Identification of the corresponding avirulence gene of *Leptosphaeria maculans* to the resistance gene (*LepR$_1$*) in *Brassica napus.*

Kaveh Ghanbarnia[1], Roger Rimmer[2], W. G. Dilantha Fernando[1]

[1]*Department of Plant Science, University of Manitoba, Winnipeg, Manitoba, R3T 2N2Canada,*
[2]*Agriculture and Agri-Food Canada, Saskatoon Research Centre, Saskatoon, SK S7N 0X2, Canada.*
Email: D_Fernando@Umanitoba.ca

Abstract

Blackleg of canola is the most important disease of canola/rapeseed worldwide. Efforts are made to understand the host-pathogen interaction by studying the interaction between resistance genes of the host and the avirulence genes of the pathogen. Previous studies showed that two double haploid canola (*Brassica napus* L.) lines, ddm-12-65-1 and ddm-12-65-2, possess a novel resistance gene (*LepR1*) against PGT (99-43 & 99-56) and PG2 (WA-74) isolates. However, these two lines are susceptible to PG2 isolate 87-41. It is thought that the avirulence of isolates WA-74, 99-43 and 99-56 on the lines ddm-12-65-1 and ddm-12-65-2 is governed by the *Avr1*/*LepR1* and the virulence of isolate 87-41 is governed by *avr1*/*LepR1*. Isolates 87-41 and 99-56 were selected as virulent and avirulent isolates based on phenotypic reactions caused by the isolates on the lines and their mating type. They were crossed and the progenies segregated based on the phenotypic reaction on the line ddm-12-6-1. The 87-41 × 99-56 *L. maculans* map will be constructed using PCR-based markers including single sequence repeats (SSR) and sequence related amplified polymorphic (SRAP) markers. Polymorphic bands will be recorded as present or absent in the parents and in the whole progeny. Once the physical distances of the *Avr* gene with its markers are identified, the genomic BAC library from *L. maculans* isolate 99-56 will be constructed. The library will be screened using the closely linked markers and a combination of markers could then be used to identify positive clones.

Key words: Avirulence, Leptosphaeria maculans, Brassica napus

Introduction

Blackleg is the most destructive pathogen of canola/rapeseed worldwide. In the *Leptosphaeria-Brassica* system, the gene-for-gene interaction has been suggested by genetic analysis of *B. napus* cv. Major and its resistance to *L. maculans* (Ferrerira *et al.,* 1995) and recent studies confirmed this system (Asan-Melayah *et al.,* 1997, Asan-Melayah *et al.,* 1998, Attard *et al.,* 2002, Balesdent *et al.,* 2002 and Rouxel *et al.,* 2003). To date six *L. maculans* AVR genes (*AvrLm1, AvrLm2, AvrLm3, AvrLm4, AvrLm7* and *alm1*) have been identified to control avirulence towards *B. napus*, two *Avrlm5* and *AvrLm6* control of avirulence toward *B. juncea* and a single gene (*AvrLm8*) control avirulence toward *B. rapa* using classical genetics on both the plant and the pathogen. Among all these genes only *AvrLm1* was cloned (Gout, et al 2006).

Avirulence gene characterization is a powerful tool for better understanding the molecular events associated with plant-pathogen interactions and also for screening matching resistance genes in the host plant. Most of studies in this area have been done based on European isolates and lines. Thus, developing closely linked markers using local isolates and lines and cloning the gene will be one of the main concerns of the recent research. Therefore the objectives of this project will be (1) to develop closely linked molecular markers (SRAP) to avirulence gene (2) map-based cloning of the target gene utilizing its genetic map.

Materials and Methods

Fungal Isolates

Two PG2 isolates 87-41and WA71 and two PGT isolates 99-56 and 99-43 were selected for mating type determination experiments and phenotype characterization.

Plant Material and Phenotype Characterization

The ddm-12-65-1 and ddm-12-65-2, canola lines used in this study possessed a newly recognized resistance gene, *LepR1*, which originated from *Brassica rapa* subsp. *sylvestris* (Yu, et al., 2005). Previous studies showed that this line is resistant to most of the PG2 isolates (i.e. WA-74) and a few PGT isolates (99-43 & 99-56) except isolate 87-41(PG2). To confirm these results, sporulating cultures of these isolates were obtained and phenotypic interaction of these isolates (*Avr* or *avr*) were determined by inoculation of cotyledons of the lines with 1 ml of 1×10^7 pycnidiospore suspension. Disease severity on cotyledons was assessed after 12 days based on a 0 to 9 scale.

In Vitro *Crosses and Mating Type Identification*

In order to genetically identify the *Avr* gene inducing resistance on cultivar possessing *LepR1* genes, crosses between isolate 87-41 as *Avirulence* (Avr) and isolates WA74, 99-43 and 99-56, as *avirulence* (avr) was performed using water–agar

and tooth-pick method (Gall, 1994, Mengistu, 1993). Cultures were incubated at 24 ºC with 24 h of cool–white fluorescent light for 7 days. To optimize condition to generate enough pseudothecia, the plates were then placed in 3 different incubators maintained at temperatures 10, 13 and 16 ºC with alternating periods of 12 h of near UV-light and darkness in both methods. The suitable opposite mating type which showed proper phenotypic reaction on cotyledons were crossed to recover progenies after random single sporing.

DNA Preparation and SRAP Markers

DNA of isolates and progenies were isolated based on protocols described previously (Attard et al., 2001). SRAP marker were used for detecting polymorphic loci in parents and progeny isolates using ABI 3600 DNA analyzer based on previously described protocols (Li and Quiros, 2001).

Mapping and BAC Library Screening

The "*Avirulence* isolate" × "*avirulence* isolate" *L. maculans* map will be constructed based on SRAP markers. Presence or absence of polymorphic bands will be recorded for the parents and whole progeny. Monogenic markers will be finally subjected to multipoint linkage analysis using the mapmaker software. Once the genetic distances of the corresponding avirulence gene to *LepR1* resistance gene with its markers is identified, the genomic BAC library from *L. maculans* isolate will be screened using the closely linked markers. Combination of markers could then be used to narrow down the positive clones.

Results

Phenotypic Reaction

The results of inoculation of the line ddm-12-65-1 with isolates 87-41, WA74, 99-43 and 99-56 showed that isolate 99-56 produce incompatible reaction and was considered as an isolate which possesses *Avr* gene. Isolate 87-41 showed a compatible reaction which is indicative of a lack of *Avr* gene. The phenotypic reaction of two other isolates (WA-74 and 99-43) on the line are different from phenotypic reaction of isolate 87-41. However, the rating score of the infected plants with these two isolates are intermediate to susceptible. These results imply that the phenotypic recognition of these isolates from virulent isolate (87-41) is difficult based on reaction on cotyledons. The experiment was repeated three times and the results have been shown in Table 1.

Table1- Mean cotyledon interaction phenotypes of line ddm-12-65-1 which possesses *LepR1* after inoculation of four different isolates

	Mean Interaction phenotype		
Isolates	Test 1	Test 2	Test 3
87-41 (PG2)	7.5	7.8	7.3
WA74 (PG2)	5.8	6.3	6.5
99-43 (PGT)	5.8	5.9	6.1
99-56 (PGT)	1.5	2.1	2.5

Note: Since two isolates WA74 and 99-43 showed intermediate phenotypic reactions and did not confirm to the previous data as avirulent isolates, they were not used in further studies.

Determination of Mating Type Using Tooth-Pick and Water-Agar Method

All the avirulence isolates (99-56, 99-43 and WA-74) were crossed with virulence isolate (87-41). The results showed that two isolates 99-56 and WA-74 belong to one mating type and two others (99-43 and 87-41) belong to another mating type group. Therefore two isolates 87-41 as a virulent and 99-56 as avirulent isolates were used in the cross for further studies based on the results of mating type determination and phenotypic reaction. One hundred single spored ascospores were obtained.

Discussion

The phenotypic reaction of four isolates WA71, 99-43 and 99-56 as *Avirulent* isolates and 87-41 as *avirulent* isolate were described previously (Yu, et al 2005). That results showed a rating score of 2.9 and 3.7 for WA 71 and 99-41 on *LepR1*-harboring line but our results showed higher scoring rate of 6.2 and 5.9 for these two isolates respectively. This discrepancy is probably due to the effect of genetic background of donor plant (*B. napus* subsp. s*ylvestris*) after only second backcross BC_2S_3, on the recurrent parent, compared to BC_2F_5 derived from DH line originated from the same cultivar used by Yu, et al(2005). They also mentioned that two isolates, WA71 and 99-43 are more aggressive than 99-56. In this study, we preferred map-based cloning strategy due to amenability of *L. maculans* to molecular genetic approach and successfulness of this method in cloning the first avirulence gene in this fungus (Gout, et al 2006). Attard et al (2002) mentioned the presence of recombination-deficient regions and transposon elements in *AvrLm1* region. They also mentioned that the organization of the markers is conserved. However, using efficient numbers of randomly used primers in SRAP marker-system can overcome this difficulty.

References

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

Asan-Melayah, D .Rouxel, T, Bertrandy,J., Letarnec,I and Balesdent, M. H. 1997. Genes for race-specific resistance against blackleg disease in *Brassica napus* . Plant Breed., 117:373-378.

Attard, A., Gourgues, M. Gout, L., Schmit, J., Roux, J., Narcy,J.P., Balesdent, M.H. & Rouxel.T.2001.Molecular characterization and polymorphism of *MinLm1*, a minisatellite from the phytopathogenic ascomycete *Leptosphaeria maculans. Curr. Genet., 40;54-64.*

Attard A., Got, L, and Rouxel, T. 2002. Analalysis of molecular markers genetically linked to the *Leptosphaeria maculans* Avirulance gene *AvrLm1* in field populations indicates a highly conserved event leading to virulence on Rlm1 genotypes. Phytopathology: Vol, 15: 672-682.

Balesdent, M. H. , A., Attard, A., Kuhn, M.L., Rouxel, T. 2002. New avirulance genes in the phytopathogenic fungus *Leptosphaeria maculans*. Phytopathology, 92:1122-1133.

Gall, C. Balesdent, M.H. Robin, P., Rouxel, T. 1994. Tetrad analysis of acid phosphatase , soluble protein patterns, and mating type in *Leptosphaeria maculans*. Phytopathology, 84:1299-1305.

Gout, L., Fudal, I.., Kuhn, M., Blaise, F., Eckert, M., Cattolico, L., Balesdent, M., and Rouxl, T. 2006. Lost in the middle of nowhere: the *avrLm1* avirulence gene of the Dothideomycete *Leptospaeria maculans*. Mol. Microbiol., 60(1): 67-80.

Li, G., and Quiros, C. F. 2001.Sequenced-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in *Brassica.*Theor. Appl. Genet. ,103: 455- 461.

Mengisto, A., Rimmer, R., Williams, P. H. 1993. Protocols for in vitro sporulation, ascospore release, sexual mating, and fertility in crosses of *Leptosphaeria maculans.*Plant Dis., 77:538-540.

Rouxel, T., Penaud, A., Pinochet, X., Brun, H., Gout, L., Delourne, R., Schmit, J. and Balesdent, M.2003. A 10-year survey of populations of *Leptosphaeria maculans* in France indicates a rapid adaptation towards in *Rlm1* resistance gene of oilseed rape. European J. Plant Pathol., 109:871-881.

Yu, F., Lydiate, D.J. and Rimmer, S.R. 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. Theor. Appl. Genet., 110: 969-979.