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Development of High-throughput Molecular Markers for Blackleg (*Leptosphaeria maculans*) Resistance Genes in *Brassica napus* for Gene Stacking

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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 (sequence related amplified polymorphism) markers and SNPs. 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 phenotype segregation in the populations supported that a single dominant resistance gene controls the resistance in 'Quinta' and "Glacier" separately. The closest SRAP marker was identified for the resistance gene in 'Quinta' by using 256 pairs of primers and screening 2,500 polymorphic loci. B342 is 1.5 cM to *Rlm1* and 6.8 cM to *Rlm3*. SNP80870 developed from the N7 orthologous region in Arabidopsis was 3 cM to *Rlm3*. A population for stacking the three resistance genes (*Rlm1*, *Rlm3* and *LepR3*) was constructed. Marker screening and disease inoculation were both used for the selection of lines with the three resistance genes.

Keywords Blackleg, Resistance Gene, Marker, Mapping, Gene Stacking, *B. Napus*

1. Introduction

Blackleg, caused by *Leptosphaeria 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. The pathogen was divided into pathogenicity groups (PG), such as PG2, PG3, and PG4 [1,2] via cotyledon inoculation test [3], A1 to A6 [4,5] with the addition of two more differential cultivars and PGT [6]. Blackleg epidemics are caused mostly by PG2 and PG3 worldwide, by PG4 in Europe and by PGT in Canada. At least sixteen resistance genes to blackleg have been genetically inferred by using the above isolates with the corresponding avirulence genes and were mapped in different sources, such as *B. napus, B. rapa, B. juncea* and Arabidopsis [6-17]. The resistance gene *LepR3* was recently cloned [18].

At least five resistance genes were mapped on linkage group N7 via genetic studies of segregating populations. *Rlm1*and *Rlm3* controlling seedling resistance to PG3 and PG2 were mapped on the DY10 linkage groups [8,12,19,20,21] that was later confirmed an equivalence to N7 [7,15,21,22]. *Rlm3* in 'Glacier', 'Columbus' and 'Maxol' [23] was linked to the field resistance *Rlm1* as a QTL locus with a genetic distance of 26 cM. *Rlm4* in 'Quinta' was linked to *Rlm1* [12] with a genetic distance of 19 cM. *Rlm2* in 'Glacier' and 'Samourai', controlling seedling resistance to PG2 isolates in these cultivars, was on linkage group LG16 [12]. At least five resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9*) in a cluster were located in this region [12]. *LmR1* in 'Sheralee', *CLmR1* in 'Cresor', *CRLMm* in 'Maluka', *CRLMrb* in RB87-62, and *cRLMj* in DH88-752 were all mapped on LG6 that also belongs to linkage group N7 [6, 21]. As quite a few genes were mapped on N7, development of closely linked markers in this hotspot region is necessary for gene identification and marker assisted selection.

Marker assisted gene stacking is intended to integrate specific genes and/or QTLs for the trait(s) into a single genotype [24] with the advantage of increasing selection efficiency for multiple genes [25-27]. There are quite a few successful examples for *gene stacking of more than two traits or pathogens* [28,29], stacking specific resistance with quantitative resistance QTLs [30] and stacking only specific resistance genes to the same pathogen [31]. Factors, such as marker distance, generations, population size and heritability [32-42] are the main elements to be considered when using marker assisted selection. However, the closeness of the molecular markers used for the selection is the key for success [25,34,38,40,43,44].

In this study, we screened the SRAP markers using the

primer combinations and developed SNPs in the N7 region using the populations constructed with 'Westar', 'Quinta' and 'Glacier' respectively. Closely linked markers were identified for two blackleg resistance genes. The gene stacking population was also constructed to produce lines with multiple resistance genes. Disease inoculation and marker screening were combined for the selection of the stacked resistance genes.

2. Materials and Methods

2.1. Construction of Populations

The plants of *B. napus* cultivars 'Westar', 'Glacier', 'Quinta', "Surpass 400' and "Cresor' 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 F_1 seeds from the mapping populations, 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 to produce F_3 seeds. The F_3 plants selfed to produce F_4 seeds for later use. For the gene stacking population, four-way crossing was adopted as 'Crèsor' × 'Quinta'//'Surpass 400' × 'Glacier'. The resulting population was screened by both the disease inoculation and the selected markers.

2.2. Phenotyping of the Populations

The F_2 and F_3 plants were inoculated separately with blackleg pycnidiospore suspension of 2×10^7 spores/ml 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 the 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 [45]. 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. '87-41" was used for the phenotyping of resistance gene *LepR3* from 'Surpass 400' [15].

2.3. Genotyping of the Populations

A modified CTAB extraction procedure described in Li and Quiros [46] was used for DNA extraction. Sequence related amplified polymorphism (SRAP) was applied in the PCR reactions with 5 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. The markers were detected using ABI 3100 Genetic Analyzer (Life Technologies, New York, USA) according to the user manual.

Table1. Primers designed for SNP development between 'Westar', 'Quinta' and 'Glacier'

Name	Primer Sequence	Genes*	Amplicon (bp)
WB26	GAACCATCCCTTTCGACGTT	At1g80870	1,300
WB27	AGCTAAATTCTCCCGCTGCT		
WB18	TGCTTGGAAAGATGATGGAAC	At1g80670	1,100
WB19	CGGGTGGAAATTCAGAGAGT		
WB20	AGGTTTGGGAGATCCCGTAG	At1g80680	1,400
WB21	TGAACTGTCGGGAGGTAGATG		
WB22	GCGTTCGCTCTACTTTCGTC	At1g80630	1,150
WB23	TTATGCCCGTACATCTGCTG		
WB24	TCCGTTGTCTACGTTTCTGG	At1g80640	1,000
WB25	CCAAAGATCCATTCTGCATCA		
WB28	CCAATTGGACCAGAGCATTA	At1g79640	1,100
WB29	TGAGCAAGCTCTAGCCCAGT		
WB30	TCCCTTTTTCATCACCGACT	At1g79670	1,300
WB31	CGAGACAACACCCCATGAGT		
WB32	AGAATCCAAGGGATGCAAAG	At1g79680	1,200
WB33	CAGCAATACGAAGACGCACT		
WB34	GCTCTGCGTTCTTTGATGG	At1g79620	850
WB35	TGTCAACTGATTATCCGCAAG		
WE15	GAAGAACTTGCAGAGCTTCAG	At1g79600	960
WE16	CTTCAGAGCATAGTAATCTCG		

* Primer sequences are from 5' – 3'. At1g79600 to At1g80870 are Arabidopsis genes.

For the SNP development, ten genes (Table 1) from a N7-orthologous-region on Arabidopsis chromosome 1 were selected for primer design [47]. The specific PCR was performed at 94°C 50 s, 50-60°C 50 s and 72°C 50 s for 35 cycles. The quality of the amplicons was visualized in the agarose gel with the BIO-RAD Gel Doc 2000. The PCR products were diluted 3 to 5 times and 2 µl was used as the sequencing template. The PCR reactions were performed at 94°C 4 min, then at 94°C 30 s, 50°C 10 s and 60°C 4 min for 25 to 30 cycles. The SNPs were identified by comparing the sequences of 'Westar', 'Glacier' and 'Quinta' using the SeqA software installed on ABI 3100 Genetic Analyzer according to the settings. The SNPs were then used to screen the segregating populations. Both the SNP and the SRAP markers were used for linkage analysis.

(V2.0) was used to generate the linkage map with LOD 4.0.

2.4. Disease Resistance Mapping

The DNA samples from 8 resistant plants and 8 susceptible plants were used to run SRAP markers first. Once a few close markers were identified, the sample number was increased to 32 to 32, or all the samples were used for marker screening. Only the closely linked markers were used to construct the genetic map. Mapmaker

3. Results

3.1. Segregation of Resistance in the Populations

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 2). 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 2). 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.

3.2. Screening of SRAP Markers

Screening of SRAP molecular markers were started by running 'Westar' × 'Quinta' population with 8 susceptible plants to 8 resistant plants from F_2. Two hundred and fifty-six primer pairs [48] were used for the initial screening. About 2,500 markers were polymorphic. One of the 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 [48]. The marker B342 was used to screen the whole population. After all the F_2 plants were screened with this marker, it showed a genetic distance of 1.5 cM to the resistance gene (Fig.1).

For the 'Glacier' resistance gene, the marker, B342, was used to screen the F_2 and F_3 generations of 'Westar' × 'Glacier'. The marker is 6.8 cM away from the resistance gene in 'Glacier' (Fig. 1).

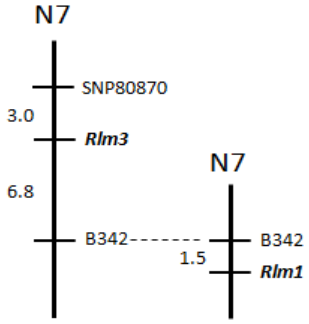

Figure 1. Localization of *Rlm1* linkage group from 'Westar' × 'Quinta' and *Rlm3* linkage group from 'Westar' × 'Glacier'. Blackleg resistance genes *Rlm1* from 'Quinta' and *Rlm3* from 'Glacier' were both mapped on the N7 linkage group. Numbers at left side show the genetic distance between the markers and the genes in centiMorgans.

Table 2. Segregation of resistance in the population*

Population	Generation	Ratio	No. of Plants			X^2	P
			R	Se	S		<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

3.3. Development of SNPs Markers

Of the ten primer pairs designed from At1g79600 to At1g80870 for the detection of SNPs in N7, one SNP ('Westar': CTTTCT; 'Quinta' and 'Glacier': CCTTTT) was found in At1g80870 by comparing the sequences of 'Westar', 'Glacier' and 'Quinta'. SNP80870 was used to screen the two populations, 'Westar' × 'Glacier' and 'Westar' × 'Quinta'. It showed a linkage to the resistance gene *Rlm3* in 'Glacier' with a genetic distance of 3.0 cM (Fig. 1). However, SNP80870 did not show any linkage to *Rlm1* in 'Quinta'.

3.4. Comparative Mapping of Resistance Genes from 'Quinta' and 'Glacier'

As the two genetic maps had a consensus marker B342, *Rlm1* and *Rlm3* both were mapped in the N7 linkage group and had a genetic distance of 11.3 cM. Marker SNP80870 was closely associated with *Rlm3* from 'Glacier'. B342 was more closely linked to *Rlm1* with a distance of 1.5 cM, while it was loosely linked to the gene *Rlm3* in a distance of 6.8 cM (Fig. 1).

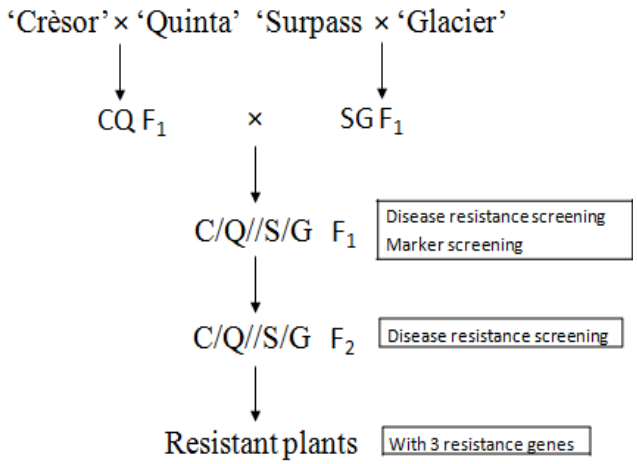

Figure 2. Blackleg resistance gene stacking procedure. 'C/Q//S/G' refers to 'Crèsor/Quinta//Surpass400/Glacier'.

3.5. Blackleg Resistance Gene Stacking

Figure 2 shows the gene stacking 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 and select the spring type of plants in later generations. The F_1s of the two combinations were then crossed to obtain the F_1 (C/Q//S/G) of the four way crossing. The F_1 (C/Q//S/G) was not treated in the cold room and was segregating in disease resistance and flower time. We inoculated their cotyledons of the 2,000 plants with both isolates 'pl 86-12' and 'Lifolle 6' each on one cotyledon. We selected only 45 plants showing resistance to both isolates, discarding the winter type plants and the other plants. After screening with B342, SNP80870 and 0127Fr382 the closest marker (0.3 cM, unpublished) to *LepR3*, 12 plants were confirmed to have the three resistance genes and were used for next generation. In the CQSG F_2 generation, 6 plants from each of the 12 families were inoculated with 87-41 and were confirmed the existence of *LepR3* in these plants. Considering the agronomic traits when harvesting these plants, we selected only three lines as the best resistance candidates for the use in breeding.

4. Discussion

In this study, we confirmed the genetic model of *Rlm1* from 'Quinta' and *Rlm3* from 'Glacier' respectively. Through the mapping of each gene, we identified very closely linked markers that can be used for automated screening of the breeding populations. The comparative mapping of the two genes indicates the N7 region containing at least 5 resistance genes would be a hotspot region for elucidating genetic control and evolution of these genes. The successful selection of lines with multiple blackleg resistance genes will broaden the genetic basis for development of resistant cultivars.

'Quinta' and 'Glacier' are winter type European cultivars with blackleg resistance [19,49]. Segregation data from both studies indicated that one dominant resistance gene controlled blackleg resistance in each cultivar. In this study, as we used the same isolate and populations as used by Keri [49], the results were consistent with the previous studies. *Rlm1* in 'Quinta' and *Rlm3* in 'Glacier' were previously mapped on the linkage group DY10 [12] that was determined to be the same as N7 [16]. The closest markers to gene *Rlm1* and *Rlm3* are at least 7 cM away, indicating the use of these markers in marker assisted selection should be very difficult. In this study, we screened 2,500 SRAP markers and the marker B342 was only 1.5 cM to *Rlm1*. Further, we developed some SNP markers and SNP80870 is 3 cM to *Rlm1*. These closer markers had been used in the marker assisted selection for blackleg resistance.

In the genetic maps, *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* were located in a 40 cM interval on linkage group DY10 [12]. Colinear markers indicated that these genes were in a gene cluster. *Rlm1* and *Rlm3* covered an interval of 34 cM. In this study, the two genes were linked by a consensus marker B342 that was linked to a marker SNP80870 developed from N7, confirming the existence of the genes on N7. *Rlm1* and *Rlm3* on N7 cover a region of 11.3 cM that is different from the interval of 34 cM on DY10. The difference in one parental line for the populations could be the major cause for the genetic difference. Similar results were also obtained for *LmR1* in 'Shiralee' and *CLmR1* in 'Cresor' [21].

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 [12]. Other genes such as *LmR1, CLmRm, CRLMrb* and *cRLMj* correspond to unknown avirulence genes. Difference in genetic background will delay the elucidation of the gene cluster. Comparative fine mapping and physical mapping will help 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 closely to *Rlm3*, there is a possibility that *LmR1* and *CLmR1* are *Rlm3*. However, genotyping by sequencing seems to be the most effective method to tackle the genes in this region.

Marker assisted gene stacking has the advantage of increasing the efficiency for variety development [25,26] in targeting multiple genes simultaneously [27] that could not easily followed by traditional breeding methods. In this study, we identified two markers for two blackleg resistance genes via high throughput SRAP and SNP development. The combination of marker screening with disease inoculation made the selection of target genotypes 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. We combined the cotyledon inoculation with marker screening at the same generation. This saved time and costs normally spent in the later generations.

Closely linked markers can increase the efficiency for disease resistance selection from decreased population sizes. The flanking markers of a major gene can increase the selection efficiency [43]. In this study, B342, as the flanking marker of only 1.5 cM for the major resistance gene locus *Rlm1* in 'Quinta', can be used as a major marker for *Rlm1* gene selection in a smaller population than the traditional breeding populations. The marker SNP80870 is 3.0 cM to *Rlm3* and can be used either alone or combined with B342 to track *Rlm3*. 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. These molecular markers could be very useful for stacking blackleg resistance gene.

Race-specific resistance genes in breeding are facing potential risks [50-53] *and could be easily overcome by a mutation in the pathogen* [54]. The *t*hree major resistance genes derived from *B. rapa* ssp. *sylvestris* and *B. juncea* were overcome by *L. maculans* in France and Australia [55-57]. In addition to finding new effective resistance genes, QTLs stacking is expected to be more effective than stacking a few single genes because they are controlled by multiple genes that could not be overcome by the pathogens simultaneously. Horizontal resistance could be the breeding objective of breeders.

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