The Australian Canola Molecular Marker Program - An update on mapping loci controlling resistance to blackleg in canola

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ABSTRACT

The current Australian Canola Molecular Marker Program (ACMMP, 2008-2013) is funded by the Australian Grains Research and Development Corporation, NSW Department of Primary Industries, University of Queensland (UQ) and Charles Sturt University, and aims to develop and validate molecular markers associated with resistance to blackleg and shatter and tolerance to drought. These traits were recognised as priorities for the Australian canola breeding programs. The ACMMP has access to several doubled haploid (DH) populations and relevant germplasm, and bioinformatics support at UQ. We mapped two DH populations of canola (*Brassica napus L)* derived from Skipton/Ag-Spectrum and BLN3347/Carousel-10 using simple sequence repeats and candidate gene based markers. Both DH populations were evaluated for resistance to blackleg at the seedling and adult plant stages. Loci associated with blackleg resistance were identified and compared with already tagged qualitative and quantitative resistance genes in canola.

Key words: Australian canola molecular marker program, molecular mapping, blackleg resistance, QTL analysis

INTRODUCTION

Canola (2n=4x=38, genome: AACC) is the third largest crop in Australia after wheat and barley, and the most significant oilseed crop. The Australian canola breeders group (CBG) and National Brassica Germplasm Improvement Program (NBGIP) have defined the following priority traits; alternative sources of blackleg resistance, drought tolerance and shatter resistance, for germplasm enhancement to support and improve the Australian canola industry.

Blackleg, caused by the fungal pathogen *Leptosphaeria maculans (Desm.) Ces. et de Not.,*(anamorph: *Phoma lingam*), is a serious disease that affects both yield and quality of canola (*Brassica napus L)*. This disease is highly prevalent in various parts of the world including in Australia and Canada. *L. maculans* is highly variable for pathogenicity which is caused by a number of virulence genes. Utilization of host resistance to blackleg is an effective approach to control yield losses in canola. To date, more than 10 major loci (*Rlm1-10 and LepR1 to LepR4*) conferring resistance to *L. maculans* have been catalogued in *Brassica napus, B. rapa ssp syvestris, B. juncea,* and *B. carinata* and most of them were mapped by different molecular markers. Various linkage mapping studies revealed that most of the resistance genes are localised on chromosome A7 (N7). Whether the loci that map on A7, are allelic or not, remains unknown. Most of the mapping studies utilised relatively small populations and different marker systems; therefore, identification of resistance genes on the basis of their linkage with molecular markers is difficult. Furthermore, the consistent ability of *L. maculans* to overcome genetic resistance genes further necessitates the identification of new sources of resistance to control blackleg.

A number of quantitative trait loci (QTLs) determining adult plant resistance have been identified (Delourme et al. 2008). However, very little information is available on the location of (especially quantitative ones) genes conferring blackleg resistance in the Australian cultivars. Molecular markers have been successfully employed to dissect qualitative and quantitative

resistance in various crops including canola. Many molecular mapping studies have utilised segregating mapping populations which may have limited value for canola improvement programs. We have utilised breeding populations for mapping blackleg resistance loci, as they are directly relevant to breeding programs. The objective of this study was to determine the inheritance and location of blackleg resistance genes, and to identify molecular markers linked with resistance loci applying a whole genome mapping approach.

MATERIALS AND METHODS

Two DH populations derived from Skipton/Ag-Spectrum (177 lines), BLN3347/Carousel-10 (62 lines) were generated via microspore culture and used for the genetic mapping of blackleg resistance loci.

Phenotyping for blackleg resistance

Three different experiments were conducted to evaluate parents and DH populations for blackleg resistance: (glasshouse experiment in 2008, field experiment in 2008, and glasshouse experiment in 2009).

Identification of the appropriate isolates for phenotyping

NBGIP has made available a number of DH populations to the ACMMP. Parental lines of the DH populations: Skipton, Ag-Spectrum, BLN3347, Carousel-10 and Surpass400 (representing "*sylvestris*" resistance, *LepR1, Rlm1*) along with standard check varieties: Beacon (representing "polygenic" resistance), Carousel, and Q2 (*Rlm3*) were evaluated for resistance to 11 single spore isolates of the blackleg pathogen (04MGPP031, 04MGPP041, 06MGPP005, 06MGPP019, 06MGPP041, 04MGPS021, 04MGPS010, 04MGPS015, 04MGPS014, 04MGPP016 and 05MGPP028) at the seedling (cotyledon) and at the adult plant stages in the glasshouse during the 2008 growing season.

Twelve seeds of each genotype were sown in plastic pots (20 cm diameter) containing a commercial potting mix. The plants were thinned to four per pot, along with one Q2 plant (susceptible check) in the centre of each pot. Each plant was inoculated on the two lobes of the two cotyledons with 10µl of a suspension containing 10^6 pycnidiospore /mL. Plants were placed in a dew chamber at 100% humidity at 20°C for 48 h and then returned to a shadehouse. Seventeen days after inoculation (dai), each inoculation point on the cotyledons was scored twice for resistance using a published rating system, where 0 = no darkening around wounds and 9 = large grey-green lesions with profuse sporulation. Average lesion score was used to rate genotypes for blackleg resistance: 0 - 3.5 = resistant; 3.6 - 4.9 = intermediate; 5.0 - 9 = susceptible.

After scoring cotyledons, plants were allowed to develop through to maturity. Plants were scored for basal internal infection (0-100%) and whether the plant died or not (0= no death, 1=death). Average adult internal infection of 0 – 35% = resistant; 36 – 49% = intermediate, and 50 – 100% = susceptible.

Screening isolates for virulence genes

DNA was isolated from mycelia of all 11 single spore isolates of *L. maculans*. DNA samples were analysed for the presence with primer-pairs specific to *AvrLm1* and *AvrLm6* genes respectively (de Wouw et al. 2009; Fudal et al. 2007; Gout et al. 2006). PCR analysis and gel electrophoresis was carried-out as described previously.

Screening DH population for blackleg resistance

(1) *Glasshouse conditions:* Both DH populations were evaluated for resistance to *L maculans* isolates 04MGPP021 and/or 06MGPP041 at the seedling stage in the glasshouse during the 2009 growing season. Each of the DH populations was tested separately in two experiments and each DH line replicated twice. A spatially optimized incomplete block design with a nested blocking structure was employed to estimate different components of variance. Inoculations and disease assessments were made as described above at the cotyledon growth stage and will be undertaken at the adult plant stage when they reach maturity.

(2) Field conditions: The two DH populations were also evaluated in the field blackleg nursery at Wagga for resistance at the adult-plant stage in 2008. A randomized design with two replicates was employed. Ten plants from each DH lines were individually scored quantitatively as having 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 % internal infection, as described above. The DH lines from Skipton/Ag-Spectrum were sown in single rows; there were 177 DH lines, the two parental lines of the DH population, plus the susceptible variety Karoo. Due to moisture stress, the germination and subsequent growth of the BLN3347/Carousel-10 population was poor and it was not suitable for reliable evaluation of blackleg resistance.

Construction of framework maps and QTL analysis

DNA was isolated from approx 10 week-old glasshouse grown seedlings using a standard phenol-chloroform method. Six hundred and eighty four simple sequence repeat (SSR) and candidate gene based markers were collated from the public domain and were investigated for polymorphism between the parents of the DH populations. The forward primers of each primer-pair were tagged with a 19bp long M13 sequence. PCR amplicons were generated using fluorescently labelled M13 dye, in 12 µl reactions as described previously (Raman et al. 2005). Amplified DNA fragments were separated on a CEQ8000 DNA sequencer (Beckman Coulter Inc.) and their sizes measured using fragment analysis software as described by Raman et al. (2005). Polymorphic marker loci were further analysed in all DH lines. Chi-square tests for segregation distortions were performed at threshold values of p=0.05. Genetic linkage maps were produced using Map Manager software using Kosambi mapping function at probability of 0.01, as described previously (Raman et al. 2009). An integrated map covering all 19 chromosomes was subsequently employed for the QTL analysis using the Map Manager version QTX20b and Cartographer.

RESULTS

Genetic variation for resistance to 11 different isolates of *L maculans* was identified between the parental lines of the DH populations (Table 1). The susceptible control, Canadian cv. Q2 (*Rlm3*) exhibited susceptibility to all isolates of *L maculans* and had a mean disease score > 5. Molecular analysis of isolates using primer-pairs specific to *AvrLm1* and *AvrLm6* loci revealed the presence of the *AvrLm1* allele (presence of 1124 bp fragment) in 04MGPP016, 04MGPP031, 04MGPP041 and 06MGPP019 and the *AvrLm6* allele in 04MGPS021 and 06MGPS019 isolates.

Table 1. Screening of parental lines for blackleg resistance at cotyledon stage using 11 isolates of *Leptosphaeria maculans* L. Lesion scores (0-9 scale) were rated as: 0-3.5=R (resistant); 3.6-3.9=I (intermediate resistant); and 4-9=S (susceptible). *Avr* and *Vir* refer to avirulence and virulence alleles of *L. maculans* respectively. ND: not determined. -: Missing data. Shaded responses show isolates used for phenotyping DH populations.

Isolate	Skipton	Ag-Spectrum	BLN3347	Carousel-10	Surpass 400	Q2	Beacon	*AvrLm1*	*AvrLm6*
04MGPP016	I	S	S	R	R	S	S	Avr	Vir
04MGPP031	S	S	S	S	R	S	S	Avr	Vir
04MGPP041	S	S	S	S	R	S	S	Avr	Vir
04MGPS010	S	S	S	S	S	S	S	Vir	Vir
04MGPS014	I	S	I	S	S	S	S	Vir	Vir
04MGPS015	I	S	S	S	S	S	S	Vir	Vir
04MGPS021	R	S	S	S	S	S	S	Vir	Avr
05MGPP028	R	-	S	-	S	S	-	ND	ND
06MGPP005	S	S	S	S	S	S	S	Vir	Vir
06MGPP019	I	S	R	R	R	S	S	Avr	Avr
06MGPP041	S	R	S	R	S	S	I	Vir	Vir

Expression of blackleg resistance to some isolates was different at the seedling and adult plant stages, when parental lines were scored at 17 dai and at maturity respectively (data not shown). Isolates 04MGPS021 and 06MGPP041 clearly exhibited contrasting phenotypes in both the parents Skipton and Ag-Spectrum (Table 1). Therefore, we used both these isolates for evaluating blackleg resistance in the DH population and to map associated loci. However in the BLN3347/Carousel-10 population, we used only one isolate 06MGPP041 for phenotyping. The mean lesion scores for BLN3347 and Carousel-10 were 6.3 and 3.3 respectively. DH lines from both populations showed clear segregation for resistance. Frequency distributions indicated that one to two genes control resistance to *L maculans* in the DH populations (Fig 1).

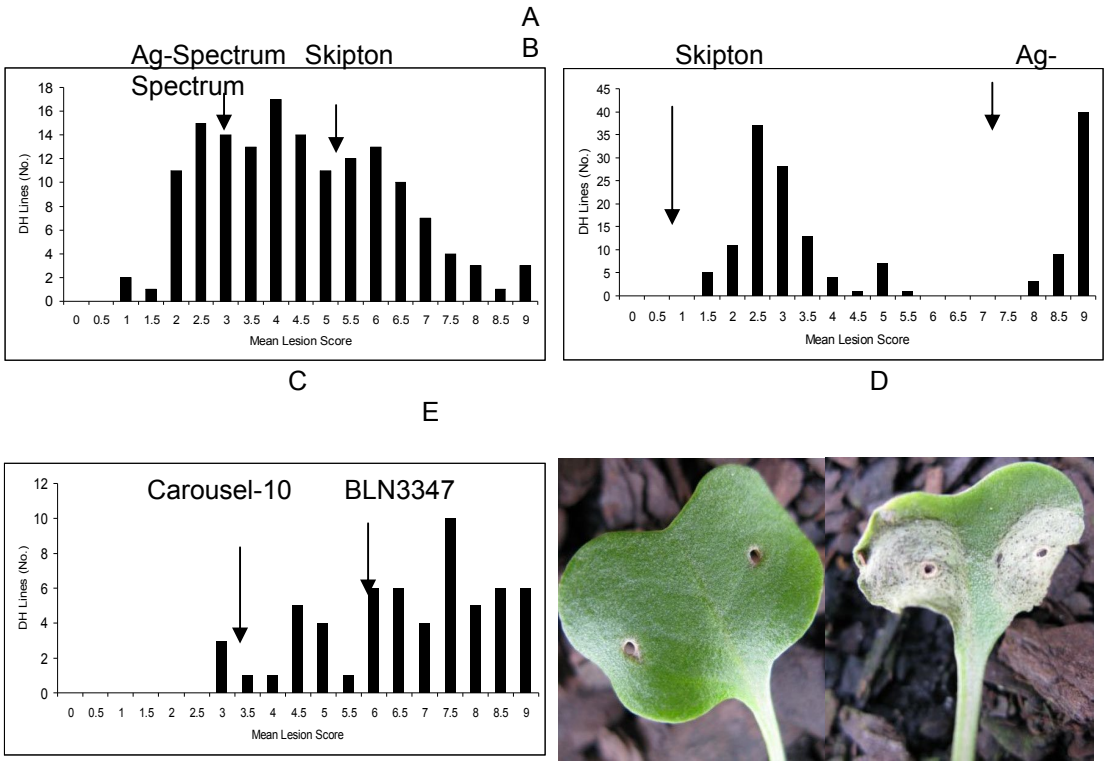

Fig 1: Phenotypic distribution of mean lesion scores at the cotyledon stage of two DH populations. DH population from Skipton/Ag-Spectrum inoculated with single spore isolate (A) 06MGPP041, and (B) 06MGPS021, and (C) DH population from BLN3347/Carousel-10 inoculated with 06MGPP041 isolate. Mean lesion scores of parental lines are shown by arrows. (D): Resistant phenotype of Skipton and (E): Susceptible phenotype of Ag-Spectrum upon inoculation with 06MGPS021 isolate (at cotyledon stage)

Linkage maps of DH populations

We screened 684 markers for polymorphisms between the parental lines of DH populations. Of which, 215 and 142 were polymorphic in Skipton/Ag-Spectrum and BLN3347/Carousel-10 derived populations, respectively. A number of markers showed segregation distortions in both the DH populations. In Skipton/Ag-Spectrum population, the 215 SSR markers mapped to 280 loci. To date, we have mapped 150 SSR marker loci in the BLN3347/Carousel10 population.

Preliminary data on genome wide scanning detected a major QTL on chromosome A7 (N7) that explained more than 21% of phenotypic variation for blackleg resistance at the cotyledon stage in the Skipton/Ag-Spectrum population. Skipton contributed an allele for increasing resistance to isolate 06MGPS021. This QTL region corresponds to a genomic region of Arabidopsis that is involved in defence against fungi and bacteria. Under field conditions, we identified three QTLs associated with blackleg resistance on chromosomes A5, C2, and C3 in 2008. The phenotypic variation explained by these individual QTL ranged from 10% to 14%. Skipton possessed both the alleles located on chromosomes C2 and C3. The resistance allele

on A5 was contributed from Spectrum. Our results suggest that seedling and adult plant (field) resistance are not highly correlated and may be controlled by different genes.

DISCUSSION

Canola improvement programs have been successful in developing varieties resistant to blackleg disease for the last 30 years in Australia. Most of these varieties were selected for resistance under field conditions. The efficiency of selection for blackleg resistance can be enhanced using marker assisted selection of targeted resistance loci over simple phenotypic selection. It is well known that field based screening is highly dependent upon weather conditions, pathogen pressure and inoculation source. Molecular marker based selection of blackleg resistance would allow culling of undesirable individuals particularly during the early stages of the breeding program and would increase selection efficiency, as field testing may be confounded with the environment leading to uneven natural infection and subsequent disease development. Molecular markers would also allow pyramiding different qualitative and quantitative genes for resistance.

In this study, we identified four genomic regions that are associated with blackleg resistance in Skipton/Ag-Spectrum. Our results also suggested that different loci control seedling and adult plant resistance to *L maculans*. Further research in combining both these QTLs in a single background is in progress. We found QTLs with small and moderate effects. These gene effects are anticipated to be additive. Various Australian canola breeding programs produce DH lines for variety development. Many of the parental lines used for producing DH lines are not completely homozygous and 'pure'. These DH populations can still be used for mapping and validation of trait-marker associations (in this case, for blackleg resistance), but these populations are not ideal for genomics studies. CMMP is producing and accessing 'new populations' that are more suited for mapping and validation of genes for various traits required by the Australian breeding programs.

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