdivided into pathogenicity groups, such as PG2, PG3, and PG4 according to differential reactions on cotyledons of *B. napus* cultivars including 'Westar', 'Quinta' and 'Glacier' (Mengistu et al. 1991, Koch et al. 1991) (Table 2.1). Among these strains, PG2 is the dominant one in Canada and PG3 in Australia and Europe. However, PG3 and PG4 were also found in some regions in Canada and the United States (Fernando and Chen 2003, Chen and Fernando 2005).

The PGs have been regrouped into A1 to A6 (Badawy et al. 1991, Kuswinanti et al. 1995). PG4 isolates were further divided into A1 and A5, PG3 into A2 and A6, and PG2 into A4 and A3. A1, A2 and A4 are virulent and A3, A5 and A6, avirulent on 'Jet Neuf', respectively. Another group PGT was added later (Rimmer 2006) (Table 2.1). In contrast, B pathogen group isolates (non aggressive) have been classified into three genetically distinct subgroups: NA1, NA2 and NA3 (Koch et al. 1991). The B group isolates have been classified as a separate species *L. biglobosa* (Shoemaker and Brun, 2001). These PG groups and subgroups were discriminated by different markers, such as isozymes, soluble proteins, RAPDs and AFLPs (Balesdent et al. 2002, Koch et al. 1991, Somda et al. 1996, Goodwin and Annis 1991).

2.5 Gene for gene concept

"For each gene determining resistance in the host, there is a corresponding gene for avirulence in the parasite with which it specifically interacts" (Flor 1942). The hypothesis describes the pathogen and host relationship, their coexistence and co-evolution. Only

with the recognition between the avirulence gene from the parasite and the resistance gene in the host, the hosts show hypersensitive reaction (resistance); otherwise the hosts are susceptible to the pathogen.

2.6 Avirulence genes in *L. maculans*

Table 2.2. List of nine avirulence genes in *L. maculans AvrLm1* to *Avrlm9* and their corresponding isolates

Avr	Isolates	References
Avrlm1	PHW1245, IBCN18, 11.26.11	Ansan-Melayah et al. 1995 Delourme et al. 2004
Avrlm2	PHW1245, IBCN18, 22.2.02	Ansan-Melayah et al. 1998 Delourme et al. 2004
Avrlm3	IBCN79	Delourme et al. 2004
Avrlm4	PHW1245, IBCN18, IBCN17	Balesdent et al. 2001, 2002
Avrlm5	PHW1245, IBCN18, 22.2.02	Balesdent et al. 2002
Avrlm6	IBCN18	Balesdent et al. 2002
Avrlm7	IBCN18, IBCN79, 22.2.02	Delourme et al. 2004
Avrlm8	IBCN18, PHW1245	Balesdent et al. 2002
Avrlm9	IBCN56	Delourme et al. 2004

L. maculans is a haploid fungus with a 34 Mb genome encoding 10,000 to 12,000 genes (Kuhn et al. 2006). From the past studies, at least 9 avirulence genes have been identified from different isolates (Table 2.2). Linkage analysis showed that these genes distributed to four independent loci. Two independent regions, *AvrLm5* and *AvrLm8*, and two clusters, *AvrLm1-AvrLm2-AvrLm6* and *AvrLm3-AvrLm4-AvrLm7*, were involved in

the host specificity to *Brassicas* (Balesdent et al. 2002). These 9 avirulence genes correspond to 9 of the resistance genes in *Brassica* species (Table 2.4) (Ansan-Melayah et al. 1998, Balesdent et al. 2001, Delmourme et al. 2004).

Molecular markers linked to the *AvrLm1* avirulence gene have been found (Attard et al. 2002). Markers for avirulence genes *AvrLm2* and *AvrLm4* have also been inferred (Ansan-Melayah et al. 1995, 1998, Balesdent et al. 2001). *AvrLm1* has been recently cloned via map based cloning (Gout et al. 2006). *AvrLm1* restored the avirulent phenotype on *Rlm1* cultivars by functional complementation of virulent isolates. *AvrLm1* is a single copy gene and the predicted protein of this gene has 205 amino acids. It contains a peptide signal indicating that the location of the gene product is extracellular.

2.7 Disease resistance genes

During the last two decades, more than 40 plant disease resistance genes have been cloned (Martin et al. 2003). Insertional mutagenesis (Bechtold et al. 2000) and map-based cloning are the main strategies employed in gene cloning. The first plant resistance gene cloned through insertional mutagenesis was the maize *Hml* (Johal and Briggs 1992). The first real *R* gene cloned via the map-based cloning method was the tomato *Pto* (Martin et al. 1993) gene. Fifty-four cloned disease resistance genes from plants are listed in Table 2.3. The conserved domains in the protein sequences of the resistance genes (*R* gene) products distinguish *R* genes into five general groups (Fig 2.3). Resistance genes, such as

Table 2.3. Cloned plant disease resistance genes with different methods

Species	Gene	Disease name	Pathogen	Method*	Reference
Arabidopsis	*Rpp27*	Downy Mildew	*Peronospora parasitica*	MBC	Mahmut et al. 2004

Arabidopsis	*Rpp1*	Downy mildew	*Hyaloperonospora parasitica*	HS	Botella et al. 1997
Arabidopsis	*Rpp5*	Downy mildew	*Hyaloperonospora parasitica*	MBC	Parker et al., 1997
Arabidopsis	*RPP13*	Downy mildew	*Hyaloperonospora parasitica*	MBC	Bittner-Eddy et al. 2000
Arabidopsis	*RPP8*	Downy mildew	*Hyaloperonospora parasitica*	MBC	McDowell et al. 1998
Arabidopsis	*RPS2*	Black specks	*Pseudomonas syringae*	MBC	Mindrinos et al. 1994
Arabidopsis	*RPM1*	Black specks	*Pseudomonas syringae*	MBC	Grant et al. 1995
Arabidopsis	*RPS4*	Black specks	*Pseudomonas syringae*	MBC	Gassmann et al. 1999
Arabidopsis	*RPS5*	Downy mildew	*Pseudomonas syringae*	MBC	Warren et al. 1998
Arabidopsis	*HRT*	Leaf distortion	*Turnip Crinkle Virus*	MBC	Cooley et al. 2000
Arabidopsis	*PAD4*	Leaf spot	*Pseudomonas syringae*	MBC	Jirage et al. 1999
Barley	*mlo*	Powdery mildew	*Blumeria graminis*	MBC	Buschges et al. 1997
Barley	*Mla1*	Powdery mildew	*Blumeria graminis*	TEA	Zhou et al. 2001
Barley	*Mla6*	Powdery mildew	*Blumeris graminis*	MBC	Wei et al. 1999
Barley	*Rpg1*	Stem rust	*Puccinia graminis*	MBC	Brueggeman et al. 2002
Flax	*M*	Rust	*Melampsora lini*	TT	Anderson et al. 1997
Flax	*L6*	Rust	*Melampsora lini*	TT	Lawrence et al., 1995
Maize	*Rp1-D*	Leaf rust	*Puccinia sorghi*	MBC	Collins et al. 1999
Maize	*Rp3*	Leaf rust	*Puccinia sorghi*	MBC	Webb et al. 2002
Maize	*HM2*	Leaf blight	*Colchliobolus carbonum*	MBC	Multani et al. 1998
Maize	*HM1*	Leaf blight	*Cochliobolus carbonum*	TT	Johal and Briggs 1992
Rice	*Xa21*	Bacterial blight	*Xanthamonas oryzae*	MBC	Song et al. 1995
Rice	*Xa1*	Bacterial blight	*Xanthamonas oryzae*	MBC	Yoshimura et al. 1998
Rice	*Xa26*	Bacterial blight	*Xanthamonas oryzae*	MBC	Sun et al. 2004
Rice	*Pi-b*	Rice blast	*Magnaporthe grisea*	MBC	Kawasaki et al. 1999
Wheat	*Lr21*	Leaf rust	*Puccinia triticina*	MBC	Huang et al. 2003
Wheat	*Lr10*	Leaf rust	*Puccinia triticina*	MBC	Feuillet et al. 2003
Wheat	*Lr1*	Leaf rust	*Puccinia triticina*	MBC	Cloutier et al. 2007
Wheat	*Pm3*	Powdery mildew	*Blumeria graminis*	MBC	Yahiaoui et al. 2004
Wheat	*Cre3*	Nematode	*Heterodera avenae*	MBC	Lagudah et al.1997
Wheat	*Lr10*	Leaf rust	*Puccinia recondita var. tritici*	MBC	Catherine et al. 1997
Wheat	*Pm3b*	Powdery mildew	*Blumeria graminis f. sp. tritici*	MBC	Yahiaoui et al. 2003
Tomato	*RX2*	PVX	*Tomato virus X PVX*	TEA	Bendahmane et al. 2000

Tomato	*I2*	Wilt	*Fusarium oxysporum*	MBC	Guus et al, 1998
Tomato	*Mi*	Nematode	*Meloidogyne javanica*	MBC	Milligan et al. 1998
Tomato	*Prf*	Necrosis	*Pseudomonas syringae pv. tomato*	MBC	Salmeron et al. 1996
Tomato	*Cf-2*	Leaf mould	*Cladosporium fulvum*	MBC	Dixon et al. 1996
Tomato	*Cf-4*	Leaf mould	*Cladosporium fulvum*	MBC	Thomas et al. 1997
Tomato	*Cf-5*	Leaf mould	*Cladosporium fulvum*	MBC	Dixon et al. 1998
Tomato	*Cf-9*	Leaf mould	*Cladosporium fulvum*	TT	Jones et al. 1994
Tomato	*Pto*	Bacterial speck	*Pseudomonas syringae*	MBC	Martin et al. 1993
Tomato	*Cf-ECP2*	Leaf mould	*Cladosporum fulvum*	MBC	Kock et al. 2003
Tomato	*Hcr9-4E*	Leaf mould	*Cladosporum fulvum*	MBC	Takken et al. 1999
Tomato	*Ve*	Wilt	*Verticillium*	MBC	Kawchuk et al. 2001
Tomato	*Pto*	Bacterial spot	*Pseudomonas syingae pv.tomato*	MBC	Martin et al. 1993
Tomato	*SW5*	Wilt	*Tomato spotted wilt virus (TSWV)*	MBC	Brommonschenkel et al. 2000
Potato	*RB*	Late blight	*Slanum Bulbocastnum*	MBC	Song et al. 2003
Tobacco	*N*	TMV	TMV	TT	Whitham et al. 1994
Lettuce	*Dm3*	Downy mildew	*Bremia lactucae*	MBC	Shen et al. 2002
Pepper	*Bs2*	Bacterial spot	*Xanthomonas campestris pv. resicatoria*	MBC	Tai et al. 1999
Sugar beet	*Hs1Pro-1*	Nematode	*Heterodera schaehtl*	MBC	Cai et al. 1997
Rye	*mlo*	Leaf lesion	*Erysiphe graminis f.sp.hordei*	MBC	Buschges et al. 1997
Rye	*Rar1*	Yellow stunt	*Barley Yellow Stunt Virus*	MBC	Lahaye et al. 1998
Rye	*Rh2*	Scald	*Rhynchosporium secalis*	MBC	Schmidt et al. 2001

* MBC, map based cloning; TEA, transit expression assay; TT, transposon tagging; HS, homologous searching

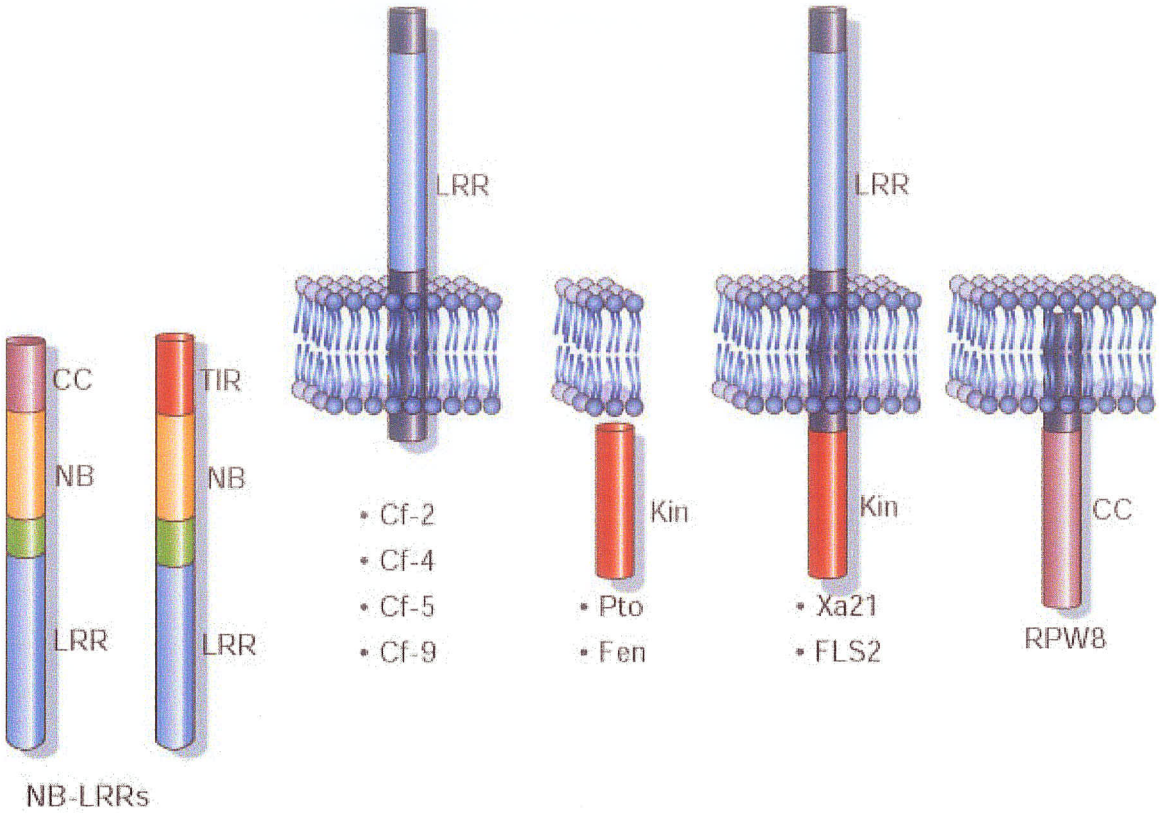


Fig. 2.3. Representation of the location and structure of the five main classes of plant disease resistance proteins. This figure is directly copied from the following article: Plant pathogens and integrated defence responses to infection (Dangl and Jones, 2001). Kin, kinase. CC, coiled coil; LRR, leucine rich repeats; NBS, nucleotide-biding site; TIR, toll and interleukin-1 receptor. Others represent different *R* genes.

Pto (Pseudomonas syringae pv. *tomato*) encodes a serine/threonine protein kinase (Martin et al. 1993). The *Cf* (*Cladosporium fulvum*) gene family in tomato represents proteins with leucine rich repeats (LRR) (Jones et al. 1994, Banerjee et al. 2001). *Xa21* (*Xanthamonas oryzae*) gene in rice codes for a structural receptor kinase with LRR motifs (Song et al. 1995). The most common *R*-genes have a nucleotide binding site (NBS) and a LRR domain (Hammond-Kosack and Jones 1997). As shown by genetic and molecular maps, 60% of the *R* genes are clustered in genomes. These clusters are each made up of a tightly linked homologous gene family (Hulbert et al. 2001). These genes confer resistance to different strains of the same pathogen or different pathogens (van der

Voort et al. 2000).

2.8 Mapped *R* genes in *B. napus* and related species

Table 2.4. Specific resistance genes in *Brassica* and other species to *L. maculans* and their map locations*

Source	Cultivar, line, or accession	Resistance gene	Map location
B. napus	'Quinta', 'Scoop'	*Rlm1*	D-Y 10*a* = N7*b*
B. napus	'Glacier', 'Bristol'	*Rlm2*	D-Y 16
B. napus	'Columbus', 'Doublol'	*Rlm3*	D-Y 10 = N7
B. napus	'Jet Neuf', 'Maluka'	*Rlm4* = *LEM1c*	D-Y 10 = N7
B. napus	French accession	*Rlm7*	D-Y 10 = N7
B. napus	'Darmor-bzh'	*Rlm9*	D-Y 10 = N7
B. juncea	'Aurea', 'Picra'	*Rlm5*	—
B. juncea	'Aurea', 'Picra'	*Rlm6* =*Jlm1d*	D-Y 17
B. rapa	French accession	*Rlm8*	
B. rapa sylvestris	Breeding line	*LepR1*	N2
B. rapa sylvestris	Breeding line	*LepR2*	N10
B. rapa sylvestris	'Surpass 400'	*LepR3*	N10
B. juncea	'AC Vulcan'	*LMJR1*	J13, G3
B. juncea	'AC Vulcan'	*LMJR2*	J18
Arabidopsis	'Col-0'	*RLM1*	$RLM1_{Col}$
Arabidopsis	'Ler-0'	*RLM2*	$RLM2_{Ler}$

* Modified after Rimmer 2006 (Refernces used : Delourme et al. 2004, Parkin et al. 1995, Ferreira et al. 1995, Chèvre et al. 1997, Bohman et al. 2004, Christianson et al. 2006)

In *B. napus*, several genes conferring cotyledon resistance and adult plant resistance to blackleg have been mapped (Ferreira et al 1995, Mayerhofer et al. 1997, Pilet et al. 1998, Rimmer et al. 1999, Zhu et al. 2003, Delourme et al. 2006). *Rlm1*, controlling seedling resistance to PG3 in 'Shiralee', 'Quinta' and 'Maxol', was mapped on N7/LG10 linkage groups (Delourme et al. 2004, Mayerhofer et al. 2005). *Rlm4* in 'Quinta' was linked to *Rlm1* (Delourme et al. 2004). *Rlm2* in 'Glacier' and 'Samourai', controlling seedling resistance in these cultivars, was on linkage group LG16 (Delourme et al. 2004). *Rlm3* in 'Maxol', resistant to isolate 19.2.01, was linked to *Rlm1*. *Rlm7* in 23-2-1 was

mapped on LG10/N7. *LepR1* on N2 and *LepR2* on N10 in DHP95 and DHP96, respectively, express resistance to isolates 87-41, 'Lefolle 5' and 'Lefolle 6' separately. *Rlm9* in 'Darmor-BZH' and 'Bristol' was mapped on N7/DY10, interacting with isolate IBCN56. *Rlm8* in 156-2-1 was from *B. rapa*. *B. juncea* was also resistant to blackleg (Keri et al. 1997). Two cultivars, 'Picra' and 'Aurea', possess *Rlm5* and *Rlm6* (Balesdent et al. 2002) separately. *LMJR1* in *B. juncea* was mapped on the linkage group J13 and *LMJR2* was mapped on the linkage group J18 (Christianson et al. 2006). 'Surpass 400' is one of the varieties containing *B. sylvestris* resistance source released by the Pacific Seed Company of Australia in 2000. The ancestry of 'Surpass 400' includes an accession of *B. napus* resynthesized from wild *B. rapa* ssp. *sylvestris* and *B. oleracea* ssp. *alboglabra* (Crouch et al. 1994, Anon. 2001), indicating that a single dominant blackleg resistance gene in 'Surpass 400' (Li et al. 2003) may be different from those in other cultivars of *B. napus*. *LepR3* in 'Surpass 400' controls seedling resistance and was mapped on N10 (Yu et al. 2004). Two independent resistance genes cloned from Arabidopsis, *RLM1* and *RLM2* from Col-0 and Ler-0 respectively, were mapped on two loci, $RLM1_{Col}$ and $RLM2_{Ler}$ (Bohman et al. 2004). *RLM1* activity is mainly dependent on At1g64070, together with a minor contribution from At1g63880 (Staal et al. 2006).

Chapter 3

Construction of an ultradense genetic recombination map in *B. napus*, consisting of 13,551 SRAP markers

This chapter was published by Sun et al. (2007). This thesis author, Zining Wang, as one of the major contributors for this mapping study, finished running and scoring approximately 7,000 SRAP markers on the map.

3.1 Abstract

Sequence related amplified polymorphism (SRAP) was used to construct an ultradense genetic recombination map for a doubled haploid (DH) population in *B. napus*. A total of 1,634 primer combinations including 12 fluorescently labeled primers and 442 unlabeled ones produced 13,551 mapped SRAP markers. All these SRAPs were assembled in 1,055 bins that were placed onto 19 linkage groups. Ten of the nineteen linkage groups were assigned to the A genome and the remaining nine to the C genome on the basis of the differential SRAP PCR amplification in two DH lines of *B. rapa* and *B. oleracea*. Furthermore, all 19 linkage groups were assigned to their corresponding N1–N19 groups of *B. napus* by comparison with 55 SSR markers used to construct previous maps in this species. In total, 1,663 crossovers were detected, resulting in a map length span of 1,604.8 cM. The marker density is 8.45 SRAPs per cM, and there could be more than one marker in 100 kb physical distance. There were four linkage groups in the A genome with more than 800 SRAP markers each, and three linkage groups in the C genome with more 1,000 SRAP markers each. Our studies suggest that a single SRAP map might be applicable to the three *Brassica* species, *B. napus, B. oleracea* and *B. rapa*. The use of this ultra-density genetic recombination map in marker development and map-based gene cloning is discussed.

Keywords: mapping, SRAP, *Brassica*, map based cloning

3.2 Introduction

During the last few decades, different kinds of molecular marker systems were developed and used for genetic mapping. Especially after PCR was invented by Kary Mullis (1983), a few PCR based marker systems were developed, such as randomly amplified polymorphic DNA (RAPD) (Williams et al. 1990), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), sequence related amplified polymorphism (SRAP) (Li and Quiros, 2001) and microsatellite (single sequence repeats, SSR) (Zietkiewicz et al. 1994) etc. DNA technology has experienced a breakthrough during the last few decades. The dideoxy termination method for DNA sequencing (Sanger et al. 1977) is the technical foundation for human, animal and crop genome sequencing projects. Single nucleotide polymorphism (SNP) was developed on the basis of sequence information. Some other marker systems, such as restriction fragment length polymorphism (RFLP) and expressed sequence tag (EST) are also very useful for marker development and comparative genomics. These marker systems can be distinguished not only by their unique detection features but also by their throughput, costs and automated detection feasibility.

Highly saturated genetic maps constructed with molecular markers are basic tools for sequencing a genome, developing specific markers linked closely to phenotypic traits for marker assisted selection (MAS) in crop breeding, and cloning of genes controlling the traits of interest through map-based strategies. Molecular markers can be derived

from a sequence polymorphism in a genome. On the basis of the whole genome sequence of rice, it has been suggested that thousands or even millions of single nucleotide polymorphisms (SNPs), insertions and deletions (INDELs) could occur in this species (Feltus et al. 2004, Shen et al. 2004). These sequence polymorphisms can be used to develop molecular markers through SNP discovery. However, after completion of genome sequencing of most major crops, enormous efforts to validate and confirm each individual SNP/INDEL will be required to fully use these markers to construct highly saturated genetic maps through SNP detection. To efficiently and effectively exploit the sequence variation in genomes, exploring other molecular markers that have similar potential to SNP detection is justified.

In potato, van Os et al. (2006) constructed an ultradense genetic recombination map containing 10,000 AFLP markers. Bowers et al. (2004) constructed a sorghum genetic map containing 2,512 sequence tagged-site (STS) loci, which can serve as a framework for the comparative study of many grains and grasses. In cotton, a genetic recombination map with 3,347 STS loci has also been assembled (Rong et al. 2004). There are no ultradense maps currently available in *Brassica* species. The most saturated map reported for the oilseed rape species *B. napus*, consisted of 1,317 RFLP markers, which was successfully used to compare the *Brassica* genomes A and C with the Arabidopsis genome (Parkin et al. 2005). SRAP is a simple marker detection method that was developed (Li and Quiros 2001). It is easily adopted to efficiently perform high throughput data collection from thousands or even millions of individuals, which is critical to any large-scale plant breeding program. It has been successfully applied in several species for different purposes (Budak et al. 2004a, b; Ferriol et al. 2003). Since

SRAP can theoretically detect any kind of sequence differences including base changes and INDELs, there are as many of these markers as SNPs in a genome. In contrast to SNPs, a primer pair combination in the SRAP protocol can detect multiple loci in a genome without previous knowledge of sequence information, such as required for SNP discovery. In this study, a high density genetic map for *B. napus* was constructed with SRAP markers using a capillary ABI 3100 DNA analyzer (Applied Biosystems, California). As genetic mapping is the first step for map based gene cloning followed by chromosome walking and complementary transformation to confirm the gene function ultimately, this map will be used to implement map-based cloning of a resistance gene *LepR3* (See Chapter 4).

3.3 Materials and methods

3.3.1 Consensus mapping population

A doubled haploid (DH) population with 58 lines was used to construct the SRAP map. This DH population was produced from a cross of a Canadian spring type canola, 'Westar', and a Chinese semi-winter type rapeseed cultivar, 'Zhongyou 821'. The diverse genetic background of these two parental lines produced a high level of polymorphism among the DH lines. *B. napus* (AC) is a natural allotetraploid that evolved naturally through the interspecific hybridization of *B. rapa* (A) and *B. oleracea* (C) (U 1935). For determining the species origin of the linkage groups on the SRAP map, a *B. rapa* DH line, 'RI16', and a *B. oleracea* DH line, 'B453' (cauliflower) were included in the analysis.

Table 3.1. DH line population and parental lines used for consensus mapping

Number	Lines	Number	Lines	Number	Lines
1	232	23	421	45	860
2	233	24	423	46	861
3	235	25	610	47	863
4	236	26	611	48	867
5	238	27	614	49	869
6	240	28	615	50	871
7	241	29	619	51	972
8	242	30	623	52	975
9	243	31	624	53	979
10	245(2)	32	628	54	881
11	248	33	629	55	882
12	342	34	653	56	885
13	344	35	757	57	889
14	346(2)	36	758	58	895
15	350	37	759	59	Zy821
16	351	38	760	60	Westar
17	376	39	761	61	B453
18	378	40	762	62	RI16
19	385	41	852		
20	386	42	853		
21	387	43	854		
22	419	44	859		

Number 1 to 58 are DH lines from 'Westar' and 'Zhongyou 821'. 'Westar' and 'ZY821 were used as parental checks and B453 (cauliflower, *B. oleracea*) and RI16 (Chinese cabbage, *B. rapa*) were used to identify the polymorphic loci in the A or C genome.

3.3.2 Primers

In the original SRAP protocol, PCR products were detected by autoradiography (Li and Quiros 2001). In the present study, SRAP products were separated with an ABI 3100 DNA analyzer, using a five-color fluorescent dye set, including 'FAM' (blue), 'VIC' (green), 'NET' (yellow) and 'PET' (red), and 'LIZ' (orange), for signal detection. The 'LIZ500' was used for labeled size standard and the other four fluorescent dye colors for labeled primers. Three primers for each dye color for a total 12 primers were synthesized and labeled by the ABI Company (Foster City, California) (Table 3.2). Since two primers

with different sequences in a SRAP PCR reaction are critical to produce a good SRAP profile, one labeled primer could be easily combined with numerous unlabeled primers having some level of sequence dissimilarity, such as the primers that came from the primer sets in our original protocol (Li and Quiros 2001). In the present study, some of the original primers, including 'EM1', 'EM2', 'EM3', 'EM5', 'EM6' and 'EM8' (the 'AATT' set), and 'ME1', 'ME2', 'ME3', 'ME4', 'ME5', 'ME6', 'ME9' and 'ME10' (the 'CCGG' set), were used. For the designing of SRAP primers, the general principles of 35–55% GC content and 17-22 nucleotides in size were followed. In total, 1,634 primer pairs were selected from the 12 labeled and 442 unlabeled primers and used for the map construction (See appendix A).

3.3.3 DNA extraction protocol and PCR amplification program

DNA was extracted with 2 × CTAB buffer as previously described (Li and Quiros 2001), but with the following major modifications. After incubation of the samples at 65°C for 90 min, an equal volume of chloroform was added to the Eppendorf tubes containing the samples, and the tubes were thoroughly vortexed. Another modification was the precipitation of DNA with a reduced amount of iso-propanol, 0.5-0.55 volume of the supernatant, and reduced centrifuge speed to 5,000 rpm for 3 min. The PCR amplification program was the same as reported in the original SRAP protocol (Li and Quiros 2001). Each PCR reaction solution contains 1.5 mM $MgCl_2$, 0.375 mM dNTPs, 0.15 μM each for the labeled primer and unlabeled primer, and 1-5 ng genomic DNA and 1U Tag polymerase in 10 μl reaction. There are five cycles at 94°C 50 s, 35°C 50 s and 72°C 50 s, followed by 30 cycles at 94°C 50 s, 50°C 50 s and 72°C 50 s. Normally, all samples in

one plate had the same labeled primer, and samples from four different color labeled primers in four plates were pooled together after running PCR reactions in a thermocycler. Two point five µl of the pooled samples was added to a 5.5 µl mixture of formamide and 500-LIZ size standard (ABI), and then denatured at 95°C for 3 min. The plates containing the samples were then loaded into the auto sampler of the ABI 3100 DNA analyzer, and the SRAP products were separated using 36-cm 16-channel arrays with a 40 min running time.

3.3.4 Data analysis

After data were collected by the ABI 3100 DNA analyzer, they were analyzed with GenScan software (ABI). Then the previously analyzed data were exported and loaded into another program, 'Genographer' (www.hordeum.oscs.montana.edu/genographer), to produce an image that looked like a gel picture. 'Genographer' allowed scoring each polymorphic locus by drawing a double-line window, and the scores were then converted directly into MAPMAKER compatible data, which were copied and pasted into a Microsoft Excel sheet. Each band showing polymorphism was scored as a dominant marker.

3.3.5 Consensus map construction

MAPMAKER v2.0 running on a Macintosh computer was used to construct the genetic recombination map (software available online at http: //www.linkage.rockefeller. edu/soft/mapmaker/). The assembled framework map contained 19 linkage groups that would represent the 19 chromosomes of *B. napus*. All markers were assigned into these

19 linkage groups based on the grouping information in the previous steps. The final version of the genetic recombination map was calculated with Mapmaker Macintosh v2.0.

3.3.6 Alignment to other maps

Ninety of the SSR markers reported by Piquemal et al. (2005) were tested in our mapping population to permit alignment of both maps. Additionally one new SSR primer pair, AGGGTTTGAGATATAGTGT, TGGCGATGAATTTGCAAGG, based on the sequence of the BAC clone (AC152123) was also used to align our SRAP linkage groups to N15, since the SSR markers on that group were not polymorphic in our mapping population. The SSR primer sequences were taken from the electronic supplementary material by Piquemal et al. (2005) and the M13 sequence (CACGACGTTGTAAAACGAC) was put at the beginning of each forward primer for the detection of PCR products. The M13 primer was labeled by the ABI Company and added to the PCR reaction mix. The components in PCR reactions and the PCR amplification program were the same as described by Piquemal et al. (2005). The PCR products were separated with an ABI 3100 DNA analyzer. After data collection and analysis, the SSR mapping data were run in Mapmaker together with 1,055 SRAP markers, of which one marker was taken from each bin on the SRAP map. All SSR markers were integrated into the corresponding SRAP marker bins on the SRAP map. The N1–N19 linkage group nomenclature on the SSR map constructed by Piquemal et al. (2005) was used to name the linkage groups on our ultradense SRAP *B. napus* map.

3.4 Results

3.4.1 Performance of labeled primers and marker distribution

Table 3.2. Summary information of the 12-labeled primers*

Primer name	Fluorescent dye	Primer sequence, 5'-3'	No. of primer combinations for each labeled primer	No. of markers for one labeled primer	Average No. of markers per primer combination
ME2	6-FAM	TGAGTCCAAACCGGAGC	236	1,422	6.03
EM2	NED	GACTGCGTACGAATTCTGC	215	1,210	5.63
DC1	PET	TAAACAATGGCTACTCAAG	184	998	5.42
EM1	VIC	GACTGCGTACGAATTCAAT	263	2,007	7.63
ODD3	6-FAM	CCAAAACCTAAAACCAGGA	70	635	9.07
SA12	NED	TTCTAGGTAATCCAACAACA	77	568	7.38
ODD20	PET	TCGTTGTTATGGCTGGAGA	76	533	7.01
GA3	VIC	TCATCTCAAACCATCTACAC	76	646	8.50
FC1	6-FAM	TCAAGGGCAGGTAAGAACAA	107	1,284	12.00
BG23	NED	ATTCAAGGAGAGTGCGTGG	111	1,430	12.88
PM88	PET	CGAAACCTCACCTCTCTCA	103	941	9.14
SA7	VIC	CGCAAGACCCACCACAA	116	1,877	16.18
Total			1,634	13,551	8.29

*Twelve primers were labeled by ABI Company as 6-FAM (blue), NED (yellow), PET (red) and VIC (green), respectively. The average number of markers for each labeled primer was calculated according to the markers and the primer combinations related to the labeled marker in each row.

A total of 1,634 primer combinations produced over 15,000 bands scored which showed polymorphism. All SRAP markers were scored as dominant. Table 3.2 illustrates the mapped SRAP markers that were inventoried on the basis of the 12-labeled primers as explained in materials and methods. The average number of mapped primers per primer combination is 8.29. The best labeled primer, 'SA7', was used in 116 primer combinations, producing 16.18 markers per primer combination on average (Table 3.2). With more than 15,000 recorded SRAP markers, and thorough grouping and regrouping of the whole data and reordering of adjacent markers by minimizing the recombination events and removing 1,005 singletons, 13,551 SRAP markers were assembled into 19

linkage groups to form a genetic recombination map. All markers were listed with their marker names and sizes, their bin positions on the map, and the primer pairs used for producing the markers. Nine of these 19 linkage groups are presumed to represent the nine chromosomes of the C genome in *B. napus* genome, based on the alignment of these linkage groups with the published N11–N19 groups cytologically assigned to their corresponding chromosomes (Howell et al. 2002).

3.4.2 Assigning linkage groups into the A and C genomes

The following banding patterns were detected in *B. napus* and the two diploid species. First, a group of bands that were amplified in *B. napus*, but not in *B. rapa* or *B. oleracea*, indicating that sequence divergence occurred between *B. napus* and its diploid parents. Second, some bands appeared in all three species, representing the highly conserved sequences in the A, C and AC genomes. The third group of the bands was amplified in *B. napus* and *B. rapa*, but not in *B. oleracea*, most of which would likely represent A-genome specific amplifications. Finally the last group of the bands was amplified in *B. napus* and *B. oleracea*, but not in *B. rapa*, thus being most likely C-genome specific. With these preliminary testing results, 5,527 mapped SRAP markers in the *B. napus* DH population were also tested in the DH *B. rapa* and *B. oleracea* lines for comparison of the bands from the A, C and AC genomes. As described previously, all bands tested could be placed into one of these four groups when the band distribution on the same linkage group were checked and counted separately (Table 3.3). A total of 2,566 (46.4%) of the 5,527 mapped marker loci only appeared in *B. napus*. On the other hand, 575 (10.4%)

Table 3.3. Distribution of mapped SRAP markers with the corresponding bands detected in *B. rapa* and *B. oleracea* for assigning linkage groups to the A and C genomes in *B. napus**

Linkage group	Grouping of mapped SRAP markers and percentage of SRAPs in one of two diploid species						
	BB	AA	BA	AB	BA+ AB	BA/(BA+ AB)*100	AB/(BA+ AB)*100
N01	127	17	95	22	117	81.2	18.8
N02	157	22	98	27	125	78.4	21.6
N03	137	12	69	24	93	74.2	25.8
N04	134	16	81	20	101	80.2	19.8
N05	201	54	120	33	153	78.4	21.6
N06	229	27	111	38	149	74.5	25.5
N07	169	35	99	71	170	58.2	41.8
N08	138	30	76	33	109	69.7	30.3
N09	68	109	74	64	138	53.6	46.4
N10	101	13	81	15	96	84.4	15.6
A genome	1,461	335	904	347	1,251	72.3	27.7
N11	119	24	27	101	128	21.1	78.9
N12	243	34	31	193	224	13.8	86.2
N13	189	45	40	168	208	19.2	80.8
N14	127	19	16	108	124	12.9	87.1
N15	51	24	24	41	65	36.9	63.1
N16	23	18	11	21	32	34.4	65.6
N17	114	22	16	88	104	15.4	84.6
N18	38	7	17	43	60	28.3	71.7
N19	201	47	46	143	189	24.3	75.7
C genome	1,105	240	228	906	1,134	20.1	79.9
Total	2,566	575	1,132	1,253	2,385	47.5	52.5

*BB representing the mapped SRAP markers without counterparts in diploid species; AA, the mapped SRAP markers with counterparts in both diploid species; BA, the mapped SRAP makers with counterparts only in *B. rapa*; AB, the mapped SRAP markers with counterparts only in *B. oleracea.*

loci were found in all three species. Some of 1,132 (20.5 %) loci appeared only in *B. rapa* and *B. napus*, and 1,253 (22.7 %), only in *B. oleracea* and *B. napus*. Those last two groups with 2,385 loci that appeared in only one of the two diploid genomes allowed us to assign the 19 linkage groups into their corresponding genomes. Eventually, when the

bands amplified in the A and AC or in the C and AC genomes on each of the 19 linkage groups were counted, 10 linkage groups had more bands (53.6–84.4%) that amplified only in the A and AC genomes of *B. rapa* and *B. napus* so these groups were assigned into the A genomes. On the remaining nine linkage groups, more bands (63.1–87.1%) were found only in the C and AC genomes, and these were assigned into the C genome.

3.4.3 Alignment of the SRAP map with other maps

The 19 linkage groups that were previously assigned into the A and C genomes were further analyzed with SSR markers including 90 of the markers on the SSR map of Piquemal et al. (2005). With these 90 SSR primer pairs, 79 SSR loci were integrated on the SRAP map. Since most SSR primer pairs produce multiple marker loci, every mapped SSR marker on the SRAP map was checked carefully and only those mapped SSR markers on the SRAP map showing similar size to that obtained by Piquemal et al. (2005) were used to assign the SRAP linkage groups. Others, which showed different sizes, were listed as new marker loci (Table 3.4). Fifteen out of the nineteen linkage groups were easily assigned into the same N number linkage groups as those on the SSR map, but there was some discrepancy for three groups, N4, N14 and N19. Six SSR markers on the Piquemal N4 linkage group mapped onto a SRAP linkage group that was assigned into the C genome, based on the differential SRAP PCR amplification in *B. rapa* and *B. oleracea*. Since N4 is a linkage group assigned to the A genome and the N4 and N14 (C genome) share a high level similarity on the RFLP map of Parkin et al. (2005), the SRAP linkage group that was integrated with the six SSR markers on the N4 linkage group of

Table 3.4. Distribution of integrated SSR markers on the SRAP map for assigning the SRAP linkage groups into N1–N19 linkage groups

Primer name	Size of PCR products[a]	Linkage group and bin position	Map position (cM) on the SRAP map	Linkage group on the SSR map	Map position (cM) on the SSR map[b]	Markers with the same order on both maps
CB10099	217	N01–11	17.4	N01	39.7	Yes
BRAS041	236	N01–16	25.7	N01	65.6	Yes
BRAS026a	198	N01–27	48	N01	96.4	Yes
BRAS083a	250	N02–17	25.6	N02	186	Yes
Na14-H11	131	N02–18	26.5	N02	175	Yes
BRAS011	222	N02–32	48.5	New		
BRAS002a	230	N03–02	0.9	*n19	34.9	Yes
CB10034	230	N03–02	0.9	New		
Na10-b11	221	N03–02	0.9	*n19	45.4	Yes
CB10036a	137	N03–35	63.1	N03	30.8	Yes
NA12-E02a	136	N03–43	74.1	N03	60.2	Yes
CB10347	214	N04–25	24.3	*n9	19.9	
CB10493a	174	N04–48	55.5	N04	170	
CB10545	98	N05–01	0	N05	5.7	Yes
BRAS002b	202	N05–21	21.6	New		
BRAS026b	179	N05–30	29.7	New		
CB10080	163	N05–44	45.1	N05	86.8	Yes
CB10229a	271	N05–59	60.5	New		
CB10229b	268	N05–64	67.8	New		
CB10487	268	N05–64	67.8	New		
Na12-D08	142	N06–11	17.5	N06	121	
BRAS026c	173	N06–46	59.2	New		
Na12-A02a	211	N07–47	75.4	New		
Na10-C06	225	N07–57	94.6	*n16	85.3	
Na12-A02b	181	N07–78	132.6	N07	98.7	
NI4-D09a	223	N08–14	15.4	*n24	20	
Ra2-E12	224	N08–27	71.2	N08	58.6	Yes
CB10364	252	N08–29	73.9	N08	18.1	Yes
CB10124a	170	N09–11	20.4	*n10	109	
NA10-A08	170	N09–20	28.5	N09	119	
CB10124b	164	N09–24	26.7	*n15	0	
CB10199a	173	N09–59	43.9	New		
Na12-H04	113	N10–01	0	N10	98.7	
CB10124c	173	N10–02	0.9	N10	109	Yes
MR156	209	N10–10	8.9	N10	76.6	Yes
CB10524	244	N10–25	25.2	N10	54	Yes
CB10079	189	N10–38	48.2	N10	6.9	Yes
Na14-F11	275	N11–08	10	New		
Ra2-F11a	285	N11–11	16.5	New		
CB10277	236	N11–30	40.4	N11	40.9	

Table 3.4. continued

Primer name	Size of PCR products[a]	Linkage group and bin position	Map position (cM) on the SRAP map	Linkage group on the SSR map	Map position (cM) on the SSR map[b]	Markers with the same order on both maps
BRAS083b	270	N12–06	7.3	New		
CB10316a	239	N12–06	7.3	N12	6.6	Yes
BRAS083c	176	N12–08	9.1	*n2	190	
CB10526	151	N12–16	18.2	New		
OI13-G05	135	N12–21	27.4	N12	28.2	Yes
BRAS123a	262	N12–48	81.3	New		
Na12-E03	281	N12–60	98.7	New		
CB10036b	143	N13–14	27.6	New		
Na14-E02	183	N13–16	33	New		
Na12-E02b	110	N13–19	36.8	N13	87.5	Yes
BRAS120a	240	N13–20	37.7	N13	89.2	Yes
BRAS120b	154	N13–21	38.6	New		
BRAS005	218	N13–31	51.1	N13	131	Yes
BRAS051	163	N13–31	51.1	N13	131	Yes
Na10-c01a	171	N13–62	100.2	N13	266	Yes
MR049	206	N13–66	105.6	N13	267	Yes
CB10316b	266	N14–14	15.3	*n4	48.1	Yes
MR036	145	N14–15	20.3	*n4	3	
BRAS123b	240	N14–20	26.7	*n4	54.8	Yes
BRAS021	222	N14–28	38.6	*n4	95.4	Yes
Na10-c01b	296	N14–34	44.9	*n4	102	Yes
CB10493b	220	N14–47	75.5	*n4	170	Yes
BGSSR2	265	N15–86	34.8	New		
CB10526a	314	N15–88	49.8	New		
Na12-A02c	193	N16–01	0	N16	59.9	Yes
CB10316c	452	N16–13	19.2	New		
CB10526b	339	N16–13	19.8	N16	26.5	Yes
Na10-c01c	224	N17–26	35.9	N17	8.7	
BRAS026d	365	N17–32	43	New		
CB10528	301	N17–33	44.8	N17	5.6	
BRAS019	176	N17–40	54.7	N17	6.7	
CB10028	186	N18–05	7.4	N18	14.4	Yes
CB10092	228	N18–08	11	N18	25.7	Yes
NI4-D09b	189	N18–18	24.6	N18	46.9	Yes
BRAS050	247	N19–03	1.8	New		
CB10199b	197	N19–18	38.9	New		
OI10-D08	196	N19–29	51.5	*n14	35.5	Yes
Ra2-F11b	212	N19–29	51.5	*n14	35.5	Yes
OI10-A09	101	N19–31	53.3	*n14	13.2	
Na12-g04	184	N19–48	72.2	*n14	72.1	Yes

[a] PCR products were separated with ABI 3100 DNA analyzer

[b] SSR map is the map of Piquemal et al (2005)

the SSR map was assigned as the N14 linkage group on the current SRAP map. However, four SSR markers on the Piquemal's N14 linkage map were integrated onto another SRAP linkage group. According to the mapped markers available at http://www.brassica.bbsrc.ac.uk/IMSORB and the mapped SSR markers at Huazhong Agricultural University (Drs Tu and Fu, personal communication), two of these four SSR markers, Na12-G04 and Ra2-F11, were mapped on the N19 linkage group by two different labs. Therefore the SRAP linkage group which has the corresponding SSR markers on the Piquemal's N14 map was assigned instead to the N19 linkage group. Unfortunately, none of the SSR markers on the Piquemal's N15 were polymorphic in our mapping population. Alternatively we used a sequence in the *B. oleracea* BAC clone (Gao et al. 2006) harboring the BoGSL-PRO gene involved in the biosynthesis of aliphatic glucosinolate to generate a SSR marker BGSSR2. The genes on this BAC clone have their counterparts on the top of the chromosome 1 in Arabidopsis. On the basis of the comparative maps of Parkin et al. (2005), this chromosome 1 region has two corresponding parts on the N15 and N18 of the C genome on the *B. napus* RFLP map. Since the N18 was assigned with the SSR markers of Piquemal et al. (2005), to a different linkage group, the only alternative was that our unassigned SRAP linkage group corresponds to N15. Furthermore, the BoGSL-PRO gene on this BAC clone has been mapped on the O5 linkage group in *B. oleracea* (Gao et al. 2006), which is equivalent to the N15 linkage group in *B. napus*. Consequently this further supports the assignment to the N15 linkage group. When the order of the integrated SSR markers on both the SRAP map and the SSR map of Piquemal et al. (2005) was compared, 25 of 27 SSR markers that were located on six linkage groups with more than three SSR markers each showed

the same order and relatively similar map length, which suggested that the current SRAP map shares the similar marker order as that on the SSR map of Piquemal et al. (2005).

3.4.4 Detection of transpositions

After all 19 SRAP linkage groups were assigned into the N1–N19 linkage group, all mapped SRAP markers which showed differential amplification in *B. rapa* and *B. oleracea* were revisited and in total five transpositions between the A and C genomes were found judged by the differential amplification in these two diploid species. The N7 linkage group contains a region from N07-62 to the end of the linkage group, spanning a genetic distance of 35.4 cM, where 29 of 32 mapped markers have their corresponding loci only in *B. oleracea*, suggesting that this region consists of one third of the N7 linkage group that originally came from the C genome. On the N16 linkage group, the region covering the bins from N16–01 to N16–16 spanning a genetic distance of 25.4 cM, contains more SRAPs markers (10 of 18) that could be amplified only in *B. rapa.* These two regions on the N7 and the N16 linkage groups are presumed to correspond to the homeologous reciprocal transposition reported by Osborn et al. (2003). Additionally with regards to the differential amplification of the mapped SRAP markers only in *B. rapa* or only in *B. oleracea*, two transpositions located on the N9 linkage groups that originally come from the C genome and another one on the N19 linkage group, from the A genome, respectively, were identified (Table 3.5).

Table 3.5. Summary of transpositions detected by the differential amplification of mapped SRAP markers in the two diploid species *B. rapa* and *B. oleracea*

Name of linkage group	Bins of transposition on the linkage group	Map length of transposition	SRAP bands amplified in *B. oleracea*, but not in *B. rapa*	SRAP bands amplified in *B. rapa* , but not in *B. oleracea*
N07	62–80	35.3	29	3
N08	37–42	10.4	8	3
N09	48–59	10.8	8	5
N16	1–16	25.4	8	10
N19	5–21	34.2	7	9

3.4.5 Recombination events and mapping

Every recombination event was checked and counted in each of the 58 DH lines on each of the 19 linkage groups. In total, 1,663 crossovers were detected according to the marker arrangement in each individual DH line on a linkage group, and the average number of recombination events per linkage group was 1.51. Since each DH line derived from a single gamete, then there are average 28.7 inferred crossovers occurring among the 58 gametes that produced the 58 DH lines for the map. If the previously assigned linkage groups of the A genome and the C genome are counted separately, there are 904 crossovers in the A genome and 759 in the C genome. There is a considerable discrepancy between the genome size and the number of crossovers in a genome, considering the genome size of 500 Mb for A genome and 600 Mb for C genome. The total map length for the *B. napus* genome is 1,604.8 cM, which includes 829.1 cM on the ten linkage groups of the A genome (N1–N10) and 775.7 cM on the nine linkage groups of the C genome (N11–N19) (Table 3.6; Fig. 3.1). The largest linkage group, N7, in the A genome spans 134.4 cM distance, and the smallest one, N18, in the C genome was 34.5 cM in size. On the basis of the mapping population with 58 DH lines, one crossover is

approximately equal to 0.9 cM of genetic distance and this is the minimum size required to separate two marker bins. On all 19 linkage groups, all 13,551 SRAP markers are assigned into 1,055 marker bins, among which there are seven bins with 100 to 188 markers. Table 3.6 illustrates the numbers of bins and markers on all 19 linkage groups, of which there are four linkage groups with 76 to 88 bins and six linkage groups with more than 800 markers per group. There are 7,120 SRAP markers on the 10 linkage groups of the A genome, and 6,221 of the C genome.

Table 3.6. Distribution of mapped SRAP markers with the corresponding bands detected in *B. rapa* and *B. oleracea* for assigning linkage groups to the A and C genomes in *B. napus*

Name of linkage group and genomes	No. of bins on linkage group	No. of markers on linkage group	Map length (cM)
N01	42	586	78.0
N02	78	824	127.3
N03	49	525	79.5
N04	51	630	60.0
N05	64	989	67.8
N06	58	930	73.6
N07	80	967	134.4
N08	32	609	76.7
N09	62	768	76.3
N10	42	508	55.5
A genome	558	7,336	829.1
N11	44	624	66.6
N12	65	1,124	110.3
N13	76	1,059	119.2
N14	48	602	78.3
N15	88	634	129.5
N16	31	157	56.2
N17	54	635	81.4
N18	27	307	34.5
N19	64	1,073	99.7
C genome	497	6,215	775.7
Total	1,055	13,551	1604.8

3.5 Discussion

Potential marker abundance in a genome, marker availability and high throughput detection at a reasonable cost are major factors determining the usefulness of a molecular marker system. Although there are several kinds of molecular marker systems, such as RFLP, AFLP, RAPD, SSR, and SNP available, each method has unique advantages and disadvantages, which have been discussed elsewhere (Peters et al. 2003). The advantages of the SRAP molecular marker detection protocol have been clearly demonstrated through construction of an ultradense genetic map in *B. napus*. This is the most saturated genetic recombination map reported in *Brassica* species to date.

The crossovers inferred from the current data display a similar range to those reported in sorghum and tomato. In a genetic recombination map for sorghum constructed with 2,512 loci, 1,376 crossovers were detected, covering a map length of 1,059.2 cM (Bowers et al. 2003). Similarly, with over 13,500 SRAP markers, 26.05 crossovers in a gamete and 0.39–2.41 crossovers per linkage group on average in *B. napus* were inferred and a genetic map with a length of 1,604.8 cM was constructed. In general, the length of a recombination map is smaller than a genetic map since the high marker density allows the identification of recombination points and removal of the singletons that come out of missing data and scoring errors. For instance, the genetic recombination map for sorghum was shortened from 1,445 cM to 1,059.2 cM in size. In line with the results in other crop plant species, the length of the current genetic recombination map was reduced compared with other published *B. napus* genetic maps that have a range of 1,968 to 2,619 cM.

The alignment of genetic maps generated by different laboratories is highly beneficial to the *Brassica* community. However, there is no generally accepted method to

align different genetic maps constructed with different kinds of markers and populations. The RFLP method is often used for the alignment of different maps (Parkin et al. 2005, Piquemal et al. 2005). There are, however, some limitations with respect to this approach. The method per se is time consuming and RFLP detection is too difficult to become a universal method. Additionally there are no standard band patterns for the probes that are available publicly. One RFLP probe generally produces multiple bands representing different loci in the allotetraploid *B. napus* species. Without a standard band pattern for a probe, it is not easy to make a reliable alignment of linkage groups from different genetic maps. Here we used SSR markers to align our SRAP map with the SSR map of Piquemal et al. (2005), which is considered the consensus map for this species. Although we could easily align 15 of the 19 linkage groups of the SRAP map with the SSR map, we had difficulty with group N15 because of lack of polymorphism for those markers in our mapping population. This problem could prove to be an obstacle for linking the maps from different laboratories. With the SRAP markers, we could detect the genomic origin of a marker in *B. napus* through the differential amplification of *B. rapa* and *B. oleracea*. This allowed us to identify syntenic differences for linkage groups N4, N14 and N19 when constructed by SSRs versus SRAP markers. These could be real differences representing structural chromosomal changes in the different *B. napus* stocks used in the two mapping populations. However, it is also possible that the RFLP markers used for assigning the Piquemal's map did not disclose the correct synteny of these groups. Additionally, we also detected some *B. napus* representing genomic rearrangements during the evolution of this species.

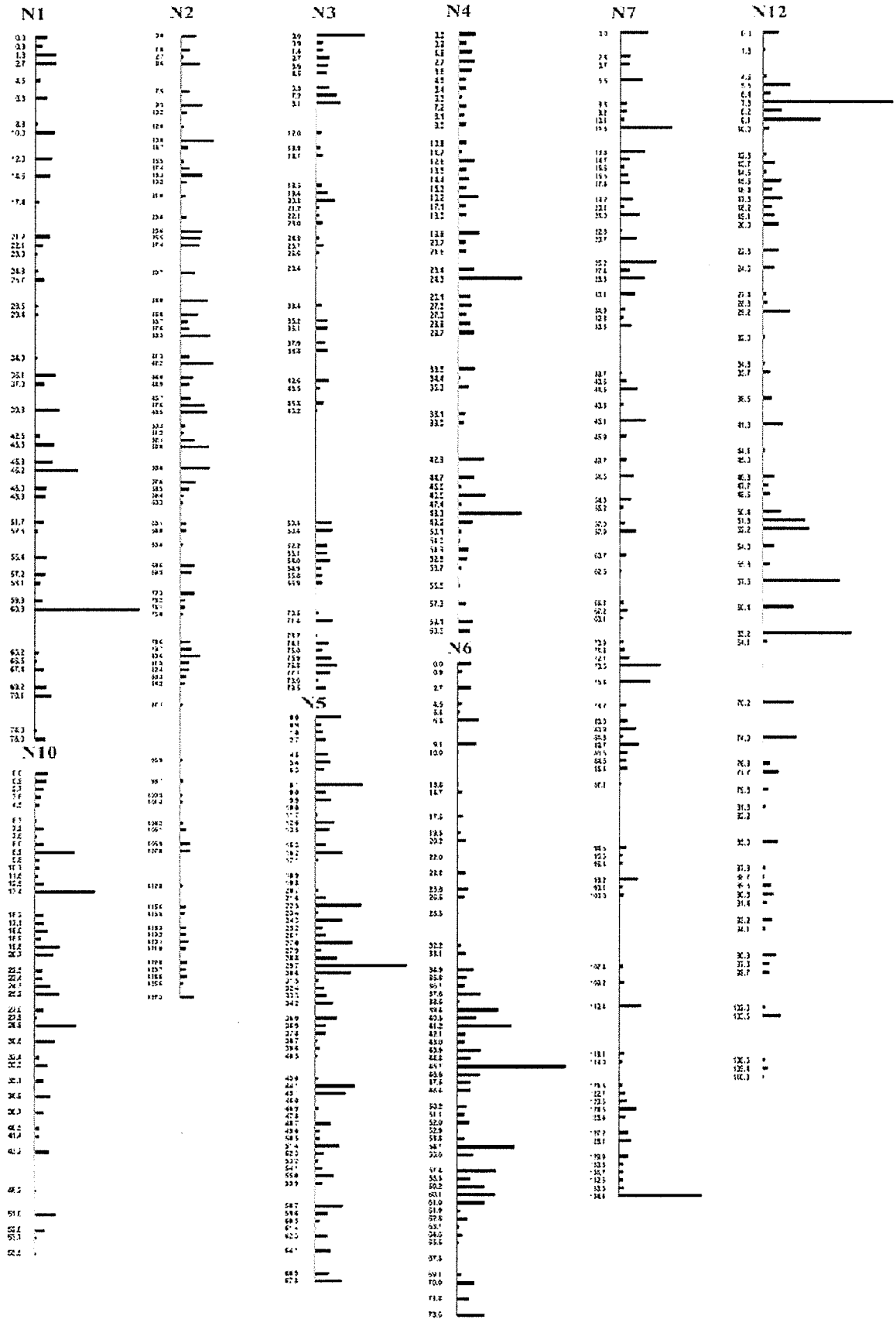
The marker bin method was used for the construction of the current ultradense genetic

map. This strategy was also used quite effectively for the construction of the AFLP map in potato (van Os et al. 2006). Due to the small size of the mapping population and limited number of crossovers occurring in the genome, the SRAP markers were organized into bins, most of which have more than one marker, and a few bins have over 100 markers. To circumvent the limitations of Mapmaker software in dealing with thousands or even millions of markers on a map, a bin map strategy can be implemented to assign new markers into existing bins.

The current genetic map provides a platform for map-based gene cloning, and this has been intensively implemented in the cloning of several genes conditioning important traits, such as resistance to blackleg disease (see the following chapter) in *B. napus* and *B. rapa* in our lab. For the location of known genes from other related species, those known genes can be first incorporated onto the genetic map. Then co-segregation analysis will allow the confirmation of the known genes involving the trait of interest. For instance, using the current genetic recombination map, a resistance gene to blackleg disease in cultivar, 'Surpass 400', introgressed from a wild *B. rapa* ssp. *sylvetris*, is being targeted. Since the population used for cloning the resistance gene is different from the mapping population, a marker was first found in the segregating population of 'Westar' and 'Surpass 400'. Then the marker was integrated on the linkage group N10 of the genetic map, and finally a dozen SRAP markers closely linked to the resistance gene were identified (Chapter 4).

Construction of a single map for three *Brassica* species is another goal of the current recombination map. Since there is a close relationship among *B. napus, B. rapa* and *B. oleracea*, the SRAP method detects corresponding markers simultaneously in these three

species. As the data illustrates, approximately 20% of the SRAP markers on the *B. napus* map are also polymorphic in *B. oleracea*, suggesting that approximately another 20% may be polymorphic in *B. rapa*. Further, the close markers on the resistance genetic map could be used for anchoring physical and genetic maps when genomic libraries, such as BAC libraries, are available. Since approximately 50% of SRAP markers locate in gene regions and it is easy to sequence most SRAP markers, the sequence information allows the identification of the corresponding regions in Arabidopsis via BLASTn analysis. This certainly will expedite gene cloning by saturating the regions harboring candidate genes with new markers.


N1
N2
N3
N4
N5
N6
N7
N10
N12

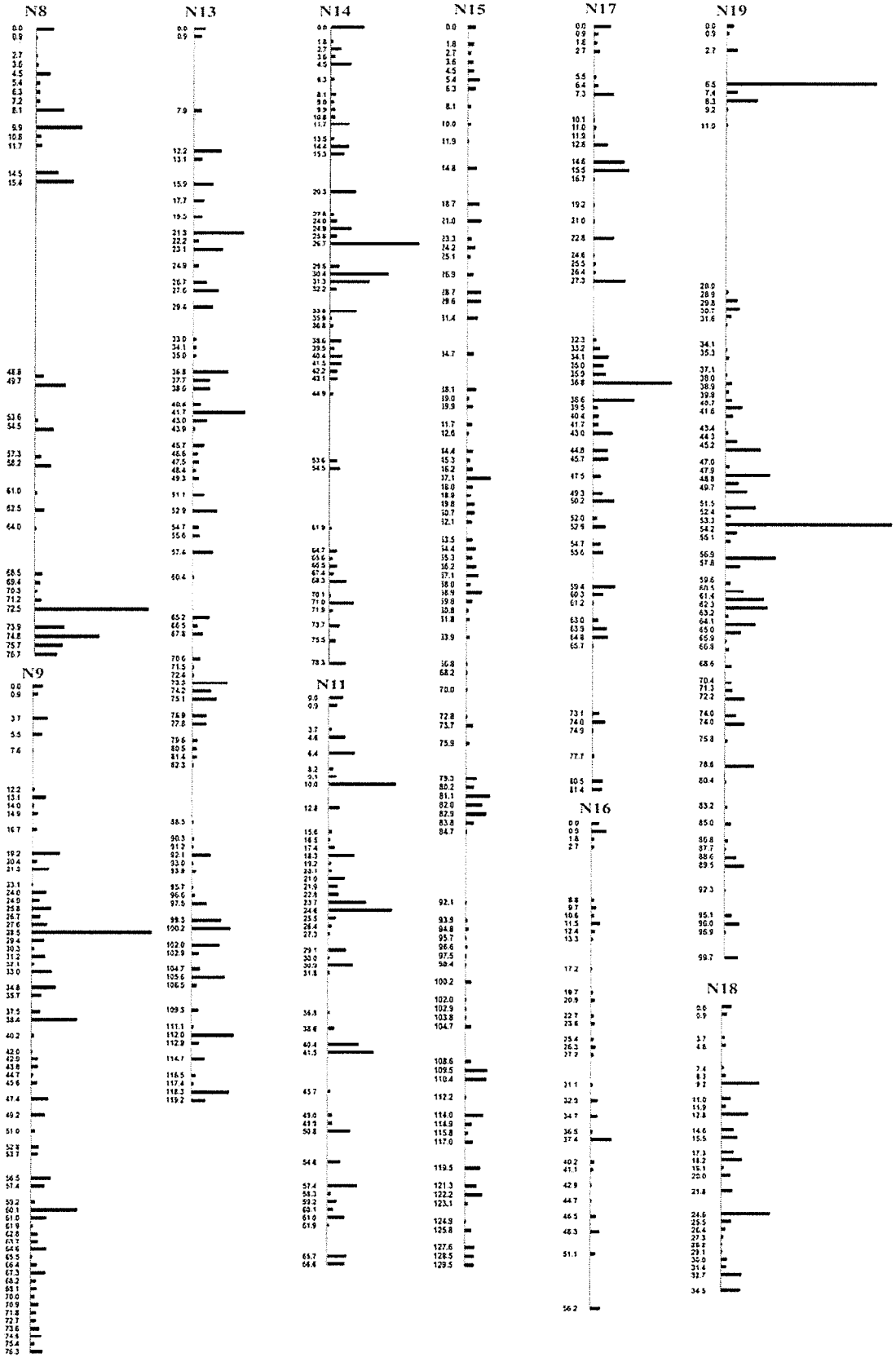

N8
N13
N14
N15
N17
N19
N9
N11
N16
N18

Fig. 3.1. High density genetic map constructed with a DH line population from a cross of 'Westar' and 'Zhongyou 821'. A total of 13,351 SRAP markers are assigned into 1,055 bins on N1-N19 linkage groups. N1-N10 linkage groups are for the A genome and N11-N19, for the C genome. The horizontal bars indicate the number of SRAP markers in the bins. The numbers at left side of each map are genetic positions of each bin by cM.

Chapter 4

Identification of a candidate resistance gene to *Leptospharea maculans* in 'Surpass 400' (*B. napus L.*)

4.1 Abstract

Map-based cloning of the resistance gene *LepR3* to blackleg in 'Surpass 400' was performed in this research. The segregation analysis of the gene tagging population showed that there was a single dominant resistance gene *LepR3* controlling the seedling resistance in 'Surpass 400'. Screening of 384 pairs of primers for SRAP identified two markers, A269 and G278 that co-segregated with the gene. Marker A269 was found to correspond to the marker 1217Ar269 on the consensus map and used as an anchoring marker on the N10 linkage group with 508 SRAP markers. A group of close markers 210Ay442, 0127Fr382 and 1128BG275 on the map co-segregated with *LepR3*. The gene tagging populations with 908 F_2 plants and 2,992 $F_{2:3}$ plants were screened with these markers and 52 recombinants were found between the marker 0127Fr382 and 1217Ar269. SRAP marker 0127Fr382 was the closest molecular marker to the resistance gene, showing a genetic distance of 0.3 cM. Sequencing of these markers suggested that there were two different regions of chromosome 5 in Arabidopsis that may contain the *LepR3*. Four SNPs that were located in four *Brassica* homologs of At5g57035, At5g57345, At5g57670 and At5g57830 were developed and used to analyze these 52 recombinants. The genetic map for these four SNPs showed the same gene order in *B. napus* as in Arabidopsis and they showed similar genetic distance to the *LepR3* gene as that of SRAP markers 1217Ar269 and G278. It was confirmed that the *LepR3* gene did not fall into this syntenic region. SNPs for another region, SNP13280, SNP13530, SNP13930, and SNP14950, were also developed with Arabidopsis genes At5g13280, At5g13530,

At5g13930, and At5g14950. Further analysis of these SNPs with these 52 recombinants showed that SNP13930 co-segregated completely with the resistance gene. The BAC clone A48M23 was identified with the primers designed using At5g13930. BAC end sequences and genes on the BAC clone showed the same gene order in Arabidopsis and *B. rapa*. Three other SNPs, SNP14060, SNP14210 and SNP14220, were developed using Arabidopsis genes At5g14060, At5g14210 and At5g14220, respectively. These four SNPs showed no recombination among these 52 recombinant populations, indicating that the *LepR3* gene would be in this small region. After searching the Arabidopsis genome in a region of about 1,060kb (At5g13290-At5g16000) containing At5g13930, At5g14060, At5g14210 and At5g14220, only At5g14210 was found to be a leucine-rich repeat transmembrane protein kinase, suggesting that the orthologous gene of At5g14210 is a good candidate for the blackleg resistance gene *LepR3* in 'Surpass 400'.

Key words: Blackleg, resistance gene, mapping, cloning, *B. napus*

4.2 Introduction

Blackleg disease (*L. maculans*) causes major yield losses in canola production in Canada, Australia and Europe (West et al. 2001). Research on fungal characteristics, genetics of plant disease resistance and pathogen-host relationships has been conducted for several decades and a considerable volume of valuable data has been collected in different areas. However, pathogen avirulence genes and plant resistance genes need to be identified to gain further understanding of plant-pathogen interactions and to control blackleg disease effectively in canola production.

There are two groups of blackleg isolates, A and B groups distinguished, on the basis of symptoms on *B. napus* (Johnson and Lewis 1994). The A pathogen group isolates have been subdivided into PG2, PG3, PG4 and PGT with respects to differential reactions on cotyledons of the *B. napus* cultivars, 'Westar', 'Quinta', 'Glacier' and 'Jet Neuf' (Koch et al. 1991, Kuswinanti et al. 1995, Balesdent et al. 2002, Rouxel et al. 2003b, Rimmer 2006). In contrast, B group isolates (non aggressive) had been classified into three genetically distinct subgroups: NA1, NA2 and NA3 (Koch et al. 1991).

Several dozen plant disease resistance genes have been cloned and classified into different groups on the basis of the conserved domains in the protein sequences of resistance gene (*R*-gene) products. *R*-genes, such as *Pto* (*Pseudomonas syringae pv. tomato*) encoding a serine/threonine protein kinase (Martin et al. 1993), belong to one group. The *Cf* (*Cladosporium fulvum*) gene family in tomato represents a group which encodes proteins with leucine rich repeats (LRR) (Jones et al. 1994, Banerjee 2001). The *Xa21* (*Xanthomonas oryzae*) gene in rice and several other genes encode a structural receptor kinase with LRR motifs (Song et al. 1995). The most common *R*-genes have a nucleotide binding site (NBS) and an LRR domain (Hammond-Kosack and Jones 1997). Map-based cloning was commonly employed for the previously described disease resistance genes and the first *R* gene cloned with this method was the tomato *PTO* gene (Martin et al. 1993).

Although at least 10 genes conferring cotyledon resistance and adult plant resistance to blackleg have been mapped in *B. napus* (Ferreira et al 1995, Maperhofer et al. 1997, Pilet et al. 1998, Rimmer 1999, Zhu et al. 2003, Delourme et al. 2006), none of them has been identified or cloned. For instance, *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* were mapped

on linkage group 10 (LG10) and *Rlm2* on LG16 (Mayerhofer et al. 1997, Delourme et al 2004), *LepR1* on linkage group N2, *LepR2* and *LepR3* on N10 (Yu et al. 2004, 2005, Rimmer 2006) respectively. More recently, *LmR1* has been fine-mapped on the N7 linkage group, equal to LG10 (Mayerhofer et al. 2005).

In this study, the *LepR3* gene in a *B. napus* cultivar, 'Surpass 400', which was a gene introgression from *B. rapa* subsp. *sylvestris* (Crouch et al. 1994, Anon. 2001), was targeted through map-based gene cloning. Sequence related amplified polymorphism (SRAP) was used to construct a fine map for the *LepR3* gene and a physical map was constructed using the Arabidopsis genome sequence as a reference.

4.3 Materials and Methods

4.3.1 Construction of segregating population

A resistant parent, "Surpass 400", and a susceptible parent, "Westar", were crossed to produce mapping populations. Nine hundred and eight F_2 and 13,644 F_3 plants were inoculated and screened with the isolate 87-41 from PG2 at the cotyledon stage. Total 3,900 plants from F_2 and F_3 were sampled for marker analysis (Table 4.1).

Table 4.1. Number of plants in the F_2 and F_3 generations inoculated and sampled for marker analysis*

Generations	Family	No. of Plants inoculated	Sample No. Each family	Plant No. for marker analysis
F_2	908	908	1	908
F_3	232 R	2784	1 R	232
	*209 S	2916	2 S	486
	*467 Se	7644	2-4 S	2274
F_3 Total		13644		2992

R: resistant S: susceptible Se: segregating

*The susceptible families and segregating families in F_3 had some replications

4.3.2 Preparation of inoculum

Pycnidial inoculum of blackleg PG2 isolate 87-41 was prepared according to the method described by Mengistu et al. (1991). The modifications were as follows. The cotyledons with lesions were hand picked and washed in sterilized distilled water for three times in a laminar flow hood. The cotyledons were then treated with 15% (V/V) bleach for 20 minutes with occasional agitation. After three washes with sterilized water each for 2 minutes, the cotyledons were transferred to Petri dishes with V8 agar medium (250 ml V8 juice, 0.5 g $CaCO_3$ and 15 g granulated agar per liter). The dishes were kept in a temperature and light controlled growth chamber at 21°C and 16 hours of light during the day, followed by 18°C and 8 hours of darkness at night. In about a week, the cotyledons were full of black pycnidia and sometimes pink pycnidiospores were released. The spores were discharged by washing and scraping the agar surface with sterilized glass. The inoculum concentration was adjusted with distilled water to 2×10^7 spores/ml from the stock solution. For each inoculation, a freshly prepared suspension was used.

4.3.3 Phenotype determination of F_2 and F_3 plants by isolate inoculation

The cotyledons were punctured with the leaf puncture forceps. Ten µl of the suspension was dropped on each puncture. The plants were kept at room temperature with light overnight for recovery. Then the plants were grown in a controlled environment growth chamber. In about 12 days, the disease symptoms were fully developed, and the disease severity was rated according to the classification of 0-9 (Williams et al. 1985). Disease severities of 0 to 6 were classified as resistant while 7 to 9 were classified as susceptible with 'Westar', 'Surpass 400' and their F_1 as controls for every inoculation. Three replicates

of F_3 families were done in the case of unclear symptoms in F_2 or F_3 plants.

4.3.4 DNA extraction and SRAP

A modified CTAB extraction procedure as described in Li and Quiros (2001) was used to extract DNA. About 0.2 gram of leaf sample in a 1.5 ml Eppendorf vial was frozen in liquid N_2 for five minutes and then ground to powder with a plastic pestle. Six hundred and fifty millilitre 2 x CTAB extraction buffer（2% CTAB, 1.4 M NaCl, 0.02 M EDTA, 0.1 M Tris）was then added to each tube and the samples were incubated at 65°C for 90 minutes. Six hundred and fifty μl chloroform was added to each vial and the vials were vortexed completely. Vials were spun at 15,000 rpm for 5 minutes. Six hundred μl supernatant was transferred to a new tube and then 400 μl of iso-propanol was added. After being shaken vigorously a few seconds, the vials were spun at 10,000 rpm for 30 seconds. The supernatant was discarded. Five hundred μl 70% ethanol was added to each vial to wash the pellet. The tubes were briefly centrifuged again and the residue solution was pipetted out. The DNA was dissolved in 500 μl distilled water.

SRAP was performed as described in Chapter 2. Five fluorescent dye colors, '6-FAM', 'VIC', 'NET', 'PET' and 'LIZ', were used for signal detection with an ABI 3100 Genetic Analyzer (Applied BioSystems, Foster City, CA, USA). Except for the 'LIZ' color as the size standard, the other four colors were used to label SRAP primers. A pair of primers，one labeled primer as above and an unlabeled primer, with the size of 16-22 nucleotides was used to amplify genomic DNA at low annealing temperature of 35°C for 5 cycles; then the annealing temperature was increased to 50°C for 35 cycles (more stringent conditions). Approximately 50 to100 bands were obtained. Most SRAP markers

were dominant. Some SRAP markers were co-dominant. The SRAP system was used to find the markers linked to the blackleg resistance. Each PCR reaction solution contained 1.5 mM $MgCl_2$, 0.375 mM dNTPs, 0.15 μM each of the labeled and unlabeled primers, and 1 to 5 ng genomic DNA and 1U Taq polymerase in a 10 μl reaction volume. The reaction cycles were 94°C 50 s, 35°C 50 s and 72°C 50 s for 5 cycles, followed by 94°C 50 s, 50°C 50 s and 72°C 50 s for 35 cycles. Two μl of SRAP mixture of blue, green, yellow and red reactions was mixed with 5.5 μl of formamide in a 96 well plate or a 384 well plate. After denaturation, the plate was loaded into an ABI3100 Genetic Analyzer system to run capillary electrophoresis. The data were collected and analyzed by Data Collection software and Genographer software separately.

For the mapping of the disease resistance gene, the ultradense genetic recombination map with 13,551 SRAP markers that was constructed with 58 DH lines from a cross of 'Westar' and 'Zhongyou 821' (Chapter 3) was employed to develop SRAP markers that were linked to the resistance gene. To use this map, the population of 'Westar' and 'Surpass 400' was screened with the same primers as used for the ultradense map construction. Samples from 8 resistant plants and 8 susceptible plants were used to perform SRAP marker analysis first. After a molecular marker was found to co-segregate with disease resistance, 64 resistant plants and 64 susceptible plants were tested to confirm linkage between the SRAP markers and the resistance gene. Once a marker was found to be linked to the resistance gene, it was used to find the corresponding SRAP molecular marker on the ultradense genetic map. After anchoring the linked molecular marker to the resistance gene on the ultradense genetic map, the SRAP molecular markers flanking the anchoring marker were used to test if they are closer SRAP markers.

4.3.5 Marker sequencing, landing on Arabidopsis chromosomes and chromosome walking using the Arabidopsis gene sequence as a reference

SRAP PCR products were separated on sequencing gels. The gels were stained with a silver staining kit (Promega, Toronto). The target markers were identified by comparing the band patterns with the marker patterns that were produced with the ABI 3100 Genetic Analyzer. DNA was eluted as described in Molecular Cloning (Sambrook and Russell, 2001). The gel slices (cut bands) were put into 1.5 ml tubes. Five hundred and fifty µl of DNA elusion buffer (0.5 M ammonium acetate, 10 mM Magnesium acetate tetrahydrate, 1 mM EDTA pH 8.0, 0.1% (V/V) SDS) was added and shaken for 24 to 48 hours at 37°C. Centrifuging was done at 14,000 rpm for 5 minutes to collect the supernatant. Two volumes of 95% ethanol were well mixed with the supernatant. After being left on ice for 10 to 20 minutes, the tubes were centrifuged at 14,000 rpm for 5 minutes to precipitate the DNA. Two hundred µl 70% ethanol was added to wash the DNA. After another centrifugation at 14,000 rpm for 3 min, the supernatant was discarded. The DNA was dried for 10 minutes and dissolved in 30 µl of ddH_2O. The DNA was reamplified by PCR and the PCR products were run in the ABI 3100 Genetic Analyzer. The PCR profile was compared with the original SRAP profile to confirm the correct position of the bands. The confirmed DNA products were sequenced using a BigDye Terminator v3.1 kit (Applied BioSystems, Toronto, Canada).

For PCR product sequencing, the product was diluted 3 to 5 times. Each reaction contained 13% of terminator, 1× reaction buffer, 0.15 µM primer, and 20 to 50 ng of PCR products. The reaction cycles were 94°C 4 min, 94°C 30 s, 50°C 10 s and 60°C 4 min for 25 to 30 cycles. For BAC sequencing cycles, 1 minute denaturation time instead of 30s was

used. The sequencing products were precipitated by adding 2 µl of 125 mM EDTA (pH 8.0) to the bottom of each well and then 20 µl 95% of ethanol to the wall of each well. The plates were centrifuged at 4,000 rpm for 35 min. The pellets were kept and the samples were dried in a Heto Vacuum Centrifuge for 10 minutes and 10 µl formamide was added to each well. After denaturation, the samples were loaded onto the ABI 3100 for sequencing. The data was collected by Data Collection software. The sequences were analyzed by ABI SeqA software.

BLAST analysis of the marker sequences was performed with the TAIR Arabidopsis database. Sequences of some SRAP markers were found to be homologous to Arabidopsis genes or genomic regions. For the SRAP marker sequences that did not match with any sequences in Arabidopsis, PCR walking (Takara. 2004) was implemented to extend their flanking regions until the extended sequences eventually landed on Arabidopsis genes.

Chromosome walking was conducted by screening a *B. rapa* BAC library with the closest SNP marker to the gene. SNP markers in *B. napus* were developed using the Arabidopsis genes in the possible candidate gene region. The Arabidopsis gene coding sequences were used to design primers that were used to amplify the DNA of 'Westar' and 'Surpass 400'. After sequencing these PCR products, SNPs were developed by comparing the two sequences. In addition, some of these primers were also used to screen a *B. rapa* BAC library for selecting the BAC clones anchoring the homologs of these Arabidopsis genes. The end sequences of BAC clones were also used to develop SNPs. These SNPs were in turn used to screen the mapping population. The direction of chromosome walking was set by co-segregation analysis of the SNPs and the plant phenotypes in the mapping population. The gene with the least recombinants was used to find a candidate gene that

shared any similar domain to known disease resistance genes such as NBS and LRR domains.

4.3.6 BAC library construction and screening

The BAC library was constructed with a *B. rapa* male sterile line 'P8-9', following the protocol available at http://www.genome.arizona.edu/agi/. The BAC cloning vector, pCCB1BAC, was purchased from Epicentre (Madison, Wisconsin). Nuclei imbedded in plugs were incubated in lysis buffer (0.5 EDTA pH 9.0-9.3, 1% Na lauryl sarcosine, 1.0 mg/ml proteinase K) at 50°C for 48 hours with gentle shaking. After washing with a Tris-EDTA buffer, the plugs were digested with HindIII restriction enzyme for 5 minutes at 37°C. The reaction was stopped by adding 5 μl EDTA (pH 8.0). The digested plugs were loaded into a 1% low melting point agarose gel and run in the BioRad CHEF-II system (Bio-Rad, Toronto). After 20 hours of running, part of the gel was stained in ethidium bromide solution. The gel with the appropriate DNA size (100 to 300 kb) was excised and loaded onto a fresh gel for a second separation. The DNA in the slices was electroeluted and dialyzed against 0.05 mM Tris-HCl (pH 9.0-9.3). Copy Control pCC1BAC cloning vectors (Epicenter, Toronto) and the DNA fragments were used for ligation. Competent *E. coli* DH10B cells (Invitrogen, Toronto) were used for transformation with a BIO-RAD Gene Pulser (BIO-RAD, Toronto). After transformation, colonies were picked up and put into 384-well plates with a QBot robotic system (Genetix, New Milton, U.K.). PCR-based screening of the BAC library was performed with plate pools, column and row pools using a robotic liquid handling system (Tecan, Toronto).

BAC DNA sequencing was done with an ABI 3100 Genetic analyzer following the ABI Company's protocol.

4.4 Results

4.4.1 The segregation of *LepR3* gene in the population

Table 4.2. Segregation of *LepR3* gene in the F_2 and F_3 populations

Generation	Expected ratio	Resistant plants	Heterozygote	Susceptible Plants	X^2, p-value
F_2	3:1	699		209	0.16
F_3	1:2:1	232	467	209	0.38

The cotyledon phenotypes of the plants were determined according to the 0 to 9 classification (Williams et al. 1985). The parental lines 'Westar', 'Surpass 400' and their F_1 plants were used as controls. The heterozygotes were determined when the F_3 families were segregating in resistance phenotype.

The segregation of the resistance gene in the F_2 and F_3 populations of 'Westar' × 'Surpass 400' showed a 3:1 ratio in the F_2 and a 1:2:1 ratio in the F_3 lines (Table 4.2), indicating that one dominant resistance gene controlled the blackleg resistance in 'Surpass 400'. This is consistent with the previous studies (Yu et al. 2004, Li and Cowling 2003).

4.4.2 Identification of linked SRAP markers and integration on the consensus map

For primer screening, 8 susceptible plants and 8 resistant plants from the mapping population were used to run SRAP molecular markers. 384 primer pairs were used for the initial screening and two SRAP markers R269 and G278 were found to co-segregate with the resistance gene (Figure 4.1) in the 16 plants tested. By comparing these two SRAP markers with the molecular markers on the ultradense genetic recombination map, it was

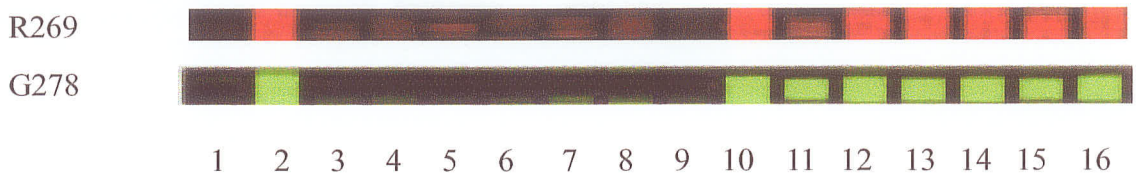


Fig.4.1. Screening of SRAP markers closely linked to *LepR3* and two marker images from the Genographer files. DNA samples were from the segregating F_3 family WS520. Lane1, Westar; 2, Surpass 400; 3 to 9, susceptible plants and 10 to 16, resistant plants.

found that R269 corresponded to a SRAP marker 1217Ar269 on N10 linkage group (see chapter 3), but there was no corresponding SRAP marker on the map to G278. There are 508 SRAP markers on N10 linkage group (Chapter 3). After searching the polymorphism of the SRAP markers flanking 1217Ar269 marker on N10 linkage group with the 'Westar' and 'Surpass 400' segregation population, 210Ay442, 0127Fr382 and 1128BG275 on the map were found to co-segregate with the resistance gene *LepR3* (Fig. 4.2). After the linked SRAP molecular markers were analyzed with 908 F_2 plants and 2,992 F_3 plants, 52 recombinants were found between the marker 0127Fr382 and 1217Ar269 (Table 4.3). SRAP marker 0127Fr382 was the closest molecular marker to the resistance gene, showing a genetic distance of 0.3 cM.

Table 4.3. Markers performance in the recombinant population*

	Primers	Resistance	Em1/pm87	EM1/BG28	DC1/SA17	Me2/PM87	Me2/PM86	DC1/pm88	Em1/pm87	SA12/PM1	Me2/PM87	DC1/SA17
	Markers		1201cg168	B278	32705R381	330	327B266	R269	1201cg174	Y442	327	32705R379
1	Westar	S	B	B	B	B	B	A	A	A	A	A
2	Westar	S	B	B	B	B	B	A	A	A	A	A
3	Surpass	R	A	A	A	A	A	B	B	B	B	B
4	Surpass	R	A	A	A	A	A	B	B	B	B	B
5	753-1	S	A	A	A	B	A	B	B	B	A	B
6	920-1	S	A	A	B	A	A	B	B	B	A	A
7	-2	S	A	A	A	A	A	B	B	B	A	A
8	932-2	S	A	A	A	B	B	B	B	B	A	A
9	964-1	S	A	A	B	B	A	A	A	A	A	A
10	-2	S	A	A	A	B	A	A	A	A	A	A
11	-3	S	A	A	B	A	A	A	A	A	B	B
12	982-1	S	A	A	A	B	A	B	B	B	A	A
13	998-1	S	A	A	B	B	A	B	B	B	A	A
14	-2	S	A	A	B	A	A	B	B	B	A	A
15	1099-1	S	A	A	B	A	A	B	B	A	B	A
16	-2	S	A	A	A	A	A	B	B	A	B	A
17	1141-1	S	A	A	A	A	B	B	B	B	A	A
18	-2	S	A	A	A	A	B	B	B	B	B	A
19	1172-1	S	A	A	A	B	A	B	A	B	A	A
20	1177-1	S	A	A	B	A	B	B	B	B	B	A
21	1181-2	S	A	A	A	B	A	B	B	A	A	B
22	1189-2	S	A	A	B	A	B	B	A	B	A	B
23	-3	S	A	A	A	A	B	B	A	B	A	A
24	1248-1	S	A	A	B	B	B	B	A	B	A	B
25	-2	S	A	A	B	B	A	B	A	B	A	B
26	1260-1	S	A	A	A	A	B	A	A	A	A	B
27	1271-1	S	A	A	A	A	B	B	B	B	A	B
28	-2	S	A	A	B	B	B	B	B	B	A	B
29	-3	S	A	A	A	A	B	B	B	B	A	B
30	-4	S	A	A	A	B	B	B	B	B	A	A

Table 4.3. continued

	Primers	Resistance	Em1/pm87	EM1/BG28	DC1/SA17	Me2/PM87	Me2/PM86	DC1/pm88	Em1/pm87	SA12/PM1	Me2/PM87	DC1/SA17
	Markers		1201cg168	B278	32705R381	330	327B266	R269	1201cg174	Y442	327	32705R379
31	72-1	R	A	A	B	B	B	A	A	A	A	B
32	-2	R	A	A	B	B	A	A	A	B	A	A
33	657-1	R	A	A	B	B	B	A	A	A	A	B
34	-2	R	B	A	B	B	A	A	A	A	A	B
35	685-1	R	A	A	B	A	B	B	A	B	B	B
36	-2	R	A	A	A	A	B	A	A	A	B	B
37	686-1	R	A	A	A	B	A	B	B	B	A	B
38	-2	R	A	A	A	B	A	A	A	A	A	B
39	699-1	R	B	B	B	B	A	A	A	A	A	B
40	731-1	R	A	A	B	B	A	A	A	B	A	A
41	757-2	R	A	A	B	A	B	A	A	A	B	A
42	815-1	R	A	A	B	B	B	B	A	B	A	B
43	-2	R	B	A	B	B	A	B	A	B	A	B
44	872-1	R	A	A	A	B	B	B	B	B	A	A
45	-2	R	A	A	B	A	B	B	B	B	B	A
46	919-1	R	A	A	B	B	A	A	A	A	A	A
47	-2	R	A	A	B	B	A	A	A	A	A	A
48	942-1	R	A	A	B	A	A	B	B	B	A	A
49	-2	R	A	A	B	A	A	B	B	B	A	A
50	969-1	R	A	A	A	B	A	B	B	B	A	B
51	-2	R	B	A	A	A	B	A	A	A	B	A
52	985-1	R	A	A	B	A	A	B	A	B	B	A
53	1049-1	R	A	A	A	A	A	A	A	A	B	B
54	-2	R	A	A	B	A	B	A	A	A	A	B
55	1177-2	R	A	A	B	A	B	B	B	B	B	A
56	1195-2	R	A	A	A	A	A	B	A	B	B	A

* S, susceptible plant; R, resistant plant; A, with a band; B,without a band

Table 4.4. Sequencing of SRAP markers and landing of these markers on the Arabidopsis genes via BLAST and PCR walking

1217Ar269: At5g57830 by PCR walking
ATGGCAACCTTAAAGCGCCTTATCTAAGATCAATTTAGGATGCAAAACTGATC
ACGAAAAGGAAAATCtGTGAGAGTTGGCTTCTTTTATTTCGATATGTCAATGTA
ACGTAACGAGCTCTCTCACTCAGCTGTACAAAGTAAGGAACATGAAATTCAG
TTTGACTTTAGGTCAAAGACTATAGTTGGGTTCAAACCCACTGAGAGAGGTG
AGGTTTCG (PM88)
210Ay442: At5g20510
TTGGGTAGAGAAACTACGTTTGGTCGCTCAACCATGGCGGACACATATCCAA
CAACTCAGCCTTTTTTTTGGTAAACACAACTCAGCCTTTATAAGTCCATTATGC
TTTGTGGTAGAGAGAAACTACTGATAGTTGCAAAATAAACAAAAATAGTTTAT
AAGATGTGTGCAAATAGCAGGTTACTTGAATCAAATGACAACGCAAGTCTATA
GTCGAGGTTTATCTTATATTCCATCTTGTTTGTTTTNCCTTTTTCGTATATTTTAT
CAAAAGGTTATCAGTTTATTTCAGCTTCCAATTTAAAAGATGACACAGTCACN
CAAAGAGGTGGGGGTTATCCAATATATAGGACGTCAAAGACAAAAATTGCTC
AGCATGAGAGGCTGCGTGTCACCAGTCAACCTATT(PM1)
1128BG275:At5g18840
CGGGAACTCCNAGATCTTGTCTCCAAGAAATACAGTCGGTCTGTCATCGTAA
GTGTTTACCTCATAACCAATAGCACAAACTTTTCAATTTTTAAATATATTATTAA
CTTGTGATCAAATATTCTTCAGATNGGTGTTTCCCTTATGGTATTCCAACAGTT
TGTGGGGATCAACGGGATTGAATTCGTACGCAGTC (EM1)
Green278: At5g57345
GCATCTCGCACGGCGCCCAGAGCCCAAACGCTAACCGCTAACGTCGCGATTG
CCAGTTTCGCCAGCTGATTCTCATTTCTCCTAACATATTATATAAATAATTCTTG
AGTTAGGTCAATTACAATTAAAACGGATAAATTTATAGAAAAGTAAAGAATATT
ACCTCGGTCAGATTCGTCTTCATGGTGTGGAGCTTTGCGTTGCATCTGATTGA
ACTCCGACGCCAGTCATTTG

The linked SRAP molecular markers G278, 1217Ar269, 210Ay442, 0127Fr382 and 1128BG275 were sequenced (Table 4.4). After BLAST analysis against the Arabidopsis database (http://www.Arabidopsis.org), the sequence of SRAP marker 1128BG275 was found to have an identity of 91% (Expect = 2e-09) to gene At5g18840 and that of G278 an identity of 88% (Expect = 1e-11) to gene At5g57354 in Arabidopsis, respectively. Unfortunately there was no significant identity between the Arabidopsis genes for the sequences of the remaining three SRAP markers, 210Ay442, 1217Ar269 and 0127Fr382. After extending the sequence of the SRAP marker to its flanking regions through PCR

walking, the flanking sequence of 1217Ar269 with an identity of 93% (Expect = e-119) with the Arabidopsis gene At5g57830. Sequences of marker 210Ay442 had an identity of 65% to the gene At5g20510.

Since the corresponding genes to the SRAP marker sequences were located in two syntenic regions in Arabidopsis (Fig. 4.2), chromosome walking was performed in both regions using the Arabidopsis genome sequence as a reference. Primers were designed from the Arabidopsis gene sequences and were shown in Table 4.5. In the first region, four Arabidopsis genes At5g57035, At5g57345, At5g57670 and At5g57830 were used to design primers and developed SNPs in *B. napus*. The SNPs were used to analyze these 52 recombinants in Table 4.4. The genetic map for these four SNPs showed the same gene order in *B. napus* as in Arabidopsis. Further, the genetic distance of these SNPs to the *LepR3* gene was similar to that of SRAP markers 1217Ar269 and G278 (Fig. 4.2). As markers 1217Ar269 and G278 were not the closest markers on the linkage map, it was confirmed that the *LepR3* gene did not fall into this syntenic region.

When the chromosome walking was done in another region with the genes flanking At5g18840 in Arabidopsis, SNPs developed with the *Brassica* orthologs of these flanking genes were found to be closer to the blackleg resistance gene *LepR3* and the target region covering *Brassica* homologs of At5g13280 and At5g14950 co-segregated closely with the resistance gene. This confirmed that the candidate gene for *LepR3* may be in this region. In total, nine SNPs that were located in nine *Brassica* homologues of Arabidopsis genes were developed (Table 4.5), and their genetic map position appeared to be in the same gene order as in Arabidopsis.

Table 4.5. PCR amplification and SNP development between 'Westar' and 'Surpass 400'*

Genes	Primers	PCR products	SNPs Westar/Surpass400
At5g13280	GAGAGTGAGCCGTACGATTT, WS13280A TGGCAATGCCTTTCAGGAGT, WS13280B	750bp	W: TCCATTGGAGATGGCTCCTCC S: TCC----------TCC
At5g13530	ATGGTTGGGAGAGTCAAGGT, WS13530A TCTGCCTTCATTGCGTTGCA, WS13530B	700bp	W: TCTAACCTTAC S: TCTAAACTTAC
At5g13930	GCTGGTGCTTCTTCTTTGGA, WB01 TAGAGAGGAACGCTGTGCAA, WB03	1150bp	W: TCTCGC(C/A)AAGGAT S: TCTCGCCAAGGAT
At5g13980	GTTTTGCTACTGGGAATCTC, WE9 GGACAACGGGGGAATCATC, WE10	1200bp	W : CAGATATTTGCT S : CAGATTTTTGCT
At5g14060	CCTGTGAGATCATCTGCACA, WS14060E1 CTCGTATGCTTGGCCAATAC, WS14060F1	890bp	W: TACTTTTTTTTGTG S: TAC--TTTTTTTGTG
At5g14210	TCAGACTCAGGTCCTGTATC, WS14210A TTTGCCTCCACAGGTCAGAT, WS14210B	970bp	W: AAACGGCCCC--TTGAACCGG S: AACCTGCCCGGTTGAACCGG
At5g14220	GTTGCAGATA GCAATGGCGT, WE45 AACTTCCCACCTACTCTTCC, WE46	440bp	W: CAGTTTTTTTCTTTTTGATAA S: CAGTTTTTTT--TTTTA--ATAA
At5g14470	GGCGATTTCTGGCAAAGATAA, WS14470A AGATGAGATGCAGAGGAGGA, WS14470B	1020bp	W : CCAAATTTTTGG S: CCAAATTTTTTG
At5g14950	CAGCTAGAGA TTGTTGGAGG, WE41 CCGAGCGAAATCAAACTGAC, WE42	890bp	W: ATGTTTTCAAAACCC S: ATGTTTTCGAAACCC
At5g57035	TCTGTAAACCGTCACACTGT, WS57035B TCCAGTCTCTTTCCTCTGAG, WS57035A	850bp	W: CCTTTTTAACTCTCTTAA S: CCCTTTTGACTCTCTTAA
At5g57345	CAGCTGGCGAAGTTGGCAAT,WS57345A ATAGTCGTCTTCGTCATCTTC, WS57345B	650bp	W: AAGGGCCTTAAAGTTT S: ANGGGCCTTAAGGTTT
At5g57670	CAACACTGCTCTTCTCTTAG, WS57670C TGAGTGGGATTAACCGGTCT, WS57670D	700bp	W: AACGTTCCCTCAACCGG S: AACGTTCCTTCAACCGG
At5g57830	ACATGCGTTGGTGTCACAAG,WS57830C ATGGAAAACTAGAACCACGC, WS57830D	850bp	W: TATCCGAAGAAGAGGAAAAT S: TCTCCGAAGAAGAGGAACAT

*W, 'Westar; S, 'Surpass 400'. Primers are from 5' to 3'.

Primers designed with the gene sequence of At5g13280, At5g13930 and At5g14950 in Arabidopsis were used to screen a *B. rapa* BAC library. The primers from At5g13930 were used to screen the library and a BAC clone A48M23 was selected. PCR products amplified from the BAC clone A48M23 were obtained with the primers designed with the sequences of At5g13840, At5g13930, At5g13950 and At5g13980 in Arabidopsis. The sequences of the PCR products were homologous to the sequences of these genes in Arabidopsis. The gene positions on this *B. rapa* BAC clone suggested that the gene order in this syntenic region would be the same as that in Arabidopsis.

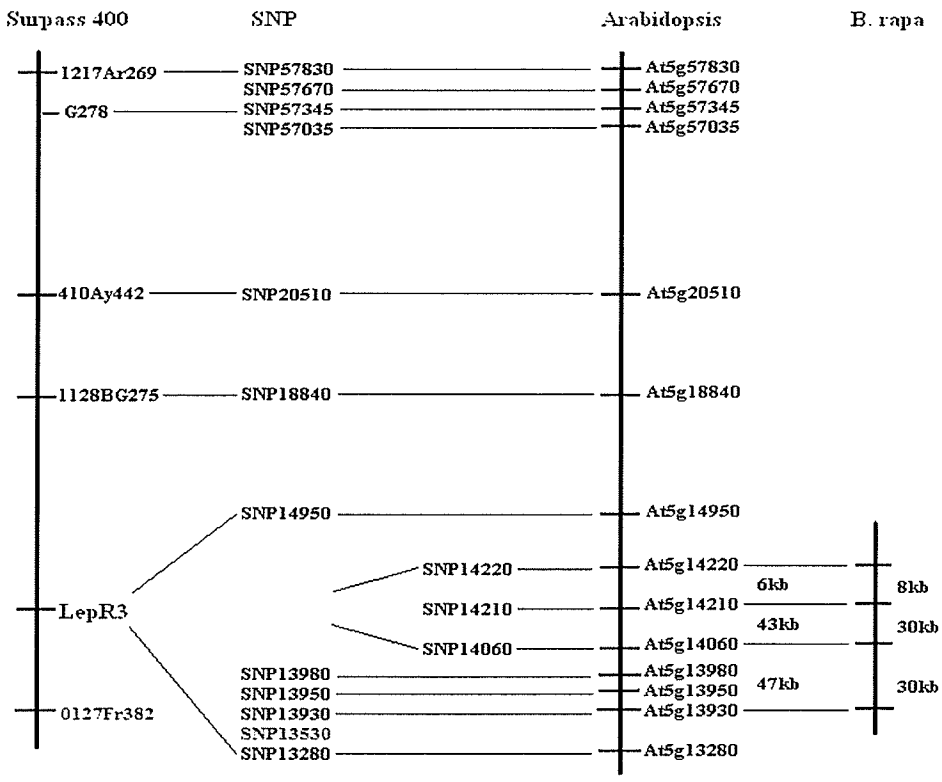


Fig. 4.2. Genetic mapping of blackleg resistance gene *LepR3*, SNP development, and candidate gene identification using Arabidopsis and *B. rapa* gene sequences as a reference

4.4.3 Identification of the candidate gene

SNP13930, SNP14060, SNP14210 and SNP14220 were developed with Arabidopsis genes At5g13930, At5g14060, At5g14210 and At5g14220, respectively (Table 4.6). These four SNPs showed no recombination in these 52 recombinants (Table 4.6), indicating that they were very close to the resistance gene *LepR3* (Fig. 4.2). After searching the Arabidopsis genome in a region about 1,060kb in length (At5g13280 - At5g16000), At5g14210 was found to be the only leucine-rich repeat transmembrane protein kinase, suggesting that the orthologous gene of At5g14210 is a good candidate for the blackleg resistance gene *LepR3* in 'Surpass 400'.

Table 4.6. SNP performance in the recombinant population*

Lines	Phenotype	SNP 13930	SNP 14060	SNP 14210	SNP 14220	SNP 57035	SNP 57345	SNP 57830
753-1	S	N	C	G	C	N	A	C
920-1	S	N	C	G	C	A	A	C
920-2	S	N	C	G	C	A	A	C
932-2	S	N	C	G	C	N	A	C
964-1	S	N	C	G	C	A	N	A
964-2	S	N	C	G	C	A	N	A
964-3	S	N	C	G	C	N	G	A
982-1	S	N	C	G	C	N	N	C
998-1	S	N	C	G	C	A	G	C
998-2	S	N	C	G	C	A	A	C
1099-1	S	N	C	G	C	A	N	C
1099-2	S	N	C	G	C	N	A	C
1141-1	S	N	C	G	C	N	A	C
1141-2	S	N	C	G	C	N	A	C
1172-1	S	N	C	G	C	A	A	C
1177-1	S	N	C	G	C	A	G	C
1181-2	S	N	C	G	C	N	A	C
1189-2	S	N	C	G	C	N	A	C
1189-3	S	N	C	G	C	A	A	C
1248-1	S	N	C	G	C	N	N	C
1248-2	S	N	C	G	C	A	N	C
1271-1	S	N	C	G	C	A	A	C
1271-2	S	N	C	G	C	A	A	C
1271-3	S	N	C	G	C	A	N	C
1271-4	S	N	C	G	C	A	N	C
1260-1	S	N	C	G	C	N	A	A
72-1	R	C	T	T	T	A	G	A
72-2	R	C	T	T	T	A	G	A
657-1	R	C	T	T	T	N	A	A
657-2	R	C	T	T	T	N	G	A
685-1	R	C	T	T	T	N	G	C
685-2	R	C	T	T	T	N	G	A
686-1	R	C	T	T	T	A	N	C
686-2	R	C	T	T	T	A	A	A
699-1	R	C	T	T	T	A	A	A
731-1	R	C	T	T	T	A	A	A
757-1	R	C	T	T	T	N	N	C
815-1	R	C	T	T	T	N	A	C
815-2	R	C	T	T	T	N	A	C
872-1	R	C	T	T	T	A	A	C
872-2	R	C	T	T	T	A	G	C
919-1	R	C	T	T	T	A	G	A
919-2	R	C	T	T	T	A	N	A
942-1	R	C	T	T	T	A	G	C
942-2	R	C	T	T	T	A	N	C
969-1	R	C	T	T	T	A	A	C
969-2	R	C	T	T	T	A	N	C
985-1	R	C	T	T	T	N	A	C
1049-1	R	C	T	T	T	A	A	A
1049-2	R	C	T	T	T	A	A	A
1177-2	R	C	T	T	T	N	N	C
1195-2	R	C	T	T	T	A	N	C

* A, T, G and C, nucleotides; N, a mixture of two nucleotides; R, resistant plants; S, susceptible plants.

4.5 Discussion

Since an ultradense genetic map constructed with over 13,551 SRAP markers is available (see chapter 3), a strategy of anchoring a SRAP marker linked to the disease resistance gene *LepR3* on the ultradense map was employed in this study. The results demonstrated that the ultradense map is very useful for map-based gene cloning. Although the gene tagging population for the blackleg disease resistance was different from the mapping population, some of the SRAP markers on the ultradense genetic recombination map also showed polymorphism in the *LepR3* gene mapping population. This allowed anchoring the SRAP markers from other populations, such as the SRAP marker R269 from the 'Westar' × 'Surpass 400' population. Using the anchored SRAP marker as a starting point, flanking SRAP markers on the ultradense map were used to pinpoint the region that contained the gene of interest. In this report, a closely linked SRAP marker with a genetic distance of 0.3 cM was easily found when the SRAP markers on the ultradense genetic map were used to do co-segregation analysis with the disease resistance gene *LepR3*.

Arabidopsis whole genome sequences should be beneficial to the gene cloning in *Brassica* crops which are closely related species to Arabidopsis. Broad conserved syntenic regions at the genome level and the gene linearity between Arabidopsis and *Brassicas* allows the use of gene order in Arabidopsis to find the candidate gene controlling the trait in *Brassica*. Since the conserved sequences between Arabidopsis and *Brassica* are mostly located in open reading frame regions, it is important for a marker system to detect gene regions. As reported by Li and Quiros (2001), nearly half of the SRAP markers were

located inside the genes. Consistent with previous results, three of five sequenced SRAP markers in this study corresponded to Arabidopsis annotated genes which facilitated the chromosome walking with the Arabidopsis genome sequence as a reference. There were two syntenic regions that were inferred with the BLAST analysis of the sequences of SRAP markers on the ultradense map against the corresponding genes in Arabidopsis. Co-segregation analysis of SNP markers and the recombinant population showed that only the region with SRAP markers closely linked to the disease resistance gene, such as the region flanked by 1128BG275 and 0127Fr382, was the correct one that may contain the candidate gene for *LepR3*.

Plant disease resistance genes share similar protein structures and domains and can be classified into different groups. In rice, there are two bacterial blight disease resistance gene loci *Xa21* and *Xa26* containing several members of the LRR receptor-like kinase (Song et al. 1995, Sun et al. 2004). In this study, after finding the SNP markers that completely co-segregate with the disease resistance gene *LepR3* among the whole population with 3,900 individuals, a search for *R* gene-like proteins was carried out in a region flanking these completely linked markers. Among over 100 genes, there is only one gene At5g14210 in Arabidopsis that has an *R*-gene structure and this gene was just located in the region that is used for finding the closely linked SNP markers. The results supported that the ortholog of At5g14210 in *B. napus* may be the correct candidate for the disease resistance gene *LepR3*. At5g14210 encodes a receptor-like kinase that has the leucine-rich repeats (LRR) and transmembrane domains.

The strategies for map-based gene cloning have been used for a few decades. However, these strategies are different from plant to plant and even lab to lab. Yahiaoui et

al. (2003) cloned genes from hexaploid wheat by referencing the BAC information of diploid and tetraploid wheat. For Arabidopsis, a standard method for map-based cloning was proposed by Jander et al. (2002). The method is to develop SNP markers between the two flanking markers by using the known Arabidopsis gene sequences. As we want to clone the gene from *B. napus* that does not have enough gene sequence information for cloning purposes, it is important to refer to the sequence information from Arabidopsis and *B. rapa*. Arabidopsis, with syntenic regions to *B. napus*, can provide the entire genome sequence information for our cloning. As *B. napus* is composed of A and C genomes, the availability of *B. rapa* sequence information from the internet is also critical. The alignment of *B. napus* and Arabidopsis sequences will reduce errors when SNPs are developed and chromosome walking performed. Comparative physical mapping between these genomes is the key step for chromosome walking and for identification of the candidate gene region because the *B. napus* genome has a lot of repeated sequences and rearrangements, making chromosome walking more difficult. As for disease resistance genes, searching for the genes with resistance gene structures or domains in the Arabidopsis gene pool will assist in the candidate identification. In this study, using the Arabidopsis gene sequences, we first developed some SNPs in a large target region, such as SNP13280, SNP13530, SNP13930, and SNP14950. We found that SNP13930 co-segregated with the resistance gene. Further development of SNPs around the At5g13930 region and the search for *R* gene structures led to the location of the candidate gene 14210 for *LepR3*.

Chaper 5

Development of high throughput detection molecular markers for blackleg (*Leptosphaeria maculans*) resistance genes in 'Quinta' and 'Glacier' (*Brassica napus*) for marker assisted selection

5.1 Abstract

'Quinta' and 'Glacier' are two important winter type canola cultivars from Europe. 'Quinta' has two independent blackleg resistance gene loci, *Rlm1* and *Rlm4*; 'Glacier' has two other genes, *Rlm2* and *Rlm3*. This study focuses on mapping the resistance genes *Rlm1* and *Rlm3* using high throughput SRAP markers and automated SNP development. The F_2 and F_3 populations were constructed with crosses of 'Westar' × 'Quinta' and 'Westar' × 'Glacier'. The populations were screened with the corresponding isolates. The disease segregation in the populations suggested that a dominant resistance gene controls the resistance to PG3 isolates in 'Quinta' and similarly, a specific resistance gene controls the blackleg resistance to the PG2 isolates. A SRAP marker was developed for the gene in 'Quinta' by using 256 pairs of primers and screening 2,500 polymorphic loci. B342 is a closely linked SRAP marker to the dominant *Rlm1* gene with a genetic distance of 1.5 cM. This marker was also used to screen the 'Westar' × 'Glacier' population and found to be linked to a resistance gene in 'Glacier' with a genetic distance of 6.8 cM. SNP primers were designed from the genes in Arabidopsis corresponding to the flanking regions of *LmR1* and *CLmR1* in *B. napus*. SNP80870 was found to be linked to the resistance gene *Rlm3* in 'Glacier' with a genetic distance of 3 cM. The resistance gene in 'Glacier' is *Rlm3* as the SNP marker was developed from the linkage group N7. The resistance gene in 'Quinta' is *Rlm1* or a gene close to *Rlm1*. Three populations for gene pyramiding were constructed. Three close markers for the related

resistance genes (*Rlm1*, *Rlm3* and *LepR3*) could be used for marker assisted gene pyramiding.

Key words: Blackleg, resistance gene, markers, mapping, pyramiding, *B. napus*

5.2 Introduction

Blackleg, caused by *L. maculans*, is one of the major diseases in *Brassica*. In order to understand the *Brassica* – *L. maculans* pathosystem, extensive studies have been performed on avirulence genes, resistance genes and their interactions during last two decades. Differential interactions were evaluated with a cotyledon inoculation test at seedling stage of the host plants (Williams and Delwiche 1979). The pathogen was divided into pathogenicity groups, including PG2, PG3, and PG4 according to the interactions with the differential cultivars, 'Westar', 'Glacier', and 'Quinta' (Mengistu et al. 1991, Koch et al. 1991). The PG groups were re-grouped into A1 to A6 (Badawy et al. 1991, Balesdent et al. 2001) with the addition of 'Lirabon' and 'Jet Neuf' as differential cultivars. PGT was added to this differential system on the basis of the unpublished survey from 1998 to 2000 (Rimmer 2006). Blackleg epidemics are caused mostly by PG2 and PG3 worldwide, by PG4 in Europe and by PGT in Canada. Sixteen resistance genes to blackleg have been genetically inferred by using isolates with the corresponding avirulence genes and these resistance genes were mapped in different sources, such as *B. napus, B. rapa, B. juncea* and Arabidopsis (Ferreira et al. 1995, Mayerhofer et al. 1997, Pilet et al. 1998, Rimmer et al. 1999, Zhu et al. 2003, Delourme et al. 2006, Rimmer 2006, Staal et al. 2006).

At least five resistance genes were mapped on linkage group N7 via genetic studies of segregating populations. As the European winter type cultivars 'Quinta' and 'Glacier' were first used for the study of specific blackleg-rapeseed interactions, a gene for gene model for PG3-'Quinta' and PG2-'Glacier' was analysed (Ansan-Melayah et al. 1995, 1998). Two resistance genes, *Rlm1* in 'Quinta' to PG3 and *Rlm2* in 'Glacier' to PG2, were inferred. Also another gene to PG2 in 'Quinta' was proposed. *Rlm1* controlling seedling resistance to PG3 isolates in 'Shiralee', 'Quinta' and 'Maxol' was mapped on the DY10 linkage groups (Mayerhofer et al. 1997, Ansan-Melayah et al. 1998, Delourme et al. 2004, Mayerhofer et al. 2005). *Rlm4* in 'Quinta' was linked to *Rlm1* (Delourme et al. 2004) and the genetic distance was about 19 cM between these two genes. *Rlm2* in 'Glacier' and 'Samourai', controlling seedling resistance to PG2 isolates in these cultivars, was on linkage group LG16 (Delourme et al. 2004). *Rlm3* in 'Glacier', 'Columbus' and 'Maxol' showing resistance to PG2 (Balesdent et al. 2002) was linked to the field resistance *Rlm1* as a QTL locus (Delourme et al. 2004). The genetic distance is 26 cM between the two loci. *Rlm3* was also mapped on the DY10 linkage group (Delourme et al. 2004). At least five resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9*) in a cluster were located on the DY10 linkage group (Delourme et al. 2004). Yu et al. (2005) and Mayerhofer et al. (2005) found that linkage group DY10/LG10 was an equivalent to N7 (Ferreira et al. 1995, Parkin et al. 1995).

Some other blackleg resistance genes corresponding to unknown avirulence genes were also identified and mapped on linkage group N7. *LmR1* from 'Sheralee' and *CLmR1* from 'Cresor' were mapped on the N7 linkage group (Mayerhofer et al. 2005). *CRLMm* from 'Maluka', *CRLMrb* from RB87-62, and *cRLMj* from DH88-752 were all resistant to

pl 86-12 and were mapped on LG6 that belongs to linkage group N7 (Rimmer 2006). As the avirulence genes in these isolates were not genetically confirmed, the resistance genes in these cultivars need to be verified if they are *Rlm4* or other genes.

These gene mappings were based on different marker systems, RFLP, RAPD and SSR in different labs and these mapped genes were from cultivars with different genetic backgrounds. It is difficult to determine if they are the same or different genes from the different maps even though they were all mapped on N7. In this study, we used SRAP and automated SNPs to map the resistance genes in 'Quinta' and 'Glacier', respectively. Closely linked markers were developed for two different blackleg resistance genes in 'Quinta' and 'Glacier'. These markers will be used for marker assisted selection in breeding and for gene pyramiding.

Marker assisted gene pyramiding is intended to integrate specific genes and/or QTLs for the trait(s) into a single genotype by marker assisted selection (Johan 2003). The advantages of this strategy are increasing line development efficiency (Dudley 1993, Lee 1995) and simultaneously targeting multiple genes (Singh et al. 2001) that could not be easily followed by traditional breeding methods. Pyramiding of Mendelian disease resistance genes into a single cultivar is proposed to be an effective way to develop improved resistance to diseases (Schaffer and Roelfs 1985). The pyramided genes in a cultivar might reduce the probability that a pathogen recombinant can overcome all the resistance genes simultaneously (Huang et al. 1997). Three kinds of resistance gene pyramiding have been used in crops. Firstly, gene pyramiding included more than two traits or pathogens. In hybrid rice, genes *Bt*, *Xa21* and *Xa7* were pyramided in the restorer line, 'Minghui 63', and *Pi1, Pi2, Qbp1* and *Qbp2* were pyramided in the maintainer line

'Zhenshan 97'. These lines increased the resistance of hybrid rice, cultivar 'Shanyou 63', to bacterial blight, blast, stem borer and brown plant hopper (He et al. 2004b). The *Xa21* gene (resistance to bacterial blight), the *Bt* fusion gene (for insect resistance) and the chitinase gene (for tolerance of sheath blight) were combined in a single rice line. Plants carrying all these genes showed resistance to bacterial blight and a high tolerance to sheath blight disease (Datta et al. 2002). This kind of disease resistance gene pyramiding is very effective because there is no inhibition between the resistances to the pathogens. The second kind of pyramiding combined specific resistance with quantitative resistance QTLs. In rice, NIL-*Gn1* increases grain number and NIL-*Ph1 (sd1)* reduces plant height. The new line carrying *Gn1* and *Ph1 (sd1)* was identified using MAS. The QTL pyramided line with the check cultivar 'Koshihikari' genetic background and NIL-*Gn1+Ph1 (sd1)*, increased the grain production by 23% and reduced the plant height by 20% compared with 'Koshihikai' (Ashikari et al. 2006). This kind of gene pyramiding is also very effective to the target traits as multiple genes were involved in the pyramiding. The third kind is to pyramid only specific resistance genes to the same pathogen. BPH (*Nilaparvata lugens Stal*) is a significant insect pest of rice (*Oryza sativa L.*). Two BPH resistance genes *Bph1* and *Bph2* (Brown plant hopper: BPH) were combined through the recombinant selection with 6 PCR-based markers. The pyramided line showed equal resistance to that of the *Bph1*-single introgression line (Sharma et al. 2004). As several genes resistant to the corresponding isolates were pyramided into the new lines, these lines may be resistant to the different isolates from the same pathogen.

Factors, such as marker distance, generations, population size and heritability are the main elements to be considered when using marker assisted selection. Linkage of markers to a gene is the key factor for MAS. Studies showed that closely linked markers for a target gene could provide more accurate selection results (Dudley 1993, Edwards and Page 1994, Hospital et al. 1997, Moreau et al. 1998, Spelman and Bovenhuis 1998). This is true when there is only one marker for QTL selection. More markers will decrease the efficiency, but increase the accuracy of gene selection. For a major gene, the flanking markers used can increase the selection efficiency (Tanksley 1983). The more closely linked markers are used, the more efficient of selection (Li et al. 2006). Selection in early generations is effective for genetic markers and can save the time and costs that are spent in later generations. Population size is also a key factor for MAS selection (Edwards et al. 1994, Gimelfarb et al. 1994a, b, 1995, Hospital et al. 1997, Whittaker et al. 1997, Zhang and Smith 1992, 1993). A larger population will increase the efficiency of MAS (Moreau et al. 1998). For quantitative traits MAS appears to be most interesting for low heritability. For an unlimited population, the computer simulation showed that the ideal heritability for quantitative traits is 0.3 to 0.4 for MAS ((Hospital et al. 1997, Moreau et al. 1998, Liu et al. 2005). For qualitative traits, such as disease resistance caused by specific resistance genes, the heritance is very high and the marker assisted selection is very effective.

In this study, in order to pyramid three specific resistance genes, two way and four way hybridizations were made for cultivars with different resistance genes. Disease inoculation and marker screening were combined to increase the efficiency of selection.

5.3 Materials and methods

5.3.1 Construction of segregating population

The seeds from *B. napus* cultivars 'Westar', 'Glacier', and 'Quinta' were planted in Metro-Mix in flats. The plants were grown in a temperature and light controlled growth chamber with 16 hours of light at 21°C, followed by 8 hours of darkness at 19°C. The plants were then transferred to 15 cm Jiffy pots at the stage of 3 to 5 true leaves and kept in the greenhouse until they were harvested. The F_1 seeds from the crosses, such as 'Westar' × 'Glacier' and 'Westar' × 'Quinta', were obtained by pollinating 'Westar' flower stigmas with 'Glacier' and 'Quinta' pollen, respectively. The F_2 seeds were obtained by selfing the F_1 plants in selfing bags. The F_2 plants were bagged and selfed to produce F_3 seeds. The F_3 plants were screened with the appropriate blackleg isolate at the cotyledon stage and selfed to produce F_4 seeds for later use.

5.3.2 Preparation of *L. maculans* inoculum

The pycnidiospores were collected from the infected cotyledons. The procedure was described in chapter 4. A suspension of 2×10^7 spores/ml was used for inoculation. Freshly prepared suspension was used for each inoculation.

5.3.3 Phenotype determination for F_2 and F_3 by isolate inoculation

The F_2 and F_3 plants were inoculated separately with blackleg isolates at the cotyledon stage in order to phenotype the segregating populations. The cotyledons were punctured with forceps. Ten µl of the suspension was dropped on each puncture. The

plants were kept at room temperature with light overnight for recovery. Then the plants were grown in a controlled growth chamber. In 12 days, the disease symptoms were fully developed, and the disease severity was rated according to the classification of 0 to 9 (Williams 1985). Disease severity 0 to 6 was classified as resistant and 7 to 9 as susceptible. Each time, the parental lines and their F_1 were used as controls. Two to 3 time replications in F_3 were needed in the case of unstable symptoms. In this study, isolates 'pl 86-12' from PG2 and 'Lifolle 6' from PG3 were used for the populations 'Westar' × 'Glacier' and 'Westar' × 'Quinta', respectively.

5.3.4 DNA extraction

A modified CTAB extraction procedure as described in Li and Quiros (2001) was used to extract DNA (See Chapter 4).

5.3.5 SRAP and PCR

SRAP is a PCR-based marker system developed by Li (2001). A pair of primers with the size of 16 to 22 nucleotides was used to amplify genomic DNA at low annealing temperature (35°C) for 5 cycles; then annealing temperature was increased to 50°C (more stringent condition) for another 35 cycles. Approximately 50 to 100 bands were obtained. Most markers were dominant and some were co-dominant. SRAP was used to screen the markers linked to blackleg disease resistance. Each PCR reaction solution contains 1.5 mM $MgCl_2$, 0.375 mM dNTPs, 0.15 μM each for the labelled and unlabeled primers, and 1 to 5 ng genomic DNA and 1 U Taq polymerase in 10 μl reaction.

For specific PCR, the reaction solution contained the same components as for SRAP. The reaction cycles are 94°C 50 s, 50-60°C 50 s and 72°C 50 s for 35 cycles. The picture of the bands in the agarose gel was taken from the BIO-RAD Gel Doc 2000.

5.3.6 Marker detection by ABI 3100 DNA Analyzer

The marker detection by ABI 3100 was fully described in chapter 4.

5.3.7 SNP development

Ten genes on Arabidopsis chromosome 1 were targeted to design primers for SNP development (Table 5.2). The background information was described (Parkin et al. 2005). The 10 disease resistance-like genes are close to the markers identified for *LmR1* and *CLmR1*. These genes on chromosome 1 of Arabidopsis correspond to a region on N7 linkage group in *B. napus*. There is a possibility that the candidate resistance gene for *LmR1* from 'Shiralee' is among these genes or genes close to this interval. As a few disease resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7,* and *Rlm9*) have been mapped on this linkage group, development of markers from this region is a shortcut for the marker development for these resistance genes.

The primers designed from each gene were used for PCR amplification of 'Westar', 'Glacier', 'Quinta' of *B. napus*, 'RI16' of *B. rapa* and '453' from *B. oleracea*. The PCR products were diluted 3 to 5 times and 2 µl was used as the sequencing template. The SNPs were detected by comparing the sequences of 'Westar', 'Glacier', 'Quinta', 'RI16' and '453'. The SNPs were then used to screen the segregating populations. The SNP markers and the SRAP markers developed were used for linkage analysis.

5.3.8 DNA sequencing

For sequencing, PCR product was diluted 3 to 5 times. Five μl reaction mix contained 1 μl terminator, 1× reaction buffer, 0.25 μl 10 μM primer, and 20 to 50 ng of PCR products. The reaction cycles were 94℃ 4 min, 94℃ 30 s, 50℃ 10 s and 60℃ 4 min for 25 to 30 cycles. For BAC sequencing cycles, we used denaturation time of 1 minute. The sequencing products were precipitated by adding 2 μl 125 mM EDTA (pH8.0) to the bottom of each plate well and then 20 μl 95% ethanol to each plate well. The plate was sealed with aluminium film and centrifuged at 4,000 rpm for 40 minutes. Immediately the film was pulled off the plate, and the plate was loaded inversely and centrifuged up to 1,000 rpm for 10 seconds. Twenty μl 70% ethanol was added to each well and the plate was centrifuged at 4,000 rpm for 20 minutes and then the inverted plate centrifuge was repeated. The plate was dried in Heto Vacuum Centrifuge for 10 minutes and 10 μl formamide was added to each well. The sample in the plate was denatured at 95°C for 5 minutes and then cooled on ice for 10 minutes. The plate was loaded into an ABI 3100 Genetic Analyzer for sequencing. The data was collected by Data Collection software. The sequences were analyzed by SeqA software.

5.3.9 Disease resistance mapping

The DNA samples from 8 resistant plants and 8 susceptible plants were used to run SRAP markers first. After finding a few close markers, the sample number was increased to 32 to 32, or more to screen the populations by the close markers. Once a marker was confirmed to be closely linked to the resistance gene, it was used to map the linked

markers from the consensus map. Mapmaker (V2.0) was used to generate the linkage map with LOD 4.0.

5.3.10 Construction of pyramiding populations

Cultivars with different resistance genes, 'Surpass 400', 'Quinta', 'Glacier' and 'Cresor', were selected for crossing. Two- and four-way crossing was adopted. For the two-way crossing, the F_1 was selfed to obtain the segregating population that was used for pyramided gene screening. For the four-way crossing, the two F_1s were crossed with each other. The double hybrid was then used for selfing. The resulting population was used for screening of disease resistance genes in pyramiding and marker assisted selection.

5.4 Results

5.4.1 The segregation of *Rlm* genes in the populations

Table 5.1. Segregation of resistance genes in the population*

Population	Generation	Ratio	No. of plants			X^2	P
			Resistant	Segregation	Susceptible		<0.05
Westar×Glacier	F_2	3:1	117		37	0.1168	3.841
	F_3	1:2:1	30	67	35	0.4242	3.841
Westar×Quinta	F_2	3:1	238		70	0.8485	3.841
	F_3	1:2:1	48	104	57	0.7798	3.841

*For 'Westar×Glacier', cotyledon inoculations were conducted with the pycnidiospore suspension of isolate pl 86-12 at 2×10^7. 'Westar×Quinta' progenies were inoculated with isolate Lifolle 6. The disease ratings followed the 0-9 scales. Ratings 0-6 were resistance and 7-9 were susceptible. Each F_3 family had 12 plants inoculated. The heterozygous family in F_3 were determined if there was phenotypic segregation.

Seedling resistance in *B. napus* cultivars to blackleg was assayed via cotyledon

inoculation under controlled environmental conditions using *L. maculans* isolate (McNabb et al. 1993). To investigate the specific seedling resistance in 'Glacier' and 'Quinta', the segregation populations of 'Westar' × 'Glacier' and 'Westar' × 'Quinta' were constructed. The plants in the populations were inoculated with a PG2 isolate 'pl 86-12' and a PG3 isolate 'Liffole 6', respectively, under growth chamber conditions.

'Glacier' and 'Quinta' were both resistant to 'pl 86-12' when they were inoculated with the isolate. However, 'Quinta' was resistant to the PG3 isolate 'Lifolle 6' while 'Glacier' was susceptible to this isolate.

For the populations of 'Westar' × 'Glacier', screened by the PG2 isolate 'pl 86-12', the parent 'Westar' was completely susceptible, F_1 and the other parent 'Glacier', resistant and the plants in the populations segregated in resistance phenotype. Segregation in the F_2 generation showed a 3:1 ratio of resistant and susceptible plants. The F_3 generation showed a 1:1:1 ratio of resistant, segregating and susceptible plants (Table 5.1). Most F_3 plants showed corresponding phenotypes to their F_2 plants. Segregation of both the populations suggested a specific dominant resistance gene model.

For the population 'Westar' × 'Quinta', screened by the PG3 isolate 'Lifolle 6', the parent 'Westar' showed complete susceptibility, F_1, intermediate resistance and the other parent 'Quinta', resistance. The plants in the populations segregated in resistance phenotype. Segregation in F_2 showed a ratio of 3 resistant to 1 susceptible and a 1:2:1 ratio for the number of resistant, segregating and susceptible plants in F_3 (Table 5.1). The phenotypes of most F_3 plants showed agreement to the corresponding F_2 plants. Segregation of both the populations suggested a specific dominant resistance gene model.

5.4.2 SRAP marker development

Screening of SRAP molecular markers were started by running 'Westar' × 'Quinta' population with 8 susceptible plants to 8 resistant plants. Two hundred and fifty-six primer pairs were used for the initial screening. About 2,500 polymorphic markers were found. One of these markers, B342, was found to co-segregate with the resistance gene in 'Quinta'. Forty-eight plants from the populations were used to test this marker. This marker was still closely linked to the plant resistance. After searching the consensus linkage mapping group N7, this marker could not be found on the map (see chapter 3). The marker B342 was used to screen the phenotypes in the population. After all the 132 F_3 plants were screened with this marker, it showed a genetic distance of 1.5 cM (3/209) to the resistance gene (Fig.5.1, Table 5.3).

For the 'Glacier' resistance gene, the marker, B342, was used to screen the F_2 and F_3 generations of 'Westar' × 'Glacier'. There were 9 recombinants from a total population of 132 F_3 plants, indicating that the marker is still 6.8 cM away from the resistance gene in 'Glacier' (Fig.5.1).

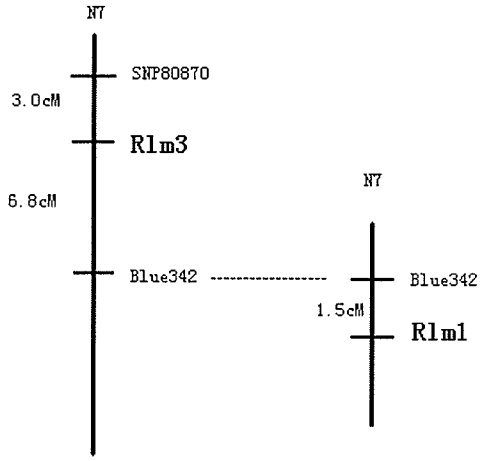


Fig. 5.1. Localization of *Rlm1* linkage group from 'Westar' × 'Quinta' and *Rlm3* linkage group from 'Westar' × 'Glacier'. Blackleg resistance genes *Rlm1* from 'Quinta' and *Rlm3* from 'Glacier' were both mapped on the N7 linkage group. Numbers at left side show the genetic distance between the markers and the genes in centiMorgans.

5.4.3 SNP development

Table 5.2. PCR amplification and SNP development between 'Westar', 'Quinta' and 'Glacier'

Genes	Primers	PCR products	SNP sequence positions*
At1g80870	GAACCATCCCTTTCGACGTT, WB26 AGCTAAATTCTCCCGCTGCT, WB27	1,300bp	W: CTTTCT Q and G: CCTTTT
At1g80670	TGCTTGGAAAGATGATGGAAC, WB18 CGGGTGGAAATTCAGAGAGT, WB19	1,100bp	
At1g80680	AGGTTTGGGAGATCCCGTAG, WB20 TGAACTGTCGGGAGGTAGATG, WB21	1,400bp	
At1g80630	GCGTTCGCTCTACTTTCGTC, WB22 TTATGCCCGTACATCTGCTG, WB23	1,150bp	
At1g80640	TCCGTTGTCTACGTTTCTGG, WB24 CCAAAGATCCATTCTGCATCA, WB25	1,000bp	
At1g79640	CCAATTGGACCAGAGCATTA, WB28 TGAGCAAGCTCTAGCCCAGT, WB29	1,100bp	
At1g79670	TCCCTTTTTCATCACCGACT, WB30 CGAGACAACACCCCATGAGT, WB31	1,300bp	
At1g79680	AGAATCCAAGGGATGCAAAG, WB32 CAGCAATACGAAGACGCACT, WB33	1,200bp	
At1g79620	GCTCTGCGTTCTTTGATGG, WB34 TGTCAACTGATTATCCGCAAG, WB35	850bp	
At1g79600	GAAGAACTTGCAGAGCTTCAG, WE15 CTTCAGAGCATAGTAATCTCG, WE16	960bp	

* W, 'Westar'; Q, 'Quinta' and Q, 'Glacier'. Primer sequences are from 5' – 3'. At1g79600 to At1g80870 are Arabidopsis genes.

The primers were designed to amplify the disease-resistance-like genes At1g79600 to At1g80870 from Arabidopsis associated with linkage group N7. One SNP was found in At1g80870 by comparing the sequences of 'Westar', 'Glacier' and 'Quinta'. In other gene sequences, a SNP was not found (Table 5.2).

SNP80870 was used to screen the two populations 'Westar' × 'Glacier' and 'Westar' × 'Quinta' (Table 5.3). It showed closer linkage to the resistance gene in 'Glacier' than to the resistance gene in 'Quinta'. It had only 4 recombinants in 'Westar' × 'Glacier' populations (132), showing a genetic distance of 3.0 cM to the resistance gene in 'Glacier' (Fig5.1). However, SNP80870 did not show any linkage to the gene in 'Quinta' (31 recombinants in 49 plants, data not shown).

5.4.4 Comparative mapping of resistance genes from 'Quinta' and 'Glacier'

As the two genetic maps had a consensus marker Blue342, these two genes both were mapped on the same linkage group. Comparative mapping of these two genes from 'Quinta' and 'Glacier' showed that they were both in the N7 region and had a loose linkage of 11.3 cM between each other. Marker SNP80870 was closely associated with the gene from 'Glacier'. Blue342 was more closely linked to the Rlm1 resistance gene from 'Quinta' with a distance of 1.5 cM, while it was loosely linked to the gene *Rlm3* from 'Glacier' in a distance of 6.8 cM (Fig. 5.1).

Table 5.3. Phenotype of the population and the SNPs*

NO.	Line	Resistance	B342	SNP80870	NO.	Line	Resistance	B342
1	Westar	S	B	N(C/T)	1	Westar	S	B
2	Glacier	R	A	T	2	Quinta	R	A
3	WG3	S	A	N	3	WQ3	S	A
4	WG5	S	B	N	4	WQ7	S	B
5	WG6	S	A	N	5	WQ9	S	B
7	WG8	S	B	N	6	WQ12	S	B
8	WG9	S	B	N	7	WQ14	S	B
9	WG10	S	B	T	8	WQ38	S	B
10	WG11	S	B	N	9	WQ73	S	B
11	WG12	S	B	N	10	WQ84	S	B
12	WG13	S	B	N	11	WQ90	S	B
13	WG21	S	B	N	12	WQ94	S	B
15	WG120	S	B	N	13	WQ96	S	B
16	WG154	S	B	N	14	WQ360-1	S	B
17	WG157	S	A	N	15	WQ366-1	S	B
18	WG158	S	A	N	16	WQ370-1	S	B
19	WG160	S	B	N	17	WQ376-1	S	B
20	WG190	S	B	N	18	WQ377-1	S	B
21	WG193	S	A	T	19	WQ381-1	S	B
22	WG196	S	A	N	20	WQ382-1	S	B
23	WG204	S	B	T	21	WQ390-1	S	B
24	WG380	S	A	N	22	WQ391-1	S	B
25	WG402	S	B	N	23	WQ396-1	S	B
26	WG417	S	A	N	24	WQ4	S	B
27	WG31-1	S	B	N	25	WQ5	S	B
29	WG54-1	S	B	N	26	WQ6	S	A
32	WG65-1	S	B	T	27	WQ10	S	B
6	WG7	R	A	T	28	WQ11	S	B
14	WG47	R	A	T	29	WQ402-1	S	B
33	WG15-2	R	A	T	30	WQ403-1	S	B
34	WG23-2	R	A	T	31	WQ411-1	S	B
35	WG24-2	R	A	T	32	WQ108-1	R	A
36	WG25-2	R	A	C	33	WQ27-1	R	A
37	WG33-1	R	A	T	34	WQ125-1	R	A
28	WG49-1	R	A	N	35	WQ385-1	R	A
30	WG59-1	R	A	T	36	WQ386-1	R	A
31	WG61-1	R	A	C	37	WQ387-1	R	A
38	WG163-1	R	A	T	38	WQ22	R	A
39	WG164-1	R	A	T	39	WQ23	R	A
40	WG166-1	R	A	T	40	WQ40	R	A
41	WG168-1	R	A	C	41	WQ50	R	A
42	WG151-1	R	A	T	42	WQ52	R	A
43	WG415-1	R	A	T	43	WQ64	R	A
44	WG94	R	B	T	44	WQ85	R	A
45	WG104	R	A	T	45	WQ101	R	A
46	WG105	R	A	T	46	WQ103	R	A
47	WG110	R	A	T	47	WQ109	R	A
48	WG15-1	R	A	T				

* WG, 'Westar' × 'Glacier'; WQ, 'Westar' × 'Quinta'; A, with a band; B, without a band; R, resistant; S, susceptible

5.4.5 Chromosome walking

Chromosome walking was initiated with two primers, WB26 and WB27, which amplified a clear band from the gene At1g80870 from 'Westar', 'Quinta' and 'Glacier', and from the *B. rapa* BAC library. One plate pool from the *B. rapa* BAC library, A55 was positive for the amplification. A55 was selected for row and column screening with the same primer pair. The BAC clone, A55E4 (about 48 kb), was verified to contain At1g80900 at one end by BAC end sequencing and At1g79600 at the other end (Fig.5.2). This clone was used as the first BAC clone for chromosome walking.

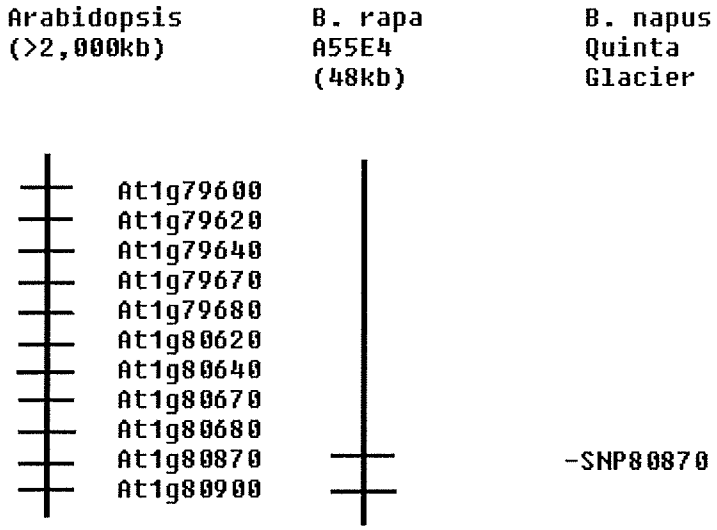


Fig 5.2. Comparative mapping of genes in the N7 linkage group. Primers from the Arabidopsis genes At1g79600 to At1g80900 were designed for PCR amplification in *B. napus*. The primers WB26 and WB27 amplified a clear band from 'Westar', 'Quinta' and 'Glacier', thus being used for the screening of the BAC library. The BAC clone A55E4 was identified as the first clone for chromosome walking.

5.4.6 Construction of pyramiding populations

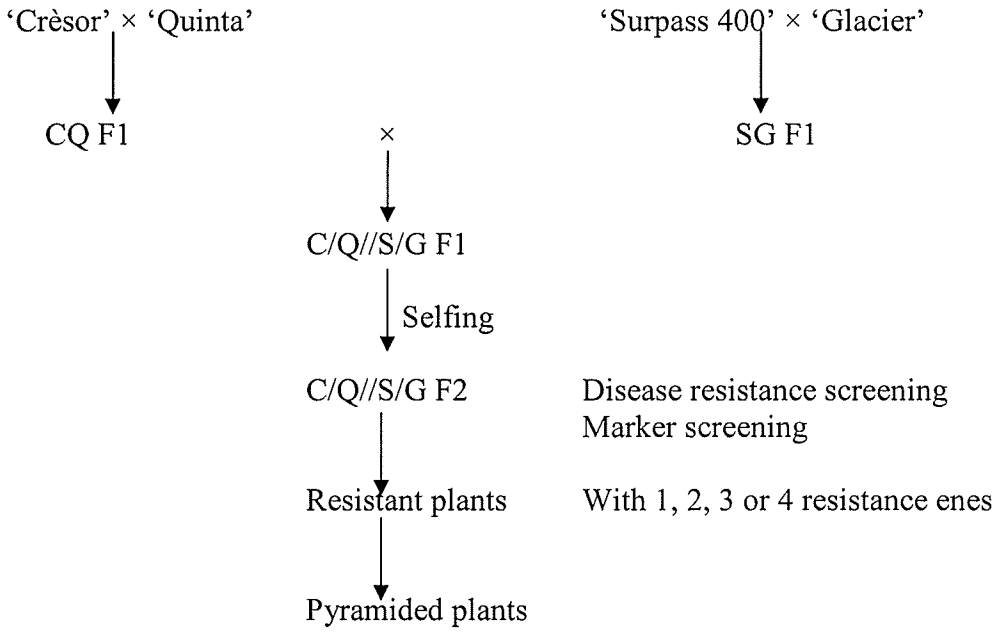


Fig. 5.3. Gene pyramiding procedure 1. 'C/Q//S/G' refers to 'Crèsor/Quinta//Surpass 400/Glacier'.

Figure 5.3 shows the proposed gene pyramiding procedure. The population was constructed with four cultivars, 'Cresor', 'Quinta', 'Surpass 400' and 'Glacier'. For the first round crossing, parental lines of both spring and winter type were used in order to reduce the time for vernalization for the F_1. The F_1s of the two combinations were then crossed to obtain the F_1 (C/Q//S/G) of the four way crossing. After selfing this F_1, we obtained the seeds of CQSG F_2 for the segregating generation. The F_2 plants were used for disease screening first in the growth chamber. The cotyledon inoculation was conducted with the corresponding isolates. The plants showing resistance to every individual gene, two genes, and three genes were selected for DNA extraction. The DNA samples were used for marker selection of these genes. By combining the results of

inoculation and marker selection, plants with a single gene (presumably *Rlm1*, *Rlm3*, or *LepR3*), two genes, and three genes will be selected. After selfing, these plants were challenged by the corresponding isolate(s) and the lines with these three genes will be identified. These lines could also be challenged with some other isolates corresponding to some other resistance genes, such as *CLmR1*. A set of differential lines was obtained and the pyramided lines with better resistance were obtained as candidate cultivars or breeding materials.

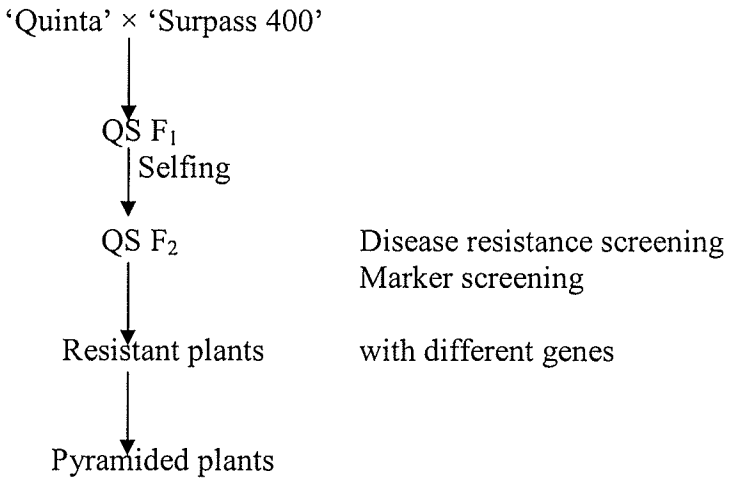


Fig. 5.4. Gene pyramiding procedure 2. 'QS' refers to 'Quinta' × 'Surpass 400'.

Figures 5.4 and 5.5 showed the gene pyramiding procedure for two additional populations. The population of 'Quinta' × 'Surpass 400' and 'Glacier' × 'Quinta' was constructed. For the population 'Quinta' × 'Surpass 400', resistance gene *Rlm1 and Rlm4* from 'Quinta' and *LepR3* from 'Surpass 400' were the target genes. In another population 'Glacier' × 'Quinta', resistance genes *Rlm1, Rlm2, Rlm3* and *Rlm4* were the genes for pyramiding. Similarly as the first cross, the F_1 plants were selfed. The F_2 plants at the cotyledon stage were inoculated with the corresponding isolates and screened by the

markers for each gene. The plants with different gene and gene combinations were selected. A set of differential lines were obtained and the pyramided lines with better resistance were obtained as candidate cultivars or breeding materials.

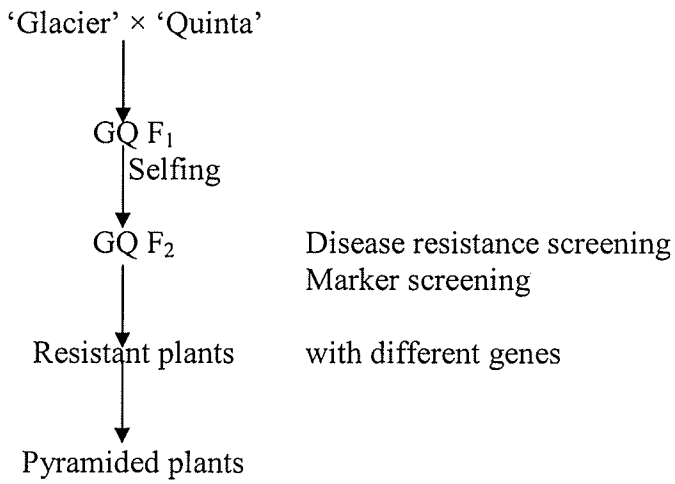


Fig. 5.5. Gene pyramiding procedure 3. 'GQ' referes to 'Glacier' × 'Quinta'.

5.5 Discussion

In this study, we have shown that the resistance to blackleg was conferred by two loci each from 'Quinta' and 'Glacier'. Segregation data from both generations indicated that one dominant resistance gene controlled blackleg resistance in 'Glacier' to the PG2 isolate. As the same isolate used by Keri (1999) was used for the same population, the results in this study were consistent with the previous ones. The isolate used in this study was different from that with which the *Rlm2* and *Rlm3* were defined (PWH245 and IBCN79). *Rlm3* was mapped on the linkage group DY10 (Delourme et al. 2004) that was determined to be the same as N7 (Yu et al. 2005) while *Rlm2* was on the linkage group DY16. The resistance gene from 'Glacier' in this study was also linked to a marker

SNP80870 developed from N7. Therefore, the resistance gene located in 'Glacier' in this study could be *Rlm3*. More evidence (Mayerhofer et al. 1997, Delourme et al. 2004, Mayerhofer et al. 2005) showed that a few resistance genes showing resistance to different isolates from PG2, PG3 and PG4 groups were mapped on the linkage group N7. In a gene cluster, *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* were located in a 35 cM interval on linkage group DY10/N7 (Delmorme et al. 2004). *Rlm1*, *Rlm4* to *Rlm3* are 26 cM and 7 cM apart, respectively, in the background of 'Quinta', 'Darmor' and 'Maxol'. In this study, the resistance gene from 'Quinta' was 11.3 cM to *Rlm3* in the 'Quinta' and 'Glacier' background. These two genes were linked by a consensus marker B342 that was linked to a marker SNP80870 developed from N7. As in 'Quinta', *Rlm1* was determined to show resistance to PG3 isolates while *Rlm4* was determined to be resistant to the isolate 21.3.01 (A5). The resistance gene in 'Quinta' in this study was also resistant to the PG3 isolate. It was presumed that the resistance gene from 'Quinta' in this study was *Rlm1* or a close locus to *Rlm1*, not *Rlm4* even though the resistance gene in 'Quinta' in this study seems to be positioned close to *Rlm4* according to the original map (Delourme et al. 2004). As to the difference of genetic distance between *Rlm1* and *Rlm3* in this study and in other studies (Delourme et al. 2004), the difference in genetic background could be the major cause. Similar results were also obtained for *LmR1* in 'Shiralee' and *CLmR1* in 'Cresor' (Mayerhofer et al. 2005).

Nine resistance genes to blackleg have been mapped on the N7 linkage group. *Rlm1, Rlm3, Rlm4 Rlm7* and *Rlm9* were defined with the avirulence genes confirmed by tetrad studies (Delourme et al. 2004). Other genes such as *LmR1, CLmRm, CRLMrb* and *cRLMj* correspond to unknown avirulence genes. As there are no commonly accepted

rules for designation of these genes, it is difficult to distinguish them. Further, the different genetic backgrounds also appear to affect the relative positions of these genes on the maps. Additionally, these maps could not be shared among labs. Comparative fine mapping and physical mapping will reveal how many different resistance genes are located in this region. In this study, SNP80870 was developed from the flanking regions of *LmR1* and *CLmR1*. As SNP80870 was linked to *Rlm3*, there is a possibility that *LmR1* and *CLmR1* are *Rlm3*.

Marker assisted gene pyramiding has the advantage of increasing line development efficiency (Dudley 1993, Lee 1995) and in targeting multiple genes simultaneously (Singh et al. 2001) that could not easily followed by traditional breeding methods. In this study, we developed two markers for two blackleg resistance genes via high throughput SRAP and automated SNP development. These genetic markers combined with disease inoculation made the selection of pyramided disease resistance gene genotypes much more efficient. However, both the inoculation of plants in each generation and the extraction of a large number of DNA samples for marker screening are tedious jobs even with a robot system. The cotyledon inoculation test combined with marker assisted selection will identify resistant plants with several resistance genes in a short time. This will save time and costs normally spent in the later generations.

Closely linked markers will also increase the efficiency for disease resistance breeding and marker assisted selection. For a major gene, the flanking markers used can increase the selection efficiency (Tanksley 1983). The markers developed in this study could be used in a gene pyramiding program. Marker B342 for the resistance gene locus

Rlm1 in 'Quinta' is 1.5 cM away from the gene. This marker can be used as a major one for *Rlm1* gene selection. The marker SNP80870 is 3.0 cM to *Rlm3*. It can be used either alone or combined with B342 to follow *Rlm3* that targets to the PG2 isolates. The population size can be decreased when the two flanking markers for each gene are used for the selection. As a consensus marker of *Rlm1* and *Rlm3*, B342 can target both genes in one population. This will increase the efficiency of selection. These molecular markers could be very useful for marker assisted disease resistance gene pyramiding.

A major challenge in developing improved blackleg resistance by pyramiding the important resistance genes from different cultivars is that the resistance genes were overcome by the changes of pathogens in some regions, such as the three major resistance genes derived from *B. rapa* ssp. *sylvestris* and *B. juncea* were overcome by *L. maculans* in France and Australia (Li et al. 2003, Rouxel et al. 2003a, Sprague et al. 2006). Race-specific resistance genes in breeding are facing potential risks (Johnson et al. 1984, Parlevliet et al. 1983, van der Plank et al. 1963, 1978) and could be easily overcome by a mutation in the pathogen (Parlevliet and Kuiper 1977). The solution for this challenge is to find new resistance genes for blackleg and to pyramid resistance genes into a cultivar. Wild *Brassicas* could be one of the sources used to identify some novel resistance genes for pyramiding. Most Arabidopsis lines are resistant to blackleg. Arabidopsis may have different resistance genes from *Brassicas*. Identifying novel blackleg resistance genes from Arabidopsis may provide great support for the canola blackleg resistance breeding.

The main goal of disease resistance gene pyramiding is to produce cultivars with improved resistance to blackleg in the field. As a global disease for canola, blackleg has a few pathogenicity groups devastating to the canola industry. As three blackleg resistance genes were overcome by the changes of blackleg pathogen, control of this disease is more urgent. As other disease management methods are not efficient for this disease, breeding of new genotypes with improved resistance is the only feasible way. In this study, specific blackleg resistance genes were combined into one line. These genes showed resistance to some of the isolates in PG2, PG3 and PG4. This kind of disease resistance gene pyramiding will reduce the great yield loss caused by blackleg disease. As several genes are involved in the resistance, this kind of resistance may be improved and last longer than when only one or two resistance genes are deployed. Furthermore, resistance QTLs are more important to gene pyramiding as they are controlled by multiple genes that could not be overcome by the pathogen simultaneously. The field resistance QTL from 'Maxol' was mapped to the *Rlm1* locus (Delourme et al, 2004). 'Samourai' carrying a resistance QTL was mapped to the *Rlm2* position. These QTLs may link to the related specific resistance genes or some other quantitative disease resistance genes. These QTLs adopted in breeding may increase the resistance of these lines.

Differential lines are very useful in discriminating the different resistance genes from the plants and the different avirulence genes from the pathogen. During the procedure of gene pyramiding, a set of differential lines with different blackleg resistance genes will be obtained. These lines will be used for the differential study of resistance. For example, the genes mapped on N7 could be differentiated by these lines. Disease resistance genetic

studies between the isolates and the differential lines as well as new plant germplasm may lead to the identification of new resistance genes.

Chapter 6

General Discussion

Consensus genetic linkage maps among related cultivated plant species are useful. They can provide information about genome organization and evolution through comparative mapping. Genetic maps can also assist in genetic studies of agronomic traits of interest through the localization of specific genes and QTLs. Further, they can identify linked genetic markers for marker assisted selection in breeding programs. An ultra-density genetic map would be very useful for the above studies and applications. Such a map should have a saturated marker distribution over all chromosomes for use with every possible trait and for use in determining involved multi-gene traits.

In *Brassica*, a few genetic maps with very low marker density have been reported. The most dense map previously reported by Parkin et al. (2005) was for the oilseed rape species *B. napus*, and consisted of 1,317 RFLP markers. This map was successfully used to compare the *Brassica* A and C genomes with the Arabidopsis genome. This map was very useful for the evolutionary studies of the A and C genomes through comparative mapping. Another consensus map reported by Lombard (2001) consisted of 540 markers distributed over 19 linkage groups and covered 2,429 cM. This map and the Parkin et al. (2005) map have relatively low abundance of markers (< 0.5 marker/cM), including RFLP, RAPD and SSR or AFLP markers for different reasons. The low marker density on linkage groups limits the application of these maps. In this study, an ultradense genetic map for *B. napus* was

constructed with 13,551 SRAP markers distributed over the N1 - N19 linkage groups. N1 - N10 corresponds to the A genome while N11 - N19 corresponds to the C genome. As the map covers 1,604.8 cM, its marker density reached 8.44 markers per cM on average. Since the genome size of *B. napus* is approximately 1,200 Mb, there was one SRAP marker in every 89 kb physical distance on average. This ultra-dense genetic map is the most saturated map in *Brassicas*. Beside marker density, this map was also aligned with other genetic maps via commonly used SSR markers for each linkage group, making it comparable to other reported maps. Even more importantly, since this map was constructed with SRAP markers, researchers can use endless SRAP primer combinations to produce numerous new markers for the map. The ready availability of SRAP markers will facilitate increasing a very level of the density of this map to saturation, such as more than 1 marker for 5 kb. The application of this map in *Brassicas* should be very broad.

Genetic maps for different traits including blackleg resistance were constructed using the anchoring markers from this ultradense map. A few blackleg resistance genes in *B. napus* have been mapped onto two linkage groups, N10 and N7 by other researchers. Three resistance genes, *Rlm2*, *LepR2* and *LepR3*, were mapped on LG N10, according to the previous studies (Yu et al. 2004, 2005; Delourme et al. 2004). On the current map, N10 contains 508 markers that span 55.5 cM, with the highest density of 9.2 markers per cM. In this study, an anchoring marker was developed for the *LepR3* resistance gene. A genetic map was constructed using markers from N10. This map allowed the identification of a few closely linked markers and finally the

candidate gene for *LepR3*. For another important linkage group N7, at least five resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9*) were reported to be located on the group (Delourme et al. 2004). Except *Rlm1* that was mapped in a different region of N7, the other four genes were believed to be closely linked in a cluster. Marker saturation is needed for this linkage group to differentiate these genes. On the current ultradense genetic map, N7 was the largest linkage group that spans 134.4 cM genetic distance with 976 markers. The marker density on this linkage group was 7.6 markers per cM. This is much higher than any previously reported marker density for N7. In this study, the N7 consensus linkage group was used to find close markers for *Rlm1* and *Rlm3* for marker assisted selection and to distinguish these genes by fine mapping. Two maps were constructed with SRAP markers and SNP markers developed separately with alternative strategies for the resistance genes *Rlm1* and *Rlm3*. The close anchoring markers for these genes were not found in this study due to time limitations. More closely linked markers for *Rlm1* and *Rlm3* will be found by screening the two populations with the markers on N7 linkage group. These two maps could be linked to the consensus map by testing the markers on the consensus map. Consequently, the N7 linkage group is important since many resistance genes were mapped on it. A comparative mapping study using N7 could reveal the relationship among these genes. Because these genes are linked with each other in a cluster covering a genetic distance of 35 cM, identification of one gene will help identify the other genes. A further screening for SRAP markers on N7 is needed.

A number of marker systems are available for marker development and genetic

mapping. RFLPs are codominant, but are expensive for detection and use isotope or fluorescently labeled probes. RAPD markers are inexpensive, but they are dominant markers and that are not very reliable. AFLPs are highly reliable, reproducible and less expensive, but too many steps for detection restricts its usage for MAS. SSR markers have been used for genetic linkage mapping. However, the high cost of initial isolation and characterization limits the application of SSR in many plant species. In recent years, the automation of single nucleotide polymorphism (SNP) has made this marker system a promising method even though it is more expensive than other systems. In our lab, the SRAP system used for genetic mapping has proved to be a high throughput marker system known for its ease of use and automation capability. SRAP and SNP are the main marker systems used in our lab.

Closely linked markers for three resistance genes, *LepR3, Rlm1* and *Rlm3* were developed for marker assisted gene pyramiding and gene cloning via different strategies. Markers for *LepR3* from 'Surpass 400' were screened by SRAP and two markers, R269 and G278, co-segregated with the resistance gene. R269 was found to correspond to the marker 1217Ar269 on the consensus map. *LepR3* was mapped on the N10 linkage group using the marker 1217Ar269 as the anchoring marker. New markers, 210Ay442, 1128BG275 and 0127Fr382 were found to be much closer to the gene and 0127Fr382 was only 0.3 cM for *LepR3*. Eventually, a SNP marker named SNP14210 was developed directly from the candidate gene. The strategy used here is to find closely linked markers based on the high throughput SRAP marker system and the high density genetic map. These markers should be very useful in MAS and

further gene cloning. For the second gene *Rlm1*, a similar strategy was used as for the *LepR3* gene marker development. After screening the population with SRAP markers for the resistance gene from 'Quinta', marker B342 was found to co-segregate with the gene. The search for markers on the consensus map produced no corresponding marker to B342. A small genetic map was generated with the most closely linked marker B342 at a genetic distance of 1.5 cM. This marker should also be very useful in MAS. A different strategy was used for the development of markers for the gene *Rlm3* in 'Glacier'. SNPs were developed using the resistance gene sequences from Arabidopsis flanking *LmR1* from 'Sheralee' and *CLmR1* from 'Cresor'. SNP80870 was linked to *Rlm3* at a distance of 3.0 cM. Additionally, B342 was also linked to *Rlm3* with a genetic distance of 6.8 cM. These two markers could be used for MAS of *Rlm3*.

Pyramiding of disease resistance genes is one of the breeding methods for producing cultivars with improved resistance. Pyramiding resistance genes to different pathogens could also be effective because each resistance gene will be effective on its corresponding pathogen. Cultivars with pyramided genes can show expected resistance to several pathogens simultaneously. Pyramiding of specific resistance genes and quantitative resistance QTLs to the same pathogen could increase the resistance of the cultivars, since the pyramided resistances are controlled by multiple genes. Pyramiding of specific resistance genes to the same pathogen can produce genotypes/lines with resistance to the different isolates of the pathogen. The resistance may last longer time as a few resistance genes could not be overcome at the

same time. In this study, we planned to pyramid three to five blackleg resistance genes, from 'Surpass 400', 'Cresor', 'Quinta' and 'Glacier'. First, what kind of resistance should a new cultivar have? As PG2, PG3, and PG4 are becoming epidemic worldwide and PGT in Canada, a new cultivar should show resistance to most of these PGs. Second, what are the important genes for pyramiding? *Rlm1* shows resistance to PG2 and PG3 isolates, *Rlm2* shows resistance to PG2 and *LepR3* to PG2 too. Other resistance genes show different resistance to different isolates. It is difficult to tell which gene is more important than the others. It is also very challenging to pyramid these genes while some of the genes have been overcome by the changes in the pathogen. Three major resistance genes (*LepR3, Rlm1* and *Rlm6*) derived from *B. rapa* ssp. *sylvestris* and *B. juncea* were overcome by *L. maculans* in France and Australia (Spraque et al. 2006). To identify some new sources with novel resistance genes for pyramiding could be a way to control the disease. However, pyramiding the resistance QTLs and important specific resistance genes may produce new cultivars with improved resistance that may last longer.

In the last two decades, 14 blackleg resistance genes in *Brassica* have been mapped, but none of them have been identified or cloned. The relatively large genome, rearrangements and repeat sequences in the *Brassica* genomes presents a lot of difficulties for positional cloning of genes from *Brassica*. The strategies for map based gene cloning have been used for several decades. However, these strategies are slightly different from plant to plant and even lab to lab. In wheat, gene cloning from hexaploid wheat refers to the BAC information of diploid and tetraploid wheat. For

Arabidopsis, a standard method for map based cloning was proposed to use Arabidopsis sequence information. For *B. napus*, no standard cloning strategy is available. By referring to the complete sequences of Arabidopsis and partial sequence of *B. rapa*, SNPs can be developed in the region flanking the resistance gene. Chromosome walking becomes less important if SNP development is successful. In this study, the first candidate gene for *Brassica* was identified through our modified strategy. Three key steps are very important to the identification of the candidate gene. First, the construction of an ultradense genetic map helped to map the resistance gene *LepR3*. Using an anchoring marker on the consensus map for *LepR3,* mapping was performed. Since the genetic map was high density, a few closely linked markers were found from the map and the gene position on the map was finely located. This facilitated the identification of gene region. Second, a BLASTn strategy helped to land the SRAP markers on the Arabidopsis chromosomes. By sequencing the markers from the high density genetic map, alignment of these sequences and the sequences from Arabidopsis and *B. rapa* helped land these markers on the Arabidopsis genes. The distribution of these genes on the Arabidopsis chromosome and the mapping order of these markers indicated the direction of chromosome walking and estimated the distance of chromosome walking required. Third, SNP development by referring to the Arabidopsis gene sequences was used to find the region of the candidate gene. In the target region, SNPs were developed with the primers designed according to the Arabidopsis gene sequences. Cosegregation analysis of these SNPs using the recombinant population showed that the candidate gene was in this region. Fourth, the

resistance genes with commonly shared structures are useful to find the candidate gene. The gene At5g14210 in the target region is a leucine-rich repeat transmembrane protein kinase that fulfills the hallmarks of a disease resistance gene. This candidate gene could be the first resistance gene found in *B. napus*. It will be very useful in the study of resistance of *Brassica* and in blackleg resistance breeding.

Chapter 7

LITERATURE CITED

Anderson PA, Lawrence GJ, Morrish BC, Ayliffe MA, Finnegan EJ, Ellis JG (1997) Inactivation of the flax rust resistance gene M associated with loss of a repeated unit within the leucine-rich repeat coding region. Plant Cell 9(4):641-651

Anon. (2001) Descriptions *Brassica napus var. oleifera* Canola. 'Varola 50' syn 'Surpass 400'. Plant Varieties J. 14:36-37

Ansan-Melayah D, Balesdent MH, Buée M, Rouxel T (1995) Genetic characterization of *avrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathology 85:1525-1529

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

Arthur L (1996) Blackleg of Canola, Biology and Management PP:1024 http://www.ag.ndsu.edu/pubs/plantsci/crops/pp1024w.htm

Ashikari M, Matsuoka M (2006) Identification, isolation and pyramiding of quantitative trait loci for rice breeding. Trends Plant Sci. 11(7):344-50

Badawy HMA, Hoppe HH, Koch E (1991) Differential reactions between the genus *Brassica* and aggressive single spore isolates of *L. maculans*. J. Phytopathology 15:109-119

Balesdent MH, Attard A, Kuhn ML, Rouxel T (2002) New avirulence genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology 92:1122-1133

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

Banerjee D, Zhang XC, Bent AF (2001) The leucine-rich repeat domain can determine effective interaction between *RPS2* and other host factors in Arabidopsis *RPS2*-mediated disease resistance. Genetics 158:439-450

Bechtold N, Jaudeau B, Jolivet S, Maba B, Vezon D, Voisin R, Pelletier G (2000) The maternal chromosome set is the target of the T-DNA in the in planta transformation of *Arabidopsis thaliana*. Genetics 155:1875-1887

Bendahmane A, Querci M, Kanyuka K, Baulcombe DC (2000) *Agrobacterium* transient expression system as a tool for the isolation of disease resistance genes: application to the *Rx2* locus in potato. Plant Journal 21(1):73-81

Bittner-Eddy PD, Crute IR, Holub ED, Beynon JL (2000) *Rpp13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. The Plant Journal 21(2):177-188

Botella MA, Parker J, Frost, LN, Bittner-Eddy PD, Beynon JL, Daniels MJ, Holub EB and Jones JDG (1998) Three genes of the Arabidopsis *RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. Plant Cell 10: 1847-1860

Bohman S, Staal J, Thomma BPHJ, Wang M, Dixelius C (2004) Characterisation of an Arabidopsis-*Leptosphaeria maculans* pathosystem: resistance partially requires camalexin biosynthesis and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J 37:9-20

Bowers JE, Abbey C, Anderson S, Chang C, Draye X, Hoppe AH, Jessup R, Lemke C, Lennington J, Li ZK, Lin YR, Liu CC, Luo LJ, Marler BS, Ming RG, Mitchell SE, Qiang D, Reischmann K, Schulze SR, Skinner DN, Wang YW, Kresovich S, Schertz KF, Paterson AH (2003) A high-density genetic recombination map of sequence tagged sites for sorghum, as a framework for comparative structural and evolutionary genomics of tropical grains and grasses. Genetics 165:367-386

Brommonschenkel SH, Frary A, Tanksley SD (2000) The broad-spectrum tospovirus resistance gene *Sw-5* of tomato is a homolog of the root-knot nematode resistance gene Mi. Molecular-Plant-Microbe-Interactions 13(10):1130-1138

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A (2002) The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. PNAS 99 (14):9328-9333

Brumby J (2006) Blackleg disease in canola on the hop. Media Release August 9. http://www.legislation.vic.gov.au/domino/Web_Notes/newmedia.nsf/

Budak H, Shearman RC, Parmaksiz I, Dweikat I (2004a) Comparative analysis of seeded and vegetative biotype buffalo grasses based on phylogenetic relationship using ISSRs, SSRs, RAPDs, and SRAPs. Theor Appl Genet 109:280-288

Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, Riordan TP, Dweikat I (2004b) Molecular characterization of buffalo grass germplasm using sequence-related amplified polymorphism markers. Theor Appl Genet 108:328-334

Buschges R, Hollricher K, Panstruga R (1997) The barley *mlo* gene: a novel control element of plant pathogen resistance. Cell 88 (5):695-705

Cai D, Kleine M, Kifle S, Harloff HJ, Sandal NN, Marcker KA, Klein-Lankhorst RM, Salentijn EMJ, Lange W, Stiekema WJ, Wyss U, Grundler FMW & Jung C (1997) Positional Cloning of a Gene for Nematode Resistance in Sugar Beet. *Science* 275: 832-834

Catherine F, Gabriele S, Beat K (1997) Molecular cloning of a new receptor-like kinase gene encoded at the *Lr10* disease resistance locus of wheat. The Plant Jonural 11(1):45-52

Chen Y, Fernando WGD (2005) First report of canola blackleg caused by pathogenicity group 4 of *Leptosphaeria maculans* in Manitoba. Plant Dis. 89:339

Chen Y, Fernando WGD (2006) Prevalence of pathogenicity groups of *Leptosphaeria maculans* in Western Canada and North Dakota, USA. Can J. Plant Pathol. 28:533-539

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

Christianson JA, Rimmer SR, Good AG, Lydiate DJ (2006) Mapping genes for resistance to *Leptospherea maculance* in *Brassica juncea*. Genome 49:30-41

Cloutier S, McCallum B, Feuillet C, Keller B, Jordan MC (2007) Cloning and characterization of wheat leaf rust resistance gene *Lr1*. January 13-17. Plant & Animal Genomes XV Conference. Town & Country Convention Center, San Diego, CA P:64

Collins N, Drake J, Ayliffe M, Sun Q, Ellis J, Hulbert S, Pryor T (1999) Molecular characterization of the maize *Rp1-D* rust resistance haplotype and its mutants. Plant Cell 11(7): 1365-1376

Cooley MB, Pathirana S, Wu HJ, Kachroo P, Klessig DF (2000) Members of the Arabidopsis *HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell 12:663-676

Crouch JH, Lewis BG, Mithen RF (1994) The effect of A genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans*. Plant Breed 112:265–278

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826-833

Datta K, Baisakh N, Thet KM, Tu J, Datta SK (2002) Pyramiding transgenes for multiple resistances in rice against bacterial blight, yellow stem borer and sheath blight. Theor Appl Genet 106(1):1-8

Delourme R, Pilet-Nayel ML, Archipiano M, Horvais R, Tanguy X, Rouxel T, Brun H, Renard M, Balesdent MH (2004) A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Phytopathology 94:578-583

Delourme R, Chèvre AM, Brun H, Rouxel T, Balesdent MH, Dias JS, Salisbury P, Renard M, Rimmer SR (2006) Major Gene and Polygenic Resistance to *Leptosphaeria maculans* in Oilseed Rape (*Brassica napus*). European Journal of Plant Pathology 114(1): 45-52

Dixon MS, Hatzixanthis K, Jones DA (1998) The tomato *Cf-5* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. Plant Cell 10:1915-1925

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K, Jones JDG (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451-459

Dudley JW (1993) Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Sci. 33:660-668

Edwards MD, Page NJ (1994) Evaluation of marker-assisted selection through computer simulation. Theor. Appl. Genet. 88:376-382

Feltus FA, Wan J, Schulze SR, Estil JC, Jiang N, Paterson AH (2004) An SNP resource for rice genetics and breeding based on subspecies *indica* and *japonica* genome alignments. Genome Res. 14:1812-1819

Fernando WGD, and Chen Y (2003) First report on the presence of *Leptosphaeria maculans* pathogenicity group-3, the causal agent of blackleg of canola in Manitoba. Plant Dis. 87:1268

Ferreira ME, Rimmer SR, Williams PH, Osborn TC (1995) Mapping loci controlling *Brassica napus* resistance to *Leptosphaeria maculans* under different screening conditions. Phytopathology 85:213-217

Ferriol M, Pico B, Nuez F (2003) Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP markers. Theor Appl Genet 107:271-282

Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. PNAS 100 (25):15253-15258

Flor HH (1942) Inheritance of pathogenicity in *Melampsora lini.* Phytopathology 32: 653-696

Gall M, Baledent H, Desthieux I, Robin P, Rouxel T (1995) Polymorphism of Tox0 *Leptosphaeria maculans* isolates as revealed by soluble protein and isozyme electrophoresis. Mycological Research 99:221-229

Gao M, Li G, Potter D, McCombie WR, Quiros CF (2006) Comparative analysis of methylthioalkylmalate synthase (*MAM*) gene family and flanking DNA sequences in *Brassica oleracea* and *Arabidopsis thaliana*. Plant Cell Rep 25:592-598

Gassmann W, Hinsch ME, Staskawicz BJ (1999) The Arabidopsis *RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. The Plant Journal 20:265-277

Gimelfarb A, Lande R (1994a) Simulation of marker-assisted selection in hybrid populations. Genet. Res. 63:39-47

Gimelfarb A, Lande R (1994b) Simulation of marker-assisted selection for non-additive traits. Genet. Res 64:127-136

Gimelfarb A, Lande R (1995) Marker-assisted selection and marker-QTL associations in hybrid populations. Theor. Appl. Genet. 91:522-528

Goodwin PH, Annis SL (1991) Rapid identification of genetic variation and pathotype of *L. maculans* by RAPD assay. Applied and Environmental Microbiology 57:2482-248

Gout L, Fudal I, Kuhn ML, Blaise F, Eckert M, Cattolico L, Balesdent MH, Rouxel T (2006) Lost in the middle of nowhere: the *AvrLm1* avirulence gene of the Dothideomycete *Leptosphaeria maculans.* Molecular Microbiology 60(1):67

Grant MR, Godiard L, Strabue E, Ashfield T, Lewald J, Sattler A, Innes RW, Dangl JL (1995) Structure of the Arabidopsis *RPM1* gene enabling dual specificity disease resistance. Science 269:843-846

Gugel RK, Petrie GA (1992) History, occurrence, impact and control of blackleg of rapeseed. Can. J. Plant Pathol 14:36-45

Guus S, Groenendijk J, Wijbrandi J, Reijans M, Groenen J, Diergaarde P, Van der Lee T, Bleeker M, Onstenk J, de Both M, Haring M, Mes J, Cornelissen B, Zabeau M, Vos P (1998) Dissection of the fusarium *I2* gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055-1068

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annual Review of Plant Physiology and Plant Molecular Biology 48:573-605

He GM, Sun CQ, Fu YC, Fu Q, Zhao KJ, Wang CL, Zhang Q, Ling ZZ, Wang XK. (2004a) Pyramiding of senescence-inhibition *IPT* gene and *Xa23* for resistance to bacterial blight in rice (*Oryza sativa L.*). Yi Chuan Xue Bao 31(8):836-41

He YQ, Li X, Zhang JF, Jiang GH, Liu SP, Chen S, Tu JM, Xu CG, Zhang QF (2004b) Gene pyramiding to improve hybrid rice by molecular marker techniques. Poster at http://www.cropscience.org.au/

Hospital F, Moreau L, Lacoudre F, Charcosset A, Gallais A (1997) More on the efficiency of marker-assisted selection. Theor. Appl. Genet. 95:1181-1189

Howell EC, Barker GC, Jones GH, Kearsey MJ, King GJ, Kop EP, Ryder CD, Teakle GR, Vicente JG, Armstrong SJ (2002) Integration of the cytogenetic and genetic linkage maps of *Brassica oleracea*. Genetics 161:1225-1234

Huang L, Brooks SA, Li WL, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. Genetics 164:655-664

Huang N, Angeles ER, Domingo J, Magpantay G, Singh S, Zhang G, Kumaravadivel N, Bennett J, Khush GS (1997) Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor. Appl. Genet. 95:313-320

Hulbert SH, Webb CA, Smith SM, Sun Q (2001) Resistance gene complexes: evolution and utilization. Annu Rev Phytopathol 39:285-312

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. Plant Physiol 129:440-450

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, and Glazebrook J (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Plant Biology 96(23):13583-13588

Johan DP, van der Voort JR (2003) The challenges in Marker Assisted Breeding. Eucarpia Leafy Vegetables: 125-130

Johal GS, Briggs SP (1992) Reductase activity encoded by the *HMI* disease resistance gene in maize. Science 258:985-987

Johnson R (1984) A critical analysis of durable resistance. Annu. Rev. Phytopathol 22: 309–330

Johnson RD, Lewis BG (1994) Variation in host range, systemic infection and epidemiology of *Leptosphaeria maculans*. Plant Pathol. 43: 269-277

Jones DA, Thomas CM, Hammond-Kosack KE, Balint-Kurti PJ, Jones JDG (1994)

Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789-793

Joosten M, De Wit P (1999) The tomato-*Cladosporiom fulcum* interaction: a versatile experimental system to study plant-pathogen interactions. Annu Rev. of Phytopatholigy 37:335-367

Kawasaki S, Nam-Soo J, Kumiko K, Osamu K, Naoki M, Yuki T (1999) Cloning of rice blast resistance gene *PI-B*. Plant & Animal Genome VII Conference, P96. San Diego, CA, January 17-21

Kawchuk ML, Hachey J, Lynch DR, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, Kokko E, Byers R, Howard RJ (2001) Tomato *Ve* disease resistance genes encode cell surface-like receptors. Proc Natl Acad Sci USA 98:6511-6515

Keri M, van den Berg CGJ, McVetty PBE, Rimmer SR (1997) Inheritance of resistance to *Leptosphaeria maculans* in *Brassica juncea*. Phytopathology 87:594-598

Keri M (1999) Genetic studies of host - pathogen interaction between *Brassica napus* and *Leptospheria maculans*. Ph.D thesis. Department of Science, University of Manitoba

Koch E, Song K, Osborn TC, Williams PH (1991) Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphisms in *Leptosphaeria maculans*. Mol Plant-Microbe Interact 4:341-349

Kock MD, Brandwagt B, Bonnema G, Wit PD, Pim L (2003) Mapping and cloning of tomato resistance gene *cf-ecp2*, targeted against a virulence factor of *cladosporium fulvum*. Plant & Animal Genomes XI Conference. January 11-15.Town & Country Convention Center. San Diego, CA P:506

Kuhn M , Gout L, Howlett BJ, Melayah D, Meyer M, Balesdent MH, Rouxel T (2006) Genetic Linkage Maps and Genomic Organization in *Leptosphaeria maculans*. European Journal of Plant Pathology 114(1):17-31

Kuswinanti T, Sock J, Hoppe HH (1995) Variation in virulence of aggressive isolates of *Leptosphaeria maculans* based on cotyledon reactions on extended differential set. In Rapeseed: Today and Tomorrow, Proceedings of the 9th International Rapeseed Congress, Cambridge, UK, 4–7 July 1995.P:1248-1250

Lagudah ES, Moullet O, Apples R (1997) Map based cloning of a gene sequence encoding a nucleotide-binding domain and a leucine-rich repeat region at the *Cre3* nematode resistance locus of wheat. Genome 40:659-665

Lahaye T, Shirasu K, Schulze-lefert P (1998) Chromosome landing at the barley *Rar1* locus. Mol Gen Gent 260:92-101

Lawrence GJ, Finnegen EJ, Ayliffe MA, Ellis JG (1995) The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N.* Plant Cell 7:1195-1206

Lee M, 1995. DNA markers in plant breeding programs. Adv. Agron. 55:265-344

Li CX, Cowling WA (2003) Identification of a single dominant allele for resistance to blackleg in *Brassica napus* 'Surpass 400'. Plant Breeding 122 (6):485-489

Li G, Quiros CF (2001) Sequece-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping & gene tagging in *Brassica*. TAG 103:455-461

Li H, Sivasithamparam K, Barbetti MJ (2003) Breakdown of a *Brassica rapa* subsp. *sylvestris* single dominant blackleg resistance gene in *B. napus* rapeseed by *Leptosphaeria maculans* field isolates in Australia. Plant Dis. 87:752

Li X (2006) Issues and strategy for marker assisted selection. icgr.caas.net.cn/973/MAS.html

Lombard V, Delourme RA (2001) consensus linkage map for rapeseed (*Brassica napus L.*): construction and integration of three individual maps from DH populations. Theor Appl Genet 103:491-507

Liu ZW, Fu TD, Liu XP, Tu JX, Chen BY (2005) The research status, factors and strategy for marker assisted selection. Chinese Bulletin of Botany 22(Supplement): 82-90

Mahmut T, Brown D, Cooper A, Woods-Tör A, Sjölander K, Jones JDG, Holub EB (2004) Arabidopsis downy mildew resistance gene *RPP27* encodes a receptor-like protein similar to *CLAVATA2* and tomato *Cf-9*. Plant Physiology 135:1100-1112

Martin G B, Brommonschenke SH, Chunwongse J, Frary A, Ganal MW, Spive R, Wu YT, Earle ED, Tanksley SD (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432-1436

Martin G, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annual Review of Plant Biology 54:23-61

Mayerhofer R, Bansal VK, Thiagarajah MR, Stringam GR, Good AG (1997) Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. Genome 40:294-301

Mayerhofer R, Wilde K, Mayerhofer M, Lydiate D, Bansal VK, Good AG, Parkin IAP (2005) Complexities of chromosome landing in a highly duplicated genome: Toward map-based cloning of a gene controlling blackleg resistance in *Brassica napus*. Genetics 171:1977-1988

McDowell JM, Dhandaydham M, Long TA, Aarts MGM, Goff S, Holub EB, Dangl JL (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of Arabidopsis. Plant Cell 10:1861-1874

McNabb WM, van den Berg CGJ, Rimmer SR (1993) Comparison of inoculation methods for selection of plants resistant to *Leptosphaeria maculans* in *Brassica napus*. Can J Plant Sci. 79:1199-1207

Mengistu A, Rimmer SR, Williams PH (1991) Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling *Brassica*s. Plant Dis 75:1279-1282

Milligan SB, Bodeau J, Yaghoobi J (1998) The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine rich repeat family of the plant genes. Plant Cell 10:1307-1319

Mindrinos M, Katagiri F, Yu GL, Ausubel FM (1994) The *A. thaliana* disease resistance gene *RPS2* encodes a protein containing a nucleotide binding site and leucine-rich repeats. Cell 78:1089-1099

Moreau L, Charcosset A, Hospital F, Gallais A (1998) Marker-assisted selection efficiency in populations of finite size. Genetics 148:1353-1365

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction. Methods in Enzymology 155:335-350

Multani DS, Meeley RB, Paterson AH (1998) Plant-pathogen microevolution: molecular basis for the origin of a fungal disease in maize. Proc Natl Acad Sci USA 95:1686-1691

Osborn TC, Butrulle DV, Sharpe AG, Pickering KJ, Parkin IA, Parker JS, Lydiate DJ (2003) Detection and effects of a homeologous reciprocal transposition in *Brassica napus*. Genetics 165:1569-1577

Parker JE, Coleman MJ, Szabò V, Frost LN, Schmidt R, van der Biezen EA, Moores T, Dean C, Daniels MJ, and Jones JD (1997) The Arabidopsis downy mildew resistance gene *RPP5* shares similarity to the toll and interleukin-1 receptors with *N* and *L6*. Plant Cell 9(6): 879-894.

Parkin, IAP, Sharpe AG, Keith DJ, Lydiate DJ (1995) Identification of the A and C genomes of amphidiploid *Brassica napus* (oilseed rape). Genome 38:1122-1133

Parkin IAP, Gulden SM, Sharp AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics 171:765-781

Parleviet JE, Kuiper HJ (1977) Resistance of some barley cultivars to leaf rust, *Puccinia hordei:* Polygenic partial resistance hidden by monogenic hypersensitivity. Neth. J. Plant Pathol 83:85-889

Parlevliet JE (1983) Race-specific resistance and cultivar-specific virulence in the barley-leaf rust pathosystem and their consequences for the breeding of leaf rust resistant barley. Euphytica 32:367-375

Peters JL, Cnudde F, Gerats T (2003) Forward genetics and map-based cloning approaches. (Review). Trends Plant Sci 8(10):484-491

Pilet ML, Delourme R, Foisset N, Renard M (1998) Identification of loci contributing to quantitative field resistance to blackleg disease, causal agent *Leptosphaeria maculons* (Desm.) Ces. et de Not., in winter rapeseed (*Brassica napus L.*). Theor. Appl. Genet. 96:23-30

Piquemal J, Cinquin E, Couton F, Rondeau C, Seignoret E (2005) Construction of an oilseed rape (*Brassica napus L.*) genetic map with SSR markers. Theor Appl Genet 111:1514-1523

Rimmer SR (2006) Resistance genes to *Leptosphaeria maculance* in *Brassica napus*. Can. J. Plant Pathol. 28:288-297

Rimmer SR, Borhan MH, Zhu B, Somers D (1999) Mapping resistance genes in *Brassica napus* to *Leptosphaeria maculans*. "New Horizons for an old crop." In: Proceedings of the 10th international rapeseed congress, Canbera, Australia

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

Rong J, Abbey C, Bowers JE, Brubaker CL, Chang C, Peng WC, Terrye AD, Ding XL, Garza JJ, Marler BS, Park CH, Pierce GJ, Rainey KM, Rastogi VK, Schulze HR, Trolinder NL, Wendel JF, Wilkins TA, Willims-Coplin TD, Wing RA, Wright RJ, Zhao XP, Zhu LH, Paterson AH (2004) A 3347-locus genetic recombination map of sequence-tagged sites reveals features of genome organization, transmission and evolution of cotton (*Gossypium*). Genetics 166:389-417

Rouxel T, Penaud A, Pinochet X, Brun H, Gout L, Delourme R, Schmidt J, Balesdent MH (2003a) A ten-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards the *Rlm1* resistance gene of oilseed rape. Eur. J. Plant Pathol 109:871-881

Rouxel T, Willner E, Coudard L, Balesdent MH (2003b) Screening and identification of resistance to *Leptosphaeria maculans* (stem canker) in *Brassica napus* accessions. Euphytica 133:219-231

Salmeron JM, Oldroyd EDG, Rommens CMT (1996) Tomato *Prf* is a member of the Leucinerich repeat class of plant disease resistance genes and lies embedded within the Pto Kinase gene cluster. Cell 86:123-133

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory Press

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors, Proc Natl Acad Sci U S A 74(12):5463-7

Schaffer JF, Roelfs AP (1985) Estimated relationship between numbers of urediniospores of *Puccinia graminis* f. sp. *tritici* and rates of occurrence of virulence. Phytopathology 75:749-750

Schmidt D, Roder MS, Dargatz H (2001) Construction of a YAC library from barley cultivar 'Franka' and identification of YAC-derived markers linked to the *Rh2* gene conferring resistance to scald (*Rhynchosporium secalis*). Genome 44(6):1031-1040

Servin B, Martin OC, Mézard M and Hospital F (2004) Toward a Theory of Marker-Assisted Gene Pyramiding. Genetics 168, 513-523

Sharma PN, Torii A, Takumi S, Mori N, Nakamura C (2004) Marker-assisted pyramiding of brown planthopper (*Nilaparvata lugens Stal*) resistance genes *Bph1* and *Bph2* on rice chromosome 12. Hereditas140(1):61-9

Shen KA, Chin DB, Arroyo-Garcia R, Ochoa OE, Lavelle DO, Wroblewski T, Meyers BC, Michelmore RW (2002) *Dm3* is one member of a large constitutively-expressed family of NBS-LRR-encoding genes. Mol. Plant Microbe Interact 15:251-261

Shen YJ, Jiang H, Jin JP, Zhang ZB, Xi B (2004) Development of genome-wide DNA polymorphism database for map-based cloning of rice genes. Plant Physiol 135:1198-1205

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

Singh S, Siduhu JS, Huang N, Vikal Y, Li Z, Brar DS, Dhaliwal HS, Khush GS (2001) Pyramiding three bacterial blight resistance genes (*xa5, xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar 'PR106'. Theor. Appl. Genet. 102:1011-1015

Somda I, Renard M, Brun H (1996) Morphology, pathogenicity and isozym variation amongst French isolates of *L. maculans* recovered from *Brassica juncea cv.* 'Picra'. Plant Pathol 45:1090-1098

Song K, Slocum MK, Osborn TC (1995) Molecular marker analysis of genes controlling morphological variation in *Brassica rapa (syn. campestris)*. Theor Appl Genet 90:1-10

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270 (5243):1804-6

Song JQ, James M, Bradeen S, Naess K, Raasch JA, Wielgus SM, Haberlach GT, Liu J, Kuang HH, Austin-Phillips S, Buell CR, Helgeson JP, Jiang JM (2003) Gene *RB* cloned from *Solanum bulbocastanum* confers broad spectrum resistance to potato late blight. PNAS 100 (16):9128-9133

Spelman R, Bovenhuis H (1998) Genetic response from marker assisted selection in an outbred populaton for differing marker bracket sizes and with two identified quantitative trait loci. Genetics 148:1389-1396

Sprague SJ, Balesdent MH, Brun H, Hayden HL, Marcroft SJ, Pinochet X, Rouxel T, Howlett BJ (2006) Major gene resistance in *Brassica napus* (oilseed rape) is overcome by changes in virulence of populations of *Leptosphaeria maculans* in France and Australia. European Journal of Plant Pathology 114:33-40

Staal J, Kaliff M, Bohman S, Dixelius C (2006) Transgressive segregation reveals two Arabidopsis TIR-NB-LRR resistance genes effective against *Leptosphaeria maculans*, causal agent of blackleg disease. Plant J. 46(2):218-30

Sun X, Cao Y, Yang Z, Xu C, Li X, Wang S, Zhang Q (2004) *Xa26*, a gene conferring resistance to *Xanthomonas oryzae pv. oryzae* in rice, encodes an LRR receptor kinase-like protein. Plant J. 37(4):517-27

Sun ZD, Wang ZN, Tu JX, Zhang JF, Li GY, McVetty PBE (2007) An ultradense genetic recombination map for *Brassica napus*, consisting of 13,551 SRAP markers. (TAG 2007, accepted)

Tai TH, Dahlbeck D, Clark ET (1999) Expression of the *Bs2* pepper gene confers resistance to bacterial spot disease in tomato. Proc Natl Acad Sci USA 96:14153-14158

Takara Company (2004) Genome Walker Universal Kit User Manual. Published August 23. Cat. No. 638904

Takken FL, Thomas CM, Joosten MH, Golstein C, Westerink N, Hille J, Nijkamp HJ, De Wit PJ, Jones JD (1999) A second gene at the tomato *Cf-4* locus confers resistance to *cladosporium fulvum* through recognition of a novel avirulence determinant Plant J. 20(3):279-88

Tanksley SD (1983) Molecular markers in plant breeding. Plant Mol. Biol. Rep. 1:3-8

Thomas CM, Jones DA, Parniske M (1997) Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognition specificity in *Cf-4* and *Cf-9*. Plant Cell 9:2209-2224

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

van der Plank JE (1963) Plant diseases: epidemics and control. Academic Press, New York

van der Plank JE (1978) Ganetic and molecular basis of pathogenesis. Springer Verlag. Berlin 166

van der Voort JR, van der Vossen E, Bakker E (2000) Two additive QTLs conferring broad-spectrum resistance in potato to *Globodera pallida* are localized on resistance gene clusters. Theor Appl Genet 101(7):1122-1130

van Os HV, Andrzejewski S, Bakker E, Barrena I, Bryan G (2006) Construction of a 10,000 marker ultradense genetic recombination map of potato: providing a framework for accelerated gene isolation and a genome-wide physical map. Genetics 173:1075-1087

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Homes M, Freijiters A, Pot J, Peleman J, Kuiper M. Zabeau M (1995) AFLP: a new concept for DNA fingerprinting. Nucl. Acids Res. 23(21): 4407-4414

Warren RF, Henk A, Mowery P, Holub E, Innes RW (1998) A mutation within the leucine-rich repeat domain of the Arabidopsis disease resistance gene *RPS5* partially suppresses multiple bacterial and downy mildew resistance genes. Plant Cell 10:1439-1452

Webb CA, Richter TE, Collins NC, Nicolas M, Trick HN, Pryor T, Hulbert SH (2002) Genetic and molecular characterization of the maize *rp3* rust resistance locus. Genetics 162:381-394

Wei F, Gobelman-Werner K, Morroll SM, Kurth J, Mao L, Wing R, Leister D, Schulze-Lefert P, Wise RP (1999) The *Mla* (Powdery Mildew) Resistance cluster is associated with three NBS-LRR gene families and suppressed recombination within a 240-kb DNA interval on chromosome 5S (1HS) of barley. Genetics 153:1929-1948

West JS, Kharbanda PD, Barbetti MJ, Fitt BDL (2001) Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology* 50, 10-27.

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene *N*: Similarity to toll and the interleukin-1 receptor. Cell 78:1011-1015

Whittaker JC, Haley CS, Thompson R (1997) Optimal weighting of information in marker-assisted selection. Genet. Res. 69:137-144

Williams PH, and Delwiche PA (1979) Screening for resistance to blackleg of Crucifers in the seedling stage. Pages 164-170 in: Proc. Eucarpia Conf. Breed. Cruciferous Crops, Wageningen, Pays-Bas, the Netherlands

Williams PH, Hill CB (1985) Crucifer Genetics Cooperative: Resource Book. Madison: Crucifer Genetics Cooperative

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, and Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531-6535

Wretblad S, Bohman S, Dixelius C (2003) Over-expression of a *Brassica nigra* cDNA gives enhanced resistance to *Leptosphaeria maculans* in *B. napus*. Molecular Plant-Microbe Interactions 16:477-484

Yahiaoui N, Srichumpa P, Dudler R, and Keller B (2004) Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. Plant J. 37:528-538

Yahiaoui N, Srichumpa P, Dudler R, Keller B (2003) Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. The Plant Journal 37:528

Yoshimura S, Yamanouchi U, Katayose (1998) Expression of *Xa1*, a bacterial blight resistance gene in rice is induced by bacterial inoculation. Proc Natl Acad Sci. 95:1663-1668

Yu F, Lydiate DJ, Rimmer SR (2005) Identification of two novel genes for black leg resistance in *Brassica napus*. Theor. Appl. Genet. 110(5):969-979

Yu FQ, Rimmer SR, Lydiate DJ (2004) Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa subsp. sylvestris*. Plant and Animal Genome XII Conference Abstracts, p520

Zhang HB (2000) Manual: Construction and Manipulation of Large-Insert Bacterial Clone Libraries. Texas A& M University

Zhang W, Smith C (1992) Computer simulation of marker-assisted selection utilizing linkage disequilibrium. Theor. Appl. Genet. 83:813-820

Zhang W, Smith C (1993) Simulation of marker-assisted selection utilizing linkage disequilibrium: the effect of several additional factors. Theor. Appl. Genet. 86:492-496

Zhou F, Kurth J, Wei FS, Elliott C, Valè G, Yahiaoui N, Keller B, Somerville S, Wise R, Schulze-Lefert P (2001) Cell-Autonomous Expression of Barley *Mla1* Confers Race-

Specific Resistance to the Powdery Mildew Fungus via a *Rar1*-Independent Signaling Pathway. Plant Cell 13 (2):337-350

Zhu B, Rimmer R (2003) Inheritance of resistance to *L. maculans* in two accessions of *B. napus*. The Canadian Journal of Plant Pathology 3:98-103

Zietkiewicz E, Rafalski A, Labuda D (1994) Genome fingerprinting by simple sequence repeats (SSR)-anchored polymerase chain reaction amplification. Genomics 20: 176-183

Appendix A: Primers used for mapping

Date	Image	ODD3 (B)	GA3 (G)	SA12 (Y)	OD20 (R)
42804	A	PM98	PM98	PM101	PM113
	B	PM100	PM113	PM104	FC6
	C	PM101	PM120	PM110	FC10
	D	PM103	PM122	ODD3	ODD3
	E	PM104	FC6	ODD8	ODD30
	F	PM110	ODD3	ODD27	ODD38
42904	A	PM113	ODD38	ODD38	ODD40
	B	PM114	ODD58	ODD40	ODD54
	C	PM116	CE4	ODD68	ODD61
	D	PM118	CE7	CE26	ODD62
	E	PM120	CE8	CE36	CE34
	F	FC6	CE16	CE37	CE35
43004	A	ODD30	SA7	SA7	SA7
	B	ODD39	GA16	GA3	SA17
	C	ODD54	GA17	GA5	SA18
	D	ODD58	GA37	GA8	GA5
	E	ODD59	GA46	GA18	GA11
	F	SA7	EM6	GA24	GA16
50204	A	GA5	ME9	GA25	GA17
	B	GA18	MC1	GA27	GA18
	C	GA19	MC3	GA31	GA19
	D	GA21	MC6	GA37	GA21
	E	GA24	MC7	GA41	GA25
	F	GA27	MC8	GA43	GA43
50304	A	GA29	LC1	EM8	GA34
	B	GA34	LC5	ME9	GA37
	C	GA37	PA2	ME10	GA39
	D	GA41	PA3	MC1	ME9
	E	GA43	MC31	MC3	ME10
	F	GA45	MC36	MC7	MC1
50404	A	GA46	PM100	MC8	MC3
	B	EM6	PM109	LC1	LC1
	C	EM8	PM110	LC5	RP3
	D	ME9	PM111	RP3	PA2
	E	ME10	PM114	PA2	MC9
	F	MC1	PM116	PA3	MC11

50504	A	MC3	PM117	PM98	MC13
	B	MC4	FC8	PM100	MC15
	C	MC7	FC9	PM103	MC23
	D	LC1	ODD25	PM111	MC25
	E	LC5	ODD26	PM114	MC26
	F	RP3	ODD39	PM115	MC27
		ME2	**EM1**	**EM2**	**DC1**
111203	A	CE2	CE2	CE6	CE3
	B	CE3	CE3	CE7	CE6
	C	CE4	CE4	CE9	CE7
	D	CE5?	CE6	CE10	CE8
	E	CE6	CE8	CE11	CE9
	F	CE7	CE10	CE12	CE10
111303	A	CE10	PM1	PM1	IPM2
	B	CE14	PM3	PM5	IPM3
	C	CE15	PM4	PM6	IPM5
	D	CE16	PM5	PM7	IPM6
	E	CE17	PM6	PM8	IPM7
	F	CE25	PM8	PM9	IPM8
111403	A				IPM9
	B				FC1
	C				FC3
	D				FC5
	E				FC7
	F				
111503	A	PM16	PM17	PM16	ODD11
	B	PM17	PM18	PM17	ODD13
	C	PM18	PM19	PM18	ODD16
	D	PM19	PM20	PM19	ODD17
	E	PM20	PM21	PM20	ODD18
	F	PM21	PM22	PM21	ODD24
111703	A	PM22	PM22	PM22	ODD27
	B	PM23	PM29	PM24	ODD28
	C	PM24	PM30	PM25	ODD29
	D	PM25	PM31	PM26	ODD42
	E	PM26	PM32	PM27	ODD44
	F	PM27	PM33	PM28	ODD46

112003	A	PM28	PM36	PM29	ODD52
	B	PM29	PM37	PM30	ODD53
	C	PM30	PM38	PM31	ODD54
	D	PM31	PM39	PM32	ODD55
	E	PM32	PM40	PM33	ODD56
	F	PM33	PM41	PM34	ODD56
112103	A	PM34	PM27	PM35	PM4
	B	PM36	PM34	PM36	PM5
	C	PM37	PM43	PM37	PM10
	D	PM38	PM45	PM38	PM15
	E	PM39	PM47	PM39	PM18
	F	PM40	PM48	PM40	PM19
112203	A	PM42	PM49	PM41	PM22
	B	PM44	PM50	PM42	PM23
	C	PM45	PM51	PM43	PM26
	D	PM46	PM52	PM45	PM28
	E	PM47	PM53	PM46	PM29
	F	PM48	PM54	PM47	PM30
112303	A	PM49	PM55	PM49	PM32
	B	PM50	PM56	PM50	PM34
	C	PM51	PM57	PM51	PM36
	D	PM52	PM58	PM52	PM37
	E	PM53	PM59	PM53	PM38
	F	PM54	PM62	PM54	PM48
112803	A	PM55	PM63	PM56	BG1
	B	PM57	PM64	PM57	BG5
	C	PM58	PM66	PM58	BG8
	D	PM59	PM67	PM59	BG9
	E	PM60	PM68	PM60	BG10
	F	PM61	PM68	PM61	BG17
112903	A	PM63	PM69	PM62	BG20
	B	PM64	PM70	PM63	BG23
	C	PM66	PM72	PM64	BG24
	D	PM69	PM73	PM67	BG25
	E	PM70	PM74	PM68	BG26
	F	PM71	PM75	PM69	BG29

120103	A	PM73	PM76	PM70	BG33
	B	PM74	PM77	PM71	BG34
	C	PM75	PM78	PM72	BG35
	D	PM76	PM79	PM73	BG37
	E	PM77	PM80	PM74	BG43
	F	PM78	PM81	PM75	BG44
120203	A	PM79	PM82	PM76	BG53
	B	PM80	PM84	PM77	BG54
	C	PM81	PM85	PM78	BG55
	D	PM82	PM86	PM79	BG60
	E	PM84	PM87	PM81	BG61
	F	PM85	PM88	PM84	BG62
120303	A	PM86	PM89	PM85	BG63
	B	PM87	PM90	PM86	BG64
	C	PM88	PM91	PM87	BG66
	D	PM89	PM92	PM88	BG68
	E	PM90	PM93	PM89	BG69
	F	PM91	PM95	PM90	BG70
120403	A	PM92	PM96	PM91	BG72
	B	PM93	BG49	PM92	BG73
	C	PM95	BG51	PM93	BG74
	D	PM96	BG53	PM94	BG75
	E	BG49	BG54	PM95	BG76
	F	BG50	BG55	PM96	BG77
120503	A	BG53	BG60	BG49	BG78
	B	BG54	BG61	BG50	BG79
	C	BG55	BG62	BG52	BG80
	D	BG56	BG63	BG54	BG81
	E	BG60	BG64	BG55	BG82
	F	BG61	BG46	BG56	BG83
120603	A	BG62	BG68	BG57	BG84
	B	BG63	BG69	BG59	BG85
	C	BG64	BG70	BG62	BG86
	D	BG66	BG72	BG63	BG87
	E	BG68	BG73	BG67	BG88
	F	BG69	BG74	BG70	BG89

120703	A	BG70	BG75	BG72	BG90
	B	BG73	BG76	BG73	BG92
	C	BG75	BG77	BG75	BG93
	D	BG76	BG78	BG77	BG94
	E	BG77	BG79	BG80	BG95
	F	BG78	BG80	BG81	BG96,CE1?
120803	A	BG80	BG81	BG82	BG46
	B	BG85	BG82	BG88	BG48
	C	BG86	BG83	BG89	ODD38
	D	BG87	BG84	BG90	ODD39
	E	BG89	BG85	BG91	ODD49
	F	BG90	BG86	BG93	ODD56
120903	A	BG83	BG87	BG1	PM49
	B	BG84	BG88	BG3	PM50
	C	BG91	BG89	BG5	PM51
	D	BG93	BG90	BG7	PM52
	E	BG94	BG91	BG9	PM53
	F	BG95	BG92	BG13	PM54
121003	A	BG1	BG1	BG13	PM55
	B	BG2	BG2	BG14	PM56
	C	BG3	BG3	BG17	PM57
	D	BG4	BG4	BG19	PM58
	E	BG5	BG5	BG21	PM59
	F	BG6	BG6	BG23	PM60
121203	A	BG7	BG7	BG24	PM61
	B	BG10	BG9	BG26	PM62
	C	BG11	BG10	BG27	PM63
	D	BG12	BG11	BG30	PM66
	E	BG13	BG12	BG31	PM67
	F	BG14	BG13	BG32	PM68
121303	A	BG16	BG14	BG34	PM69
	B	BG17	BG16	BG35	PM70
	C	BG18	BG17	BG37	PM71
	D	BG19	BG18	BG38	PM72
	E	BG20	BG19	BG39	PM73
	F	BG21	BG20	BG41	PM74

121403	A	BG22	BG21	BG43	PM75
	B	BG23	BG22	BG44	PM76
	C	BG24	BG23	BG45	PM77
	D	BG25	BG24	BG46	PM78
	E	BG26	BG25	BG47	PM79
	F	BG27	BG26	PM82	PM80
121503	A	BG28	BG27	IPM1	PM81
	B	BG29	BG28	IPM2	PM82
	C	BG30	BG29	IPM3	PM84
	D	BG31	BG30	IPM4	PM85
	E	BG32	BG31	IPM5	PM86
	F	BG33	BG32	IPM6	PM87
121603	A	BG34	BG33	IPM7	PM88
	B	BG35	BG34	IPM8	PM89
	C	BG37	BG35	IPM9	PM90
	D	BG38	BG37	IPM10	PM91
	E	BG39	BG38	FC1	PM92
	F	BG40	BG39	FC2	PM93
121703	A	BG42	BG40	IPM7	PM88
	B	BG43	BG41	IPM8	PM89
	C	BG44	BG42	IPM9	PM90
	D	BG45	BG43	IPM10	PM91
	E	BG46	BG44	FC1	PM92
	F	BG48	BG45	FC2	PM93
121803	A	IPM1	BG46	FC3	PM95
	B	IPM2	BG47	FC4	PM96
	C	IPM4	BG48	FC5	CE2
	D	IPM5	IPM1	FC6	CE3
	E	IPM6	IPM2	FC7	CE4
	F	IPM7	IPM3	FC8	CE6
122103	A	IPM8	IPM4	FC9	CE7
	B	FC1	IPM5	FC10	CE8
	C	FC2	IPM6	ODD11	CE10
	D	FC3	IPM7	ODD13	CE11
	E	FC4	IPM8	ODD17	CE12
	F	FC6	IPM9	ODD18	CE13

121603	A	ODD3	ODD15	ODD12	ODD10
	B	ODD8	ODD20	ODD13	ODD15
	C	ODD17	ODD22	ODD15	ODD23
	D	ODD26	ODD24	ODD3	ODD24
	E	ODD34	MC7	ODD8	ODD30
	F	ODD44	MC8	ODD17	ODD34
123103	A	FC7	FC1	ODD24	CE14
	B	FC9	FC2	ODD25	CE15
	C	FC10	FC3	ODD27	CE16
	D	ODD11	FC4	ODD28	CE17
	E	ODD13	FC5	ODD29	CE18
	F	ODD17	FC6	ODD34	CE19
10104	A	ODD24	FC7	ODD37	CE20
	B	ODD25	FC8	ODD38	CE23
	C	ODD28	FC9	ODD39	CE24
	D	ODD38	FC10	ODD40	CE25
	E	ODD39	ODD11	ODD41	CE26
	F	ODD40	ODD13	ODD42	CE27
11804	A	ODD41	ODD17	ODD44	CE28
	B	ODD44	ODD24	ODD47	CE29
	C	ODD47	ODD25	ODD48	CE30
	D	ODD48	ODD27	ODD49	CE32
	E	ODD49	ODD28	ODD52	CE33
	F	ODD52	ODD29	ODD53	CE34
11904	A	ODD53	ODD29	ODD53	CE35
	B	ODD54	ODD34	ODD54	CE36
	C	ODD55	ODD37	ODD55	CE37
	D	ODD56	ODD38	ODD56	CE38
	E	CE23	ODD39	CE13	CE39
	F	CE24	ODD40	CE14	CE40
12004	A	CE25	ODD41	CE15	SA1
	B	CE26	ODD42	CE16	SA2
	C	CE27	ODD44	CE17	SA3
	D	CE28	ODD47	CE18	SA4
	E	CE29	ODD48	CE19	SA5
	F	CE30	ODD49	CE20	SA6

12104	A	CE32	ODD52	CE23	SA7
	B	CE33	ODD53	CE24	SA8
	C	CE34	ODD54	CE25	SA9
	D	CE35	ODD55	CE26	SA10
	E	CE36	ODD56	CE27	SA11
	F	CE37	CE10	CE28	GA10
12204	A	CE38	CE11	CE29	GA10
	B	CE39	CE12	CE30	GA11
	C	CE40	CE13	CE32	GA12
	D	SA1	CE14	CE33	GA13
	E	SA2	CE15	CE34	GA14
	F	SA3	CE16	CE35	GA15
12404	A	SA4	CE18	CE36	GA7
	B	SA5	CE19	CE37	GA8
	C	SA6	CE20	CE38	GA9
	D	SA7	CE23	CE39	GA10
	E	SA8	CE24	CE40	GA11
	F	SA9	CE25	SA1	GA12
12504	A	SA10	CE26	SA2	GA13
	B	SA11	CE27	SA3	GA14
	C	SA12	CE28	SA4	GA15
	D	SA13	CE29	SA5	GA16
	E	SA14	CE30	SA6	GA17
	F	SA15	CE32	SA7	GA18
12704	A	SA16	CE33	SA8	SA12
	B	SA17	CE34	SA9	SA13
	C	SA18	CE35	SA10	SA14
	D	SA19	CE36	SA11	SA15
	E	SA20	CE37	SA12	SA16
	F	SA21	CE38	SA13	SA17
12804	A	GA1	CE39	SA14	SA18
	B	GA2	CE40	SA15	SA19
	C	GA3	SA1	SA16	SA20
	D	GA4	SA2	SA17	SA21
	E	GA5	SA3	SA18	GA19
	F	GA6	SA4	SA19	GA20

12904	A	GA7	SA5	SA20	GA21
	B	GA8	SA6	SA21	GA22
	C	GA9	SA7	GA1	GA23
	D	GA10	SA8	GA2	GA24
	E	GA11	SA9	GA3	GA25
	F	GA12	SA10	GA4	GA26
13004	A	GA13	SA11	GA5	GA27
	B	GA14	SA12	GA6	GA28
	C	GA15	SA13	GA7	GA29
	D	GA16	SA14	GA8	GA30
	E	GA17	SA15	GA9	GA31
	F	GA18	SA16	GA10	GA32
13104	A	GA19	SA17	GA11	GA33
	B	GA20	SA18	GA12	GA34
	C	GA21	SA19	GA13	GA35
	D	GA22	SA20	GA14	GA36
	E	GA23	SA21	GA15	GA37
	F	GA24	GA1	GA16	GA38
20104	A	GA25	GA2	GA16	GA39
	B	GA26	GA3	GA17	GA40
	C	GA27	GA4	GA18	GA41
	D	GA28	GA5	GA19	GA42
	E	GA29	GA6	GA20	GA43
	F	GA30	GA7	GA21	GA44
20204	A	GA31	GA8	GA22	GA45
	B	GA32	GA9	GA23	MC1
	C	GA33	GA10	GA24	MC2
	D	GA34	GA11	GA25	MC3
	E	GA35	GA12	GA26	MC4
	F	GA36	GA13	GA27	MC5
20304	A	GA37	GA14	GA28	
	B	GA38	GA15	GA29	
	C	GA39	GA16	GA30	
	D	GA41	GA17	GA31	
	E	GA42	GA18	GA32	
	F	GA43	GA19	GA33	

20404	A	GA44	GA20	GA34	MC5
	B	GA45	GA21	GA35	MC6
	C	GA46	GA22	GA36	MC7
	D	MC1	GA23	GA37	MC8
	E	MC2	GA24	GA38	MC9
	F	MC3	GA25	GA39	MC10
20504	A	RP1	GA26	GA40	RP1
	B	RP2	GA27	GA41	RP2
	C	RP3	GA28	GA42	RP3
	D	NY1	GA29	GA43	NY1
	E	NY2	GA30	GA44	NY2
	F	LUM1	GA31	GA45	LUM1
20604	A	LUM2	GA32	RP1	LUM2
	B	LUM3	GA33	RP2	LUM3
	C	LUM4	GA34	RP3	LUM4
	D	LC1	GA35	NY1	LC1
	E	LC2	GA36	NY2	LC2
	F	LC3	GA37	LUM1	LC3
20704	A	LC4	GA38	ME1	ME1
	B	LC5	GA39	ME2	ME2
	C	ALPN?1	GA40	ME3	ME3
	D	ALPN?2	GA41	ME4	ME4
	E	CUL1	GA42	ME5	ME5
	F	CUL1501?	GA43	ME6	ME6
				SA12	**OD20**
20904	A	EM3	GA44	GA44	GA44
	B	EM4	GA45	GA45	GA45
	C	EM5	GA46	GA46	GA46
	D	EM6	ME1	ME1	ME1
	E	EM8	ME2	ME2	ME2
	F	TE1	ME3	ME3	ME3
		ODD3	**GA3**	**SA12**	**OD20**
21004	A	PM1	PM1	PM1	PM1
	B	PM3	PM3	PM3	PM3
	C	PM4	PM4	PM4	PM4
	D	PM5	PM5	PM5	PM5
	E	PM6	PM6	PM6	PM6
	F	PM7	PM7	PM7	PM7

21104	A	PM10	PM10	PM10	PM10
	B	PM11	PM11	PM11	PM11
	C	PM12	PM12	PM12	PM12
	D	PM14	PM14	PM14	PM14
	E	PM15	PM15	PM15	PM15
	F	PM16	PM16	PM16	PM16
21204	A	PM17	PM17	PM17	PM17
	B	PM18	PM18	PM18	PM18
	C	PM19	PM19	PM19	PM19
	D	PM20	PM20	PM20	PM20
	E	PM21	PM21	PM21	PM21
	F	PM29	PM29	PM29	PM29
21304	A	PM30	PM30	PM30	PM30
	B	PM31	PM31	PM31	PM31
	C	PM32	PM32	PM32	PM32
	D	PM34	PM34	PM34	PM34
	E	PM35	PM35	PM35	PM35
	F	PM36	PM36	PM36	PM36
21404	A	PM37	PM37	PM37	PM37
	B	PM38	PM38	PM38	PM38
	C	PM40	PM40	PM40	PM40
	D	PM41	PM41	PM41	PM41
	E	PM43	PM43	PM43	PM43
	F	PM45	PM45	PM45	PM45
21504	A	PM47	PM47	PM47	PM47
	B	PM49	PM49	PM49	PM49
	C	PM50	PM50	PM50	PM50
	D	PM51	PM51	PM51	PM51
	E	PM52	PM52	PM52	PM52
	F	PM55	PM55	PM55	PM55
21604	A	PM55	PM55	PM55	PM55
	B	PM56	PM56	PM56	PM56
	C	PM57	PM57	PM57	PM57
	D	PM58	PM58	PM58	PM58
	E	PM59	PM59	PM59	PM59
	F	PM60	PM60	PM60	PM60

32404	A	PM61	PM61	PM61	PM61
	B	PM62	PM62	PM62	PM62
	C	PM63	PM63	PM63	PM63
	D	PM64	PM64	PM64	PM64
	E	PM65	PM65	PM65	PM65
	F	PM66	PM66	PM66	PM66
32504	A	PM67	PM67	PM67	PM67
	B	PM68	PM68	PM68	PM68
	C	PM69	PM69	PM69	PM69
	D	PM70	PM70	PM70	PM70
	E	PM71	PM71	PM71	PM71
	F	PM72	PM72	PM72	PM72
		FC1	**SA7**	**BG23**	**PM88**
71105	A	BG2	BG1	BG3	BG1
	B	BG4	BG2	BG4	BG2
	C	BG6	BG5	BG5	BG4
	D	BG11	BG6	BG6	BG5
	E	BG12	BG8	BG7	BG8
	F	BG13	BG9	BG9	BG9
71805	A	BG14	BG11	BG11	BG10
	B	BG16	BG12	BG14	BG11
	C	BG18	BG13	BG18	BG12
	D	BG23	BG14	BG19	BG13
	E	BG25	BG16	BG21	BG16
	F	BG28	BG17	BG24	BG18
72005	A	BG29	BG18	BG25	BG20
	B	BG30	BG19	BG27	BG23
	C	BG35	BG20	BG28	BG24
	D	BG37	BG22	BG29	BG25
	E	BG40	BG23	BG30	BG26
	F	BG43	BG24	BG31	BG27
72205	A	BG48	BG25	BG32	BG32
	B	BG49	BG26	BG33	BG33
	C	BG50	BG29	BG35	BG35
	D	BG54	BG31	BG37	BG37
	E	BG55	BG32	BG38	BG38
	F	BG56	BG33	BG41	BG41

80505	A	BG59	BG34	BG43	BG41
	B	BG60	BG35	BG44	BG48
	C	BG64	BG37	BG48	BG49
	D	BG66	BG38	BG50	BG53
	E	BG67	BG39	BG51	BG55
	F	BG68	BG40	BG52	BG59
80905	A	BG69	BG41	BG59	BG61
	B	BG70	BG43	BG60	BG62
	C	BG72	BG45	BG62	BG63
	D	BG73	BG47	BG63	BG67
	E	BG84	BG48	BG66	BG68
	F	BG85	BG49	BG67	BG70
81105	A	PM13	BG52	BG68	BG73
	B	PM14	BG53	BG69	BG84
	C	PM 15	BG54	BG70	BG85
	D	PM 16	BG55	BG71	BG95
	E	PM 17	BG56	BG72	PM4
	F	PM 19	BG59	BG73	PM5
81205	A	PM20	BG60	BG80	PM5
	B	PM28	BG61	BG82	Pm6
	C	PM57	BG62	BG85	PM9
	D	PM58	BG63	PM4	PM14
	E	PM59	BG64	PM5	PM15
	F	PM60	BG66	PM8	PM17
81505	A	PM60	BG67	PM17	PM18
	B	Pm70	BG68	Pm18	Pm19
	C	PM73	BG69	PM29	PM20
	D	PM77	BG70	PM32	PM26
	E	PM79	BG71	PM33	PM27
	F	PM80	BG72	PM34	PM28
81705	A	PM87	BG73	PM37	PM31
	B	Pm89	BG74	Pm47	Pm32
	C	PM90	BG76	PM49	PM34
	D	PM95	BG80	PM52	PM36
	E	PM96	BG83	PM53	PM37
	F	PM101	BG84	PM55	PM38

81905	A	PM104	BG85	PM56	PM39
	B	Pm116	BG86	PM58	PM40
	C	PM118	BG88	PM59	PM41
	D	PM119	BG92	PM60	PM45
	E	PM120	BG95	PM63	PM46
	F	PM88	PM1	PM64	PM52
82005	A	FD1	PM75	PM89	PM54
	B	FD4	PM80	PM90	PM56
	C	FD5	PM81	PM92	PM57
	D	FE2	PM82	PM95	PM58
	E	FE3	PM85	PM96	PM60
	F	FE5	PM87	PM100	PM63
82205	A	FE8	PM8	PM101	PM66
	B	FE9	PM14	PM108	PM68
	C	FE16	PM15	PM109	PM70
	D	FE25	PM16	PM113	PM72
	E	FE26	PM17	PM114	PM75
	F	FE6	PM18	PM115	PM77
82405	A	FE30	PM19	PM116	PM78
	B	FE31	PM20	PM117	PM79
	C	FE41	PM22	PM118	PM80
	D	FE43	PM23	PM119	PM81
	E	FE46	PM26	PM120	PM82
	F	FE48	PM27	PM122	PM84
82505	A	ODD2	PM28	FD1	PM85
	B	ODD3	PM29	FD3	PM87
	C	ODD4	PM30	FD4	PM89
	D	ODD6	PM31	FD5	PM91
	E	BG61	PM32	FE1	PM92
	F	BG62	PM34	FE2	PM97
82605	A	FD1	PM36	FE3	PM98
	B	FD4	PM37	FE4	PM99
	C	FD5	PM38	FE5	PM104
	D	FE2	PM39	FE6	PM110
	E	FE3	PM40	FE7	PM117
	F	FE5	PM41	FE8	PM118

		ME2	**SA7**	**BG23**	**PM88**
82905	A	PM116	PM45	FE15	FD4
	B	PM118	PM46	FE16	FD5
	C	PM119	PM48	FE22	FE3
	D	PM120	PM49	FE25	FE13
	E	FD1	PM50	FE30	FE15
	F	FD4	PM52	FE32	FE16
83005	A	FD5	PM53	FE40	FE19
	B	FE2	PM55	FE41	FE30
	C	FE3	PM56	FE42	FE31
	D	FE5	PM57	FE43	FE42
	E	FE6	PM58	FE44	FE43
	F	FE8	PM59	FE45	FE44
		FC1	**SA7**	**BG23**	**PM88**
90105	A	FE9	PM63	FE46	FE49
	B	FE16	PM64	FE48	FE48
	C	FE25	PM66	FE50	ODD2
	D	FE26	PM67	ODD4	ODD3
	E	FE30	PM68	ODD8	ODD4
	F	FE31	PM70	PM98	ODD5
90205	A	BG2	PM72	BG4	PM56
	B	BG13	PM73	BG59	PM57
	C	BG50	PM74	BG24	PM58
	D	BG23	PM75	BG31	PM60
	E	BG37	PM76	BG52	PM63
	F	BG43	PM77	BG71	PM87

Primers in bold are the ones labeled with florescent. To find the primer pair for a marker, such as 1217Ar269, one can find the date 121703, the image A at the same row, the labeled primer DC1 for r (red), and the unlabeled primer PM88.