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RAPD markers associated with resistance to blackleg disease in *Brassica* species

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Blackleg, caused by *Leptosphaeria maculans*, is a serious disease of *Brassica* species. Genetic analysis of resistance to *L. maculans* was carried out with 15 accessions from the USDA *Brassica* germplasm collections, representing diploids (A, C), and tetraploid (AC) genomes, respectively; and 9 cultivars from the National Winter Canola Variety Trials (NWCVT) all carrying AC genomes. All genotypes were screened for blackleg disease at the cotyledonary stage. The results indicated that 46% of the 24 genotypes were resistant, while 54% were susceptible. On the other hand, adult plant screening revealed that all the public genotypes were resistant. In an effort to identify molecular markers associated with resistance to blackleg disease, all genotypes were screened with 13 RAPD and 8 SSR markers producing 169 amplified products. Six RAPD markers (OPB01, OPE03, OPE16, OPF10, OPE12, and OPI01) were polymorphic, while the SSR markers were monomorphic. Chi-square analysis indicated that 5 amplified fragments (OPE03-4000, OPE16-1100, OPE16-1300, OPE16-1900, and OPI01-280) from RAPD primers were significantly associated with blackleg resistance. Thus this study demonstrated that RAPD primers could be effectively used to identify DNA markers that are associated with blackleg disease resistance, and that resistance to *L. maculans* might also exist in the A and C genomes.

Key words: *Leptosphaeria maculans*, Pathogenicity, Innoculum, *Brassica napus*.

INTRODUCTION

Leptosphaeria maculans (anamorph Phoma lingam) causes blackleg disease in *Brassica species*, resulting in substantial yield loss worldwide. Blackleg disease is economically important in many areas where oilseed rape is grown such as North America, Europe and Australia. The most severe epidemics occurred in Australia in the early 1970's, where the disease curtailed development of the emerging oilseed rape industry (West et al., 2001). Severe epidemics have also been reported in Canada and Europe (Gugel and Petrie, 1992). *L. maculans* is ca-

pable of causing blackleg disease on different winter and spring cultivars of oilseed rape grown under wide range of climates and despite different agricultural practices. The ascospores and conidia adhere to the cotyledons or young leaves and they germinate in humid or wet conditions to produce hyphae that cause infection (West et al., 2001). Infections can be spread through airborne ascospores, infected seeds and stubbles, as well as rain-splashed conidia. The fungus predominantly enters the leaves via stomatal pores and wounds (Chen and Howlett, 1996).

The genomic relationships among *Brassica* species are usually represented by the U triangle (U, 1935). *Brassica nigra* (BB), *Brassica oleracea* (CC), and *Brassica rapa* (AA) are the primary diploid species, while *Brassica carinata* (BBCC), *Brassica juncea* (AABB) and *Brassica napus* (AACC) are amphidiploids that result from hybridization between corresponding pairs of the diploid

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Abbreviations: NWCVT, National Winter Canola Variety Trials, PI, Pathological index, PDA, Potato Dextrose Agar, PG2, Pathogenicity group 2.

species. A number of different sources of partial resistance to blackleg disease have been used successfully in *Brassica* breeding programs. However, the most promising source of resistance genes have been identified in *Brassica* 'B' genome of *B. nigra, B. carinata*, or *B. juncea*; that confer complete resistance to blackleg disease (Balesdent et al., 2001).

Several efforts have been made to transfer the B genome resistance into *Brassica napus (B. napus)* through interspecific hybridization (Brun et al., 2000; Ansan-Melayah et al., 1998; Chevre et al., 1997). Two types of *L. maculans* resistance are expressed during specific stages of plant growth. Seedling infection can lead to a systematic, latent infection. But, adult plant infection is the most damaging symptom in terms of yield losses (Johnson and Lewis, 1994). Currently, there are resistant genes (loci) from different sources that confer resistance to *L. maculans* (Rimmer et al., 1995; Eke et al., 1995; Ansan-Melayah et al., 1998). These genes have been identified and characterized, and linkage maps have been developed (Dion et al., 1995; Wang et al., 1999a). Most importantly, the genes of resistance have been found to exist in the B genome of *Brassica species* (Pilet et al., 2001; Axelsson et al., 2000).

Recent advances in molecular marker techniques provide the possibilities to improve the agronomic characteristics of *Brassica* crops. Molecular plant bree-ders have investigated the genome organization, inter-genomic relationships, and genome evolution among *Brassica species*. In the past years, molecular markers, such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), Single Seq-uence Repeats (SSRs), and restriction fragment length polymer-phism (RFLP), have been used to identify and characterize important molecular traits in *Brassica species* (Pongam et al.*,* 1998; Chevre et al., 1997; Veld-boom and Lee, 1996a). According to Struss et al. (1991), different B genomes were analyzed by the creation of addition lines containing one B chromosome on an oil-seed rape genetic background. Using this material, Che-vre et al., (1996) found out that one chromosome of *B. nigra* among the five analyzed, carried resistance genes that were effect-tive at the cotyledon and adult stage.

L. maculans can be controlled effectively by the use of host resistance and some common resistance sources in a breeding program. The identification of markers associated with different disease resistance genes has been achieved by the use of bulked segregant analysis in combination with molecular markers (Chevre et al.*,* 1997). There are also markers that have been developed for marker-assisted selection (MAS) for disease resistant genes (Warren et al., 1999; Sigareva and Earle, 1999). Some of these markers are very specific and an example is SCAR (sequence characterized amplified region). Therefore, molecular markers linked to resistant genes must be developed so that, the process of breeding for resistance to blackleg disease can be facilitated.

In the present study, we report that resistance to *L. maculans* might also exist in the A and C genomes of *Brassica* species and not only on the B genome as earlier reported. Genetic analysis of resistance to *L. maculans* was carried out with 15 accessions from the USDA *Brassica* germplasm collections, representing diploid (AA and CC) and tetraploid (AACC) genomes, respectively; and 9 cultivars from the National Winter Canola Variety Trials (NWCVT) all carrying AC genomes. We hypothesized that there was no correlation between the DNA fragments amplified in resistant and susceptible genotypes carrying the A and C genomes. To test this hypothesis, all the 24 genotypes were screened with 13 RAPD and 8 SSR markers.

MATERIALS AND METHODS

Plant materials

Twenty-four genotypes, including fifteen accessions from the USDA germplasm collections (Fort Collins, Colorado) and nine cultivars of canola from the National winter Canola Variety Trials (NWCVT, Kansas) were selected for this study (Table 1). These genotypes were selected because they represented A and C genomes of four species; *Brassica napus, Brassica napus var. napobrassica, Brassica rapa, and Brassica oleracea var. virids* that were of interest in this study. They were grown in separate trays in the greenhouse at the Department of Plant and Soil Sciences, Alabama A&M University in a random complete block. Trays were tagged and numbered based on the genotype.

Fungal isolate

Isolate Leroy of *L. maculans*, belonging to pathogenicity group 2 (PG2), was obtained from Dr. Ginette Seguin-Swartz (Agriculture Canada Research Station, Saskatoon, Saskatchewan, Canada). This isolate is considered to be representative of the predominantly aggressive pathotypes occurring in Canada. Innoculum was prepared from mycelium collected from two weeks old cultures grown in Petri plates at room temperature on PDA agar medium. After two weeks of incubation the isolate was cut out of the petri dish with a sterile blade. It was placed in 200 ml sterile distilled water and blended for about 1 min. The innoculum was then transferred into a clean beaker, and one drop of the inoculum was placed onto a homogenizer and placed under the microscope to determine the concentration of the innoculum. The mycelium suspension was adjusted to a concentration of 1×10^4 mycelium ml^{-1}.

Cotyledon inoculation and Greenhouse disease reaction evaluation

To evaluate the disease reaction of individual plants, seeds were planted separately in small pots filled with potting soil. Three seeds were planted in a random complete block for each genotype and all plants were maintained in the greenhouse in four replicates. Nine days after seeding, the entire surfaces of cotyledons were wounded with a rough ended tong and droplets of mycelium suspension were spread onto the wound. The inoculated cotyledons were then placed in a growth chamber for 48 hrs, to allow the fungus to infect the plants under high humidity. Disease scoring was rated using a 0 to 7 scale and two scores were taken. The first one was taken 7 days after the inoculation, and the second one was taken 12 days

after the inoculation. Disease rating of 0 - 5 was defined as resistant, and 6 - 7 as susceptible.

Genomic DNA Extraction

Genomic DNA of all genotypes and accessions were isolated from the leaves of all cultivars using a modified protocol developed by Edwards et al. (1991). Leaves were harvested when the plants were 5 - 6 weeks-old and a total of 25 plants were sampled for each genotype or accession.

RAPD PCR Analysis

RAPD primers were purchased from MWG-Biotech (High Point, NC). The primers were of 10 nucleotides in length with the GC content of 60 to 70%. They were selected because they have been identified to be specific for chromosome 4 in B genome of *Brassica nigra,* and they are linked to the gene of resistance to blackleg disease (Chevre et al., 1997). PCR reactions were carried out in 25 µL volumes containing the following: 16.75 µL of sterile distilled water, 0.25 µL of gold *Taq* DNA polymerase enzyme (Promega, Madison, WI), 1µL of 10x dNTP (Promega), and 1 µL of 10 pmol/uL of each primer (MWG-Biotech), 2.5 µL of 15 mM MgCl$_2$, 2.5 µL of 10x PCR buffer, 1 µL of 25 ng DNA template. The reactions were performed in a PTC-200 Peltier Thermal Cycler. The Thermal Cycler was programmed for 45 cycles of 1 min at 94°C for DNA denaturation, 1 min at 32°C and 34°C depending on the annealing temperature of each primer, and 2 min of 72°C for primer extension. An additional 7 min extension was followed at 72°C at the end of the program.

Five micro-liters of amplified PCR products were mixed with blue dye (0.25% bromophenol blue, 0.25% xylene cynol FF, 15% Ficoll) and loaded onto 1% agarose gels prepared in 1x TBE buffer. The DNA size marker used for the gels was Bench-top 1 kb DNA ladder (Promega, Madison, WI-Catalog no. G7541). Gel electrophoresis was carried out at 40-45 V for about 5-6hrs in a standard horizontal electrophoresis unit.

The electrophoresed PCR amplified fragments were stained with ethidium bromide for 15 min on a shaker and then destained for 25 min. The gels were visualized under ultraviolet light and the pictures were taken using AlphaImager v5.5 2000 (Alpha Innotech Corporation, San Leandro, CA).

SSR DNA fingerprinting

Genomic DNA was amplified with SSR loci primer pairs SSRNa10-A08, SSRNa10-D07, SSRNa10-D09, SSRNa10-F06, SSRNa10-G10, SSRNa12-A01, SSRNa12-A02 and SSRNa12-A07 (MWG-Biotech, NC). These primers were mapped to linkage groups (O3, O4, O6, N6, and N7) (Lowe et al., 2003; Sebastian et al*.,* 2000; Sharpe et al*.,* 1995) www.Brassica.info. A modified PCR procedure reported by Williams et al. (1990) was followed with a PTC-200 Peltier Thermal Cycler. The PCR amplification reactions contained 10X PCR buffer, 2.0 mM MgCl$_2$, 200 µM of each dNTP, 0.15 µM of 3' and 5' primers, 25 ng template DNA, and 0.5 units Taq DNA polymerase in 25 µL. A touchdown PCR was carried out. Cycling consisted of a 1 min denaturation at 94°C, 1 min annealing at 55, 60, or 65, depending on the optimum annealing temperature of a primer pair, and 2 min extension at 72°C for 35 cycles. The PCR products were separated on 6% polyacrylamide gels in 1X TBE buffer at 250 V for 2 h. Following electrophoresis, the amplified PCR fragments were stained with ethidium bromide. The gels were visualized under ultraviolet light and the pictures were taken using AlphaImager v5.5 2000 (Alpha Innotech Corporation, San Leandro, CA). Fragment size was estimated by a means of a 100-base pair

DNA ladder (GibcoBRL, Life Technologies, Carlsbad, CA) using gel image software (Alpha Imager).

RESULTS

Twenty-four cultivars, which included 15 accessions germplasm, and 9 public or cultivated genotypes that produced consistent amplifications, were analyzed (Table 1). Six polymorphic RAPD primers (OPB01, OPE03, OPE12, OPE16, OPF10, and OPI01), and 8 SSR primers (SSRNa10-A08,   SSRNa10-D07,   SSRNa10-D09, SSRNa10-F06,   SSRNa10-G10,   SSRNa12-A01, SSRNa12-A02 and SSRNa12-A07) that consistently amplified these 24 cultivars were analyzed. The number and size of amplified products were determined for each of the primer using AlphaEaseFC version 4.0.1. Each DNA fragment was examined for size in base pairs and the amplified products were scored as "+" and non-amplification as "-" using AlphaEaseFC version 4.0.1.

Chi–square test was used to evaluate the frequency distribution of each fragment in susceptible and resistant genotypes. Chi–square test was performed to study the relationship between the fragments amplified in resistant and susceptible genotypes. The hypothesis tested was that there is no correlation between the fragment amplified in resistant genotypes and fragments amplified in susceptible genotypes. If the fragments amplified in resistant and susceptible genotypes are independent, the expected number of amplified resistant genotypes is equal to the total number of resistant genotypes times the total number of amplified products (including both resistant and susceptible) divided by the grand total (including amplified and non-amplified resistant and susceptible genotypes). Every other expected class was calculated the same way. The following chi – square formula for1degree of freedom was applied to these fragments:

$$\text{Adjusted } \chi^2 = \sum \frac{(|\text{Observed} - \text{Expected}| - 0.5)^2}{\text{Expected}}$$

where, observed and expected represent the number resistant or susceptible seedling based on response to pathogenicity.

The values obtained were referred to chi – square critical value under 1 degree of freedom [the degree of freedom equals (r-1) x (c-1) = 1], the chi –square value of 6.826 at 99% confidence was expected. If the values obtained were greater than 6.826, then the hypothesis of independence was rejected and the conclusion was that, there is a correlation between the resistant genotypes with a given fragment.

Field and Greenhouse evaluation of Blackleg disease

The distribution of disease reactions in the 24 genotypes at the cotyledon and adult stages is shown in Table 1.

Table 1. List of Plant Materials used in this study indicating the accession number, species, and their disease ratings based on response to blackleg at seedling and adult stage (S = Susceptible R = Resistant).

Accession	Genome	Species	Response to Blackleg in seedling stage	Mean Disease rating at the seedling level	Response to Blackleg in adult stage
NSL6102	AACC	*B. napus var. napobrassica*	R	3	_
NSL6106	AA	*Brassica rapa*	S	6	_
NSL6112	AA	*Brassica rapa*	S	7	_
NSL6114	AA	*Brassica rapa*	R	5	_
NSL6116	AA	*Brassica rapa*	S	6	_
NSL6117	AA	*Brassica rapa*	S	7	_
NSL6125	AA	*Brassica rapa*	S	6	_
NSL6558	AA	*Brassica rapa*	S	6	_
NSL6560	AA	*Brassica rapa*	R	1	_
NSL34675	AA	*Brassica rapa*	S	6	_
NSL42980	AA	*Brassica rapa*	R	1	_
NSL167291	AA	*Brassica rapa*	R	1	_
NSL6145	CC	*Brassica oleracea var .virids*	S	7	_
NSL6146	CC	*Brassica oleracea var. virids*	R	5	_
NSL6153	CC	*Brassica oleracea var. virids*	S	7	_
Abilene	AACC	*Brassica napus*	R	5	R
Arctic	AACC	*Brassica napus*	S	7	R
Baldur	AACC	*Brassica napus*	R	5	R
Jetton	AACC	*Brassica napus*	S	6	R
Maestro	AACC	*Brassica napus*	S	6	R
Plainsman	AACC	*Brassica napus*	R	4	R
Rasmus	AACC	*Brassica napus*	S	6	R
Talent	AACC	*Brassica napus*	R	2	R
Viking	AACC	*Brassica napus*	R	4	R
Falcon*	AACC	*Brassica napus*	R	1	R
Westar*	AACC	*Brassica napus*	S	7	S

*Resistant and susceptible check cultivars (Falcon and Westar), respectively.

The disease reaction of the nine cultivated genotypes (Abilene, Arctic, Baldur, Jetton, Maestro, Plainsman, Rasmus, Talent, and Viking) tested at the adult stages in blackleg nursery indicated that they were resistant to the pathotypes of *L. maculans* present in southeastern region of the USA. These cultivars are referred to as public genotypes because they have been officially released to the public for production purposes. Seven cultivars; Arctic, Jetton, Plainsman, Rasmus, Talent, Titan, and Viking had a pathological index (PI) of 0, meaning that they were resistant to the disease at the adult stage. Cultivars, Abilene, Baldur, Banjo, Ceres, Kronos, Maestro, and Wotan registered a PI of 1, which also classified them as resistant. When classified in discrete resistant or susceptible categories some plants were susceptible at the cotyledon level, but resistant at the adult stage (Table 1).

Similar results have been reported by Dion et al. (1995) for single gene expressing adult plant resistance in cultivar cresor. Several cultivars, Arctic, Jetton, Maestro, and Rasmus were susceptible at the cotyledon level, but resistant at the adult stage. This might indicate that a single dominant gene controls resistance at the adult stage. The remaining cultivars; Abilene, Baldur, Plainsman, Talent, and Viking showed consistent disease reactions (resistant) at cotyledon and adult plant stages. This result might support the hypothesis of two linked genes, one controlling cotyledon resistance, and one controlling adult plant resistance (Ferreira et al., 1995). The plants that were consistently resistant might have both sets of genes. Reaction of the susceptible check cultivar (Westar) used in this study indicated the effectiveness and virulence of the pathogen.

Seven days after inoculation, seedlings of the resistant check cultivar (Falcon) were vigorous and pathogen-free compared to the response of the susceptible check (Table 1).

Fragment Identification

In this study, 6 RAPD primers (Table 2) that are specific to chromosome B4 of *Brassica nigra* were analyzed; they are associated to a blackleg resistant gene (Chevre et al., 1997). The size of the fragments amplified by the RAPD primers ranged from 125 bp to 4000 bp. Large fragments (>4000 bp) produced by some primers are justified because due to the use of gold *Taq* for PCR amplification. Gold *Taq* is known to be very robust, and it can produce up to 8000 bp fragment (Promega, Madison, WI). Eight SSR primers were randomly chosen from 5 linkage groups (O3, O4, O6, N6, and N7) published in *Brassicca* website (www.Brassica.info; Lowe et al., 2003; Sebastian et al., 2000). All these primers generated DNA fingerprints in different species of *Brassica* plants (*B. rapa*, *B .napus*, *B. napus var. napobrassica*, and *B. oleracea var. virids*). The purpose of this study was to determine the relationship between the amplified products in the resistant genotypes and susceptible genotypes by identifying the DNA fragments (markers) that are significantly associated with the blackleg resistance.

DNA polymorphism generated with primer OPE03

Primer OPE03 produced 6 polymorphic bands ranging in size from 600 bp to 4000 bp (Table 2). Among these 6 fragments, the 4000 bp (Figure 1b) was found to be significantly associated with the blackleg disease resistance rating at the cotyledon stage. The number of bands in each accession or genotype ranged from 1 (accessions NSL: 6102, 6106, 6112, 6114, 6116, 6117, 6558, 42980, 167291) to 5 (accession NSL 6146).

In most of the public genotypes, primer OPE03 generated 2-3 polymorphic bands. All the public genotypes had 2100 bp and 4000 bp fragments, but they lacked 600bp, 850bp and 1100bp.

DNA polymorphism generated with primer OPE16

Primer OPE16 revealed 13 polymorphic bands ranging in size from 600 bp to 2500 bp (Table 2). Out of the 13 fragments, 3 of them i.e. 1100 bp, 1300 bp, and 1900 bp (Figure 1c) were found to be significantly associated with the blackleg disease resistance rating. The number of fragments amplified in each accession ranged from 1 (Jetton) to 10 (Viking). Most of the accessions and genotypes produced 2 to 5 fragments.

Some fragments generated with primer OPE16 were specific to some public genotypes and/or USDA accessions. For example, 1000 bp, 2100 bp, and 2500 bp frag-

ments were amplified in the public genotypes, while none of the USDA accessions produced them; the same applies to the 600 bp fragment that was amplified in the USDA accessions, but not in the public genotypes. Talent and Viking were the only genotypes with 1000 bp. On the other hand, a 900-bp fragment was found only in the USDA genotypes.

DNA polymorphism generated with primer OPI01

Primer OPI01 revealed 13 polymorphic bands ranging in size from 280 bp to 2500 bp (Table 2). Out of the 13 fragments, 280 bp (Figure 1a) was found to be significantly associated with the blackleg disease resistance rating. The number of bands in each USDA accession or public genotype ranged from 1 (NSL: 6112, 6153, Jetton) to 9 (Arctic, Talent). Most of the USDA accessions and public genotypes had 3 to 6 fragments along with the common fragments of 280 bp and 1400 bp.

The polymorphisms generated by primer OPI01 showed that some fragments could be used to identify either some USDA accessions or public genotypes. For example, 1000 bp, 2000 bp, and 2100 bp fragments were amplified in public genotypes but not in USDA accessions. On the other hand 900 bp, 1500 bp, and 1600 bp fragments were amplified in USDA accessions, but not in public genotypes.

Relationship between amplified fragments and blackleg disease resistance

The polymorphism information content (PIC value) indicates that some primers were more polymorphic than others (Table 2) and that polymorphism was closely associated or unique to each accession or genotype. Therefore, fragments generated by the 6 RAPD primers and 8 SSR primers were tested in SAS for their association with resistance to blackleg disease using chi-square test. This was based on the frequency distribution for each amplified fragment in the resistant and susceptible accessions or genotypes.

These results (Table 2) indicate that 5 specific DNA fragments generated by primer OPE03, OPI01, and OPE16 were significantly correlated with the resistance to blackleg disease rating as follows: (1) Primer OPE03 generated a significant fragment that is correlated with the disease rating at 4000 bp. The chi-square value obtained was 17.1834 with the P-value of 0.0001; (2) Primer OPE16 generated 3 significant fragments: 1100 bp, 1300 bp, and 1900 bp that were correlated with the disease rating. The chi-square values obtained are 8.0607, 20.3077, and 8.3916 with the P-values of 0.0001, 0.0045, and 0.0038, respectively; (3) Primer OPI01 generated a significant fragment correlated with the resistance rating at 280 bp. The chi-square value obtained was 12.1846 with the P-value of 0.0005. There was no significant relationship between SSR fragments and blackleg

Table 2. Polymorphic RAPD primers with PCR fragment amplification in 24 *Brassica* accessions and genotypes comprising of genomes A and C.

Primer name and sequence	No. of bands scored	Approximate band size (bp)	PIC[1] value	Fragments[2] of significance	Analytical methods[3]	
					χ^2 & P-value	R^2 & P-value
OPB01 5'-GTTTCGCTCC-3'	9	125 - 3500	0.97	None	-	-
OPE03 5'-CCAGATGCAC-3'	6	600 – 4000	0.94	4000	17.18 0.0001**	0.35 0.05*
OPE12 5'-TTATCGCCCC-3'	9	690 – 2000	0.88	None	-	-
OPE16 5'-GGTGACTGTG-3'	13	600 – 2500	0.86	1100 1300 1900	8.06 0.0045* 20.30 0.0001** 8.06 0.0038*	0.48 0.02* 0.65 0.002* 0.42 0.03*
OPF10 5'-GGAAGCTTGG-3'	12	400 – 3500	0.78	None	-	-
OPI01 5'-ACCTGGACAC-3'	12	280 - 2500	0.92	280	12.18 0.0005*	0.71 0.0001**

[1]PIC: Polymorphism Information Content.
[2](Fragments of Significance; FOS): PCR fragments significantly associated with blackleg resistance at the seedling stage.
[3]Chi-square tests and regression analysis used to determine relationship between FOS and disease rating at the seedling stage.
*, **Significant at 0.05 and 0.01 levels, respectively.

disease rating.

Regression Analysis

Association studies between RAPD bands and traits of interest have been reported. For precision in establishment of trait-related DNA markers, Virk et al. (1995) have used RAPD analysis of highly diverse accessions of rice in multiple regression analysis to determine associations between the DNA marker(s) and quantitative traits. In this study, regression analysis (Table 2) was undertaken to determine the association of specific DNA fragments with the prediction of the levels of disease infection. For example, linear regression showed that the RAPD band (at 280 bp) obtained with OPI01 primer has a highly significant regression coefficient for disease resistance (Table 2). This might indicate that the 280-bp fragment is associated with plants exhibiting high activity of disease resistance. This 280-bp fragment from the primer OPI01 could be used as a marker in germplasm screening for blackleg disease resistance in different *Brassica* species. Regression analysis showed a good agreement between DNA fragments and the similarity coefficients obtained from this study. The coefficient of determination (r^2) was between 0.3495 and 0.7059 for all the fragments (Table 2).

DISCUSSION

The results in this study indicate that RAPD primers used to find DNA markers were shown to be significantly associated with the resistance to blackleg disease rating in 24 genotypes of *Brassica* species carrying the A and C genomes. According to Chevre et al. (1997), these RAPD primers were previously used to screen the B genome of *B. nigra*, and *B. juncea*, which is believed to contain genes of blackleg resistance. These results presented through correlation and regression analysis indicate that these RAPD primers can also be used to find resistant genotypes in the A and C genomes. This implies that resistance to blackleg disease is not only localized in the B genome, but it may also exist within the diverse range of *Brassica* species carrying the A and C genomes. This study was carried out with 15 accessions from USDA *Brassica* germplasm collections, representing diploids A, C, and tetraploid AC genomes, respectively; and 9 cultivated cultivars from the National Winter Canola Variety Trials (NWCVT) all carrying AC genomes. Greenhouse screening of all the 24 genotypes indicated that 45.84% were resistant at the cotyledon stage.

Cotyledon tests have been reported to provide an efficient method to select for resistance in *B. juncea* (Chevre et al. 1997). Accessions such as NSL 6560, NSL 167291, and NSL 42980, which have the diploid genome A had a mean disease rating of 1 (Table 1), meaning that they were highly resistant to blackleg disease. Tetraploids with the AC genomes such as Viking and Talent were also rated as resistant. Five RAPD fragments were significantly correlated with the resistance ratings among the 169 fragments tested. The fragments OPE03-4000, OPI01-280, OPE16-1100, OPE16-1300, and OPE16-1900 can therefore be used as a selection criterion for blackleg resistance trait. Among the five fragments found, OPI01-280 may be used as a good candidate in blackleg screening. This is because of its higher R-value of 0.7059. Fragment OPE03-4000 gave a lower R-value of about 0.3495; therefore, it may not qualify to be a good candi-
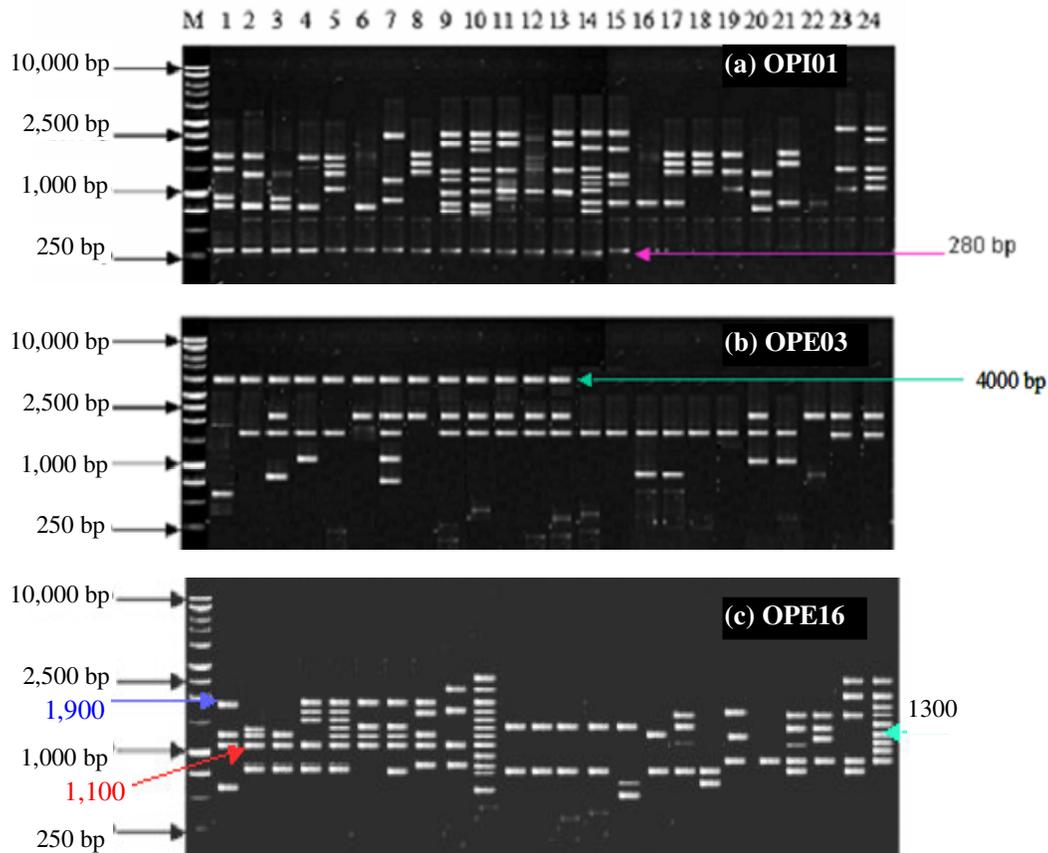
Figure 1. DNA amplification and fragments generated by different primers in Brassica species with genomes A and C: (a) OPI01: M= 1 kb DNA ladder; Lane 1 to 24 represents the following accessions and cultivars: NSL 6102, NSL 6106, NSL 6114, NSL 6116, NSL 6560, NSL 42980, NSL167291, NSL6146, Abilene, Arctic, Baldur, Jetton, Plainsman, Talent, Viking, NSL6112, NSL6117, NSL6125, NSL6558, NSL34675, NSL6145, NSL6153, Maestro and Rasmus, respectively. (b) OPE03: M= 1 kb DNA ladder; Lane 1 to 24 represents: NSL6102, NSL6114, NSL6125, NSL6560, NSL42980, NSL167291, NSL6146, Abilene, Baldur, Maestro, Plainsman, Talent, Viking, NSL6106, NSL6112, NSL6116, NSL6117, NSL6558, NSL34675, NSL6145, NSL6153, Arctic, Jetton and Rasmus, respectively. (c) OPE16: M= 1 kb DNA ladder; Lanes 1 to 24 represent: Lane 1 to 24 represents the following accessions and cultivars: NSL6102, NSL6114, NSL6560, NSL34675, NSL42980, NSL167291, NSL6146, Abilene, Arctic, Viking, NSL6106, NSL6112, NSL6116, NSL6117, NSL6125, NSL6558, NSL6145, NSL6153, Baldur, Jetton, Maestro, Plainsman, Rasmus and Talent, respectively.

date for screening purposes.

Through genetic analysis of reaction to *L. maculans* in *Brassica* species, both monogenic and polygenic models to explain heritability of resistance have been reported (Rimmer and van den Berg 1992). Genetic analysis in this study did not investigate which genes confer resistance at the adult and cotyledon stage. But, some plants were susceptible at the cotyledon and resistant at the adult stage, while others were consistently resistant in both adult and cotyledon stage. This response indicates that different resistant genes or alleles are expressed at different growth stages of the plant. Further study will be carried out to confirm whether there are single dominant genes that confer resistance on cotyledon and at the adult plant stage as earlier reported by other scientists. Dion et al. (1995) reported that there is a single gene for

adult plant resistance in cultivar Cresor. Their results demonstrated that genetic control of resistance to *L. maculans* differs within *Brassica* species based on growth stage at which the plant was inoculated.

Resistance reaction to *L. maculans* can be influenced in *Brassica* by environmental conditions, different sources of resistance, as well as different genetic backgrounds (Dion et al*.,* 1995). This is well illustrated by the complexity of resistance factors in plants tested under different conditions. Ferreira et al*.,* (1995) reported that in cultivar Major, a single major locus (*LEMI1*) and four other genomic regions were associated with cotyledon and adult plant resistance to PHW1245, which is a PG2 isolate. This may suggest that resistance genes could be operating at different developmental stages of plants, under varied environmental conditions or in response to differe-

nt virulence of pathogen populations. This makes it difficult to select appropriate plants for blackleg resistance in a segregating population in breeding programs. Therefore, DNA based markers such as RAPD and SSRs can be used to study the introgression of resistance genes without screening with the pathogen and irrespective of environmental conditions. In the follow up to this study, a SCAR marker will be developed by sequencing some of the identified RAPD fragments. Thus, the SCAR marker will be useful for selecting resistant plants in segregating populations. However, this study shows that RAPD fragments have the ability to predict disease rating in the resistant genotypes, and that resistance may also exist either in the A or C genomes and not only on the B genome as earlier reported.

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