Blackleg resistance in rapeseed: phenotypic screen, molecular markers and genome wide linkage and association mapping

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ABSTRACT

High-throughput methods for screening rapeseed germplasm for blackleg resistance were developed and subsequently utilised for mapping qualitative and/or quantitative trait loci in the doubled haploid populations derived from Skipton/Ag-Spectrum, Maxol*1/Westar10, Columbus*3/Westar10, and BLN2762/Surpass400. We have now identified and validated molecular markers associated with blackleg resistance in these populations and anticipate that these molecular markers will position Australian canola breeding programs to perform marker assisted selection and enrich desirable alleles for blackleg resistance in their germplasm.

The identification and validation of qualitative and quantitative trait loci has been restricted exclusively to bi-parental mapping/breeding populations. We expect that genome wide association analysis, especially in contemporary breeding germplasm and elite cultivars, may lead to a more effective marker implementation strategy in rapeseed breeding programs, as more than two alleles (unlike in bi-parental population) can be sampled simultaneously and it does not require structured populations. In order to test this approach, we performed association analysis of 181 accessions of *Brassica napus*, one of *B. rapa*, three of *B. juncea* and two of *B. carinata* collected from different parts of the world. All accessions were evaluated for blackleg resistance at both seedling and adult plant stages using single spore isolates and were subsequently genotyped with 1513 Diversity Array Technology (DArT) and SSR markers. Association mapping identified several genomic regions associated with blackleg resistance, including those identified with QTL analysis. Our results confirmed that the genome wide association mapping approach can complement 'traditional' QTL analysis. Precise knowledge of intra-and inter-genomic duplications and other chromosomal rearrangements, such as inversions, is required to enhance our understanding of the genetic network underlying blackleg resistance in rapeseed.

Key word: Blackleg resistance – canola – germplasm – molecular markers – linkage mapping

INTRODUCTION

Blackleg, caused by *Leptosphaeria maculans* (Desm.), is one of the most serious diseases of rapeseed (*Brassica napus* L, 2n=4x=38, genomes: AACC) in Australia, Europe and Canada. Under severe epiphytotic conditions, this disease can account for 90% of yield losses (Marcroft and Bluett 2008). It is widely accepted that without effective genetic resistance to blackleg, there will be no rapeseed (canola) industry in Australia. This disease can be managed following best agronomic practices such as crop rotation, stubble management and application of fungicides. However, deployment of genetic resistance is the most cost-effective and environmentally friendly strategy to minimise yield losses.

Genetic variation for blackleg resistance exists within Brassica species and has been exploited, especially from *B. rapa,* in breeding programs worldwide. As a result, several varieties resistant to blackleg have been released for commercial cultivation, especially in Australia,

France and Canada. During the last few decades, significant achievements have been made to characterise the blackleg pathogen, and to understand the inheritance of resistance and pathogen-host interactions. In addition to several quantitative trait loci, 14 major loci (*Rlm1-10* and *LepR1* to *LepR4*), conferring resistance to specific races of *L. maculans,* have been identified on chromosomes A2, A7, A8, and A10 (Delourme et al. 2006; Rimmer 2006; Yu et al. 2005; Yu et al. 2008).

In recent years, molecular markers have become an integral part of plant breeding programs to enhance selection efficiency of target traits. In rapeseed, a number of qualitative and quantitative genes conferring blackleg resistance have been tagged using molecular markers in different mapping populations (Delourme et al. 2006; Dusabenyagasani and Fernando 2008; Yu et al. 2005; Yu et al. 2008). These studies revealed that at least five resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9*) are localised in a cluster in a 35 centiMorgans genomic region on chromosome A7 (Delourme et al. 2004; Delourme et al. 2006). In order to use genetic markers for routine marker assisted selection in rapeseed breeding programs, blackleg resistance-molecular marker associations need to be identified and validated in diverse genetic backgrounds. Furthermore, high-throughput and reliable methods amenable for screening large populations for blackleg resistance are also required to validate such associations, in order to make rapid genetic gains in the breeding programs.

Within the Australian Canola Molecular Marker Program, we (i) developed the protocol for screening rapeseed germplasm for blackleg resistance, (2) determined the inheritance and genetic locations of blackleg resistance loci in the doubled haploid populations derived from Skipton/Ag-Spectrum, Maxol*1/Westar10, Columbus*3/Westar10, and BLN2762/Surpass400, and (3) identified and validated molecular markers linked with resistance loci, using QTL and genome wide-association mapping approaches (GWAS).

MATERIALS AND METHODS

In order to develop a method suitable for phenotyping several thousand lines within a few months, we raised the rapeseed plants in plastic trays (7 x 8 wells) under glasshouse conditions. Seven seedlings/genotype/replication were raised in the pre-soaked Jiffy discs. Approximately two-wk old seedlings were used for inoculations. Each lobe of the cotyledons was punched and then inoculated with the spore suspension of the *L. maculans* isolate(s).

Molecular markers were genetically mapped in four DH populations, derived from Skipton/Ag-Spectrum, Maxol*1/Westar10, Columbus*3/Westar10, and BLN2762/Surpass400, in order to identify blackleg resistance loci. The populations derived from Skipton/Ag-Spectrum and BLN2762/Surpass400 were constructed via microspore culture at the Wagga Wagga Agricultural Institute by Mr Ross Smithard. DH lines from Maxol*1/Westar10, and Columbus*3/Westar10 were developed by Ms Denise Barbulescu at the Victorian Department of Primary Industries, Horsham. In addition, diverse germplasm comprising 181 accessions of *Brassica napus*, one of *B. rapa*, three of *B. juncea* and two of *B. carinata* collected from different parts of the world were utilised for genome wide association mapping to (i) identify and validate trait-marker associations identified in the above bi-parental populations and (ii) identify novel alleles associated with blackleg resistance.

Phenotyping for blackleg resistance: Plants were screened at the cotyledon and adult plant growth stages under shade house, glasshouse and field conditions, in 2009, 2010 and 2011, using single spore *L. maculans* isolates procured from the University of Melbourne (kindly provided by Dr Angela Van de Wouw, University of Melbourne) and Marcroft Grain Pathology labs, Horsham. Inoculum was prepared by subculturing the *L. maculans* isolates on 10% V8 agar plates. The spores were dislodged into distilled water and spore concentration was adjusted to 5 x 10^6 spores/mL. We inoculated at least 28 cotyledon lobes/genotype/replication, and each inoculation site was inoculated with 10 µl of a fungal spore suspension. Plants were placed in a dew chamber at 100% relative humidity, at 18-20°C, for 48 h, and then returned to a glasshouse maintained at 18°C. After 2-3 wks of inoculation, each inoculation point was scored for resistance using the rating system of Koch et al. (1991). All experiments were replicated according to statistically valid designs made using the DiGGer program (Coombes 2002). Some populations were also evaluated for blackleg resistance at the adult plant stage under

glasshouse/field conditions. Up to ten plants from each DH line were scored for percent internal infection using 0 (resistant)-100 (most susceptible) scales and or using digital software.

Marker analysis: In order to map resistance loci that are effective at seedling and adult plant stages, we constructed linkage maps of Skipton/Ag-Spectrum (Raman et al. 2010) and BLN2762/Surpass400 (unpublished), which were further employed for QTL analysis. Blackleg resistance loci in Columbus and Maxol have been mapped previously on chromosome A7 (Delourme et al 2006). Therefore, we analysed markers only specific to chromosome A7, in the DH lines from Columbus*3/Westar and Maxol*1/Westar, and then determined the linkage between resistance loci and the molecular markers.

Validation of blackleg resistance-marker associations: We employed a genome wide association mapping approach to identify and validate blackleg resistance in a set of germplasm comprising 187 diverse accessions. All accessions were evaluated for blackleg resistance at both seedling and adult plant stages using 12 single spore isolates (unpublished). In this study, we only present results on isolate 04MGPS021. Accessions were subsequently genotyped with 1513 Diversity Array Technology (DArT) and SSR markers as described previously (Raman et al 2010). Markers that had excessive missing data were removed before genetic analysis. Association mapping was carried out using different algorithms such as general linear model, mixed model and SMQ as described previously.

RESULTS AND DISCUSSION

Protocol for phenotyping blackleg resistance: The phenotyping method was found to be suitable for screening a large number of genotypes. The plastic trays proved to be suitable for supporting healthy plants over a long period of time, even up to maturity. It was necessary to clip young leaves regularly to divert the seedling's energy toward cotyledon growth. Seedlings raised in Jiffy pots were suitable for transplanting with 100 per cent survival under shade house/glasshouse/birdcage/field conditions. Currently, we are utilising this protocol to screen thousands of individuals for blackleg resistance at Wagga Wagga.

Mapping blackleg resistance loci: In a DH population from Skipton/Ag-Spectrum, we identified both major and minor quantitative loci for blackleg resistance (Raman et al. 2009; Raman et al. 2010). A major locus was identified on chromosome A7 that exhibited significant association with blackleg resistance scored at the cotyledon stage. A suite of SSR markers were identified; one of them mapped approximately 0.8 cM from blackleg resistance locus. In addition, we identified several QTLs on chromosomes A2, A9, A10, C1, C2, C3 and C6 for blackleg resistance evaluated under field conditions in years 2008 and 2009. These QTLs are planned for validating their stability in different environments.

In the Maxol*1/Westar and Columbus*3/Westar DH populations, we identified two loci for blackleg resistance (*Rlm1* and *Rlm3*), as reported previously. We used a differential set of *L. maculans* isolates developed by the University of Melbourne and INRA France to characterise host resistance genes in these lines.

In the BLN2762/Surpass400 population, we identified at least two major genes for blackleg resistance, evaluated using single spore isolates. These genes are located on chromosomes A7 and A10. Previously, the *LepR3* gene from Surpass400 has been mapped on A10. We have accessed tightly linked markers developed under the blackleg Consortium Canada (kindly provided by Drs Derek Lydiate and Nick Larkans) and confirmed the linkage between blackleg resistance and molecular markers that we have identified in BLN2762/Surpass400 population. Our results confirmed that one of the blackleg resistance loci, maps within the *Lep3* genomic region on A10 identified in the Westar/Surpass400 (Yu et al 2007). Under field conditions, *LepR3* does not seem to confer resistance to blackleg, as all the DH lines from BLN2762/Surpass400 were susceptible under one environment in 2010. On the other hand, a significant effect of the resistance locus located on chromosome A10 was observed under field conditions, in an independent experiment in 2009.

Validation of linkage between blackleg resistance loci and molecular markers: The genome-wide association mapping study using 1513 markers enabled us to identify and validate

genomic loci associated with blackleg resistance. To our knowledge, this is the first GWAS performed for blackleg resistance evaluated at the seedling stage.

We detected significant marker - race-specific blackleg resistance associations (P<0.01) at the seedling and adult plant stages. Loci for resistance were located on chromosomes A1, A2, A3, A5, A6, A7, A10, C1, and C2. In our previous mapping study, we could only identify a significant marker-blackleg resistance association (isolate 04MGPS021) on chromosome A7 in a DH population derived from Skipton/Ag-Spectrum. This suggests that association mapping is an efficient approach for identifying novel loci/alleles associated with blackleg resistance in diverse germplasm. Several genomic regions associated with blackleg resistance that we identified, are consistent with map locations of blackleg resistance genes on chromosomes A2, A7 and A10 detected in previous studies. We also identified superior molecular marker allele(s) associated with blackleg resistance to isolate 04MGPS021 that has been captured by the Australian canola breeding programs and therefore is likely to facilitate marker assisted selection. Desirable alleles may be enriched to develop elite germplasm having new gene/allele combinations for blackleg resistance. Results on linkage and genome wide association mapping studies will be discussed in context to intra-and inter-genomic duplications and other chromosomal rearrangements in order to enhance our understanding of the genetic network underlying blackleg resistance in rapeseed.

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REFERENCES

Coombes NE (2002) The reactive tabu search for efficient correlated experimental designs. PhD thesis, John Moores University, Liverpool UK

Delourme R, Chevre 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 114:41-52

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

Dusabenyagasani M, Fernando WGD (2008) Development of a SCAR marker to track canola resistance against blackleg caused by *Leptosphaeria maculans* pathogenicity group 3. Plant Disease 92:903-908

Marcroft S, Bluett C (2008) Blackleg of canola. Agriculture notes, State of Victoria, Department of Primary Industries, May 2008, AG1352, ISSN 1329-8062.

Raman H, Raman R, Rehman A, Taylor B, Wratten N, Marcroft S, Lindbeck K, Van De Wouw A, Batley J, Edwards D, Stiller J, Luckett D (2009) The Australian Canola Molecular Marker Program-An Update on mapping loci controlling resistance to blackleg in canola. Proceeding of 16th Australian Research Assembly on Brassicas. pp 157-161

Raman R, Belinda Taylor SM, Paul Eckermann, Ata Rehman, Kurt Lindbeck, David Luckett, Neil Wratten, Jiri Stiller, Jacqueline Batley, David Edwards, Raman H (2010) Genetic map construction and localisation of qualitative and quantitative loci for blackleg resistance in canola (*Brassica napus* l.). Proceedings of the 17th Crucifer Genetics Workshop, 5-8 Sept, Saskatoon, Canada

Rimmer SR (2006) Resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Can J Plant Pathol-Rev Can Phytopathol 28:S288-S297

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

Yu F, Lydiate DJ, Rimmer SR (2008) Identification and mapping of a third blackleg resistance locus in *Brassica napus* derived from *B. rapa* subsp. *sylvestris*. Genome 51:64-72