Theor Appl Genet (2012) 125:405–418
DOI 10.1007/s00122-012-1842-6

ORIGINAL PAPER

Molecular mapping of qualitative and quantitative loci for resistance to *Leptosphaeria maculans* causing blackleg disease in canola (*Brassica napus* L.)

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Received: 24 January 2011 / Accepted: 5 March 2012 / Published online: 28 March 2012

Abstract Blackleg, caused by *Leptosphaeria maculans*, is one of the most important diseases of oilseed and vegetable crucifiers worldwide. The present study describes (1) the construction of a genetic linkage map, comprising 255 markers, based upon simple sequence repeats (SSR), sequence-related amplified polymorphism, sequence tagged sites, and EST-SSRs and (2) the localization of qualitative (race-specific) and quantitative (race non-specific) trait loci controlling blackleg resistance in a doubled-haploid population derived from the Australian canola (*Brassica napus* L.) cultivars Skipton and Ag-Spectrum using the whole-genome average interval mapping approach. Marker regression analyses revealed that at least 14 genomic regions with LOD $\geq$ 2.0 were associated with qualitative and quantitative blackleg resistance, explaining 4.6–88.9 % of genotypic variation. A major qualitative locus, designated *RlmSkipton* (*Rlm4*), was mapped on chromosome A7, within 0.8 cM of the SSR marker *Xbrms075*. Alignment of the molecular markers underlying this QTL region with the genome sequence data of *B. rapa* L. suggests that *RlmSkipton* is located approximately 80 kb from the *Xbrms075* locus. Molecular marker-*RlmSkipton* linkage was further validated in an F_2 population from Skipton/Ag-Spectrum. Our results show that SSR markers linked to consistent genomic regions are suitable for enrichment of favourable alleles for blackleg resistance in canola breeding programs.

Communicated by H. Becker.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-012-1842-6) contains supplementary material, which is available to authorized users.

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Introduction

Blackleg, syn. Phoma stem canker, caused by the devastating hemi-biotrophic fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. & de Not., [anamorph: *Phoma lingam* (Tode:Fr.) Desm.], is a serious disease that affects both yield and quality of oilseed and vegetable *Brassicas* including canola (*Brassica napus L.*, $2n = 4x = 38$, genomes: AACC). This disease is prevalent worldwide and causes yield losses of millions of tonnes in Europe, North America, Australia, and Africa (Fitt et al. 2006; Howlett 2004; West et al. 2001). Predictions suggest that the severity and range of blackleg epidemics will increase under changing climatic conditions (Evans et al. 2008). In

Australia, this disease can cause yield losses of up to 90 % under severe epiphytotics (Marcroft and Bluett 2008). The pathogen not only kills seedlings and young plants, but also grows systemically within the host and leads to stem canker development (Hammond et al. 1985).

Specific resistance genes in the host interact with corresponding avirulence (*AvrLm*) genes of the pathogen in a gene-for-gene manner (Ansan-Melayah et al. 1998; Flor 1942). Australian populations of *L. maculans* have a high level of genetic variability as compared with European and North American isolates (Kutcher et al. 1993), along with a high diversity of avirulence genes (Balesdent et al. 2005). Blackleg disease severity has been controlled through various management options, including diverse crop rotations, stubble management, and the application of fungicides. However, the latter is not economically feasible for lower-yielding environments where canola is often grown. Furthermore, the application of fungicides poses a risk to growers and the environment. Utilization of durable host resistance (Johnson 1984) to blackleg is recognized as an effective approach to control yield losses in *Brassicas*.

Host resistance genes have been catalogued using differential sets of *L. maculans* isolates and/or using molecular markers (Delourme et al. 2006a; Rouxel et al. 2003b). These studies evaluated blackleg resistance on the basis of pathogen infection on cotyledons, stem (canker), and survival under field conditions. To date, 14 major loci (*Rlm1-10* and *LepR1* to *LepR4*) conferring resistance to specific races of *L. maculans* have been identified (Delourme et al. 2006a; Rimmer 2006; Yu et al. 2005, 2008).

Previous linkage mapping studies revealed that at least five resistance genes (*Rlm1*, *Rlm3*, *Rlm4*, *Rlm 7,* and *Rlm9*) are localised in a cluster within a 40-cM genomic region on chromosome A7 (Delourme et al. 2004, 2006a; Dion et al. 1995; Ferreira et al. 1995; Mayerhofer et al. 1997; Rimmer 2006; Rimmer et al. 1999). This genomic region showed extensive inter- and intra-genomic duplications, as well as intra-chromosomal tandem duplications (Mayerhofer et al. 2005). Whether some of these R genes are allelic remains unknown.

The effectiveness of resistance for some of the major genes has decreased in some cultivars within a few years of their release, limiting their usefulness in managing blackleg disease (Chen et al. 1996; Kutcher et al. 2007; Li et al. 2003; Rouxel et al. 2003a). Recently, Brun et al. (2010) demonstrated that a major resistance gene (*Rlm6*) is more durable when expressed in a genetic background that has quantitative resistance, indicating the need to identify, and combine, both existing and new qualitative and quantitative genes for blackleg resistance.

In this study, we describe (1) the construction of a linkage map of the *B. napus* doubled-haploid (DH) population derived from Skipton/Ag-Spectrum designated as 'SASDH', (2) the determination of the inheritance and location of blackleg resistance genes, and (3) the identification of molecular markers linked with resistance loci, applying a whole-genome mapping approach, with the aim of providing canola breeders with tools for routine marker-assisted selection.

Materials and methods

Plant material

The DH population, SASDH, used for the genetic mapping of blackleg resistance loci consisted of 186 lines derived from a cross between the blackleg resistant cultivars Skipton [Barossa (*Rlm4*)/BLN356-3///58410K/Shiralee (*Rlm4*)//Cobra) and Ag-Spectrum (Eureka (*Rlm4*)/ZE6]. This population was generated via microspore culture at the Wagga Wagga Agricultural Institute and showed segregation for several traits of agronomic importance such as blackleg resistance, flowering time, and carbon isotope discrimination (Luckett et al. 2011; Raman et al. 2011).

Inoculum preparation

Eleven single-spore isolates were acquired from the national blackleg isolate collection, University of Melbourne, Australia (Electronic supplementary material 1). Inoculum was prepared by subculturing the *L. maculans* isolates on 10 % V8 agar plates. Approximately 2 weeks later, pycnidiospores were collected in 10 ml of sterile water by dislodging the pycnidia. The spore suspension was filtered through muslin cloth and spore concentration adjusted to 10^6 per mL using a haemocytometer. All isolates were also screened for the presence of the three cloned *Avr* genes *AvrLm1*, *AvrLm4,* and *AvrLm6* using either PCR-based markers and or whole-genome sequence data (Electronic supplementary material 1).

Single-spore isolate screen

The parental lines of SASDH along with other check lines were screened for resistance at the cotyledon and adult plant stages against the 11 single-spore *L. maculans* isolates (Electronic supplementary material 1). The two isolates, 04MGPS021 and 06MGPP041, which were each found to be virulent and avirulent against one of the parental canola lines, were selected for evaluating the DH lines for resistance to *L. maculans*.

Twelve seeds of each genotype from SASDH and parental lines (Table 4) 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 (the

susceptible control) in the centre of each pot. Each genotype had two replicates. Both cotyledons of each seedling were punctured with a pair of bent tweezers and both lobes were inoculated with 10 µl of a suspension containing 10^6 pycnidiospore/mL. Plants were placed in a dew chamber at 100 % relative humidity, at 20 °C, for 48 h, and then returned to a shade house. Seventeen days after inoculation, each inoculation point on the cotyledons was scored for resistance using the rating system of Koch et al. (1991), where 0 = no darkening around wounds and 9 = large gray-green lesions with profuse sporulation. The same plants were then allowed to develop to maturity and scored for resistance by assessing plant mortality and internal infection of the crown. Plants were severed at the crown with a pair of secateurs to enable the crown to be visually inspected for blackleg symptoms. Plants were scored for basal internal infection (0–100 % area discolouration). Average internal infection was rated as 0–35 % = resistant; 36–49 % = intermediate and 50–100 % = susceptible.

A spatially optimized incomplete block design with a nested blocking structure was employed to estimate different variance components. This design was made using the spatial design search program DiGGer (Coombes 2002) assuming positive correlation between neighboring pots in rows and columns and allowing for random row and column effects within a column-pair of benches. Twenty-five of the DH lines were duplicated and 137 lines evaluated by a single replicate.

Field screen

Parental and SASDH lines were screened, in both 2008 and 2009, in blackleg nurseries at Wagga Wagga, New South Wales. The blackleg nurseries consisted of 6-month-old canola stubble from the previous year's crop. Stubble sourced from a mixture of triazine-tolerant varieties in 2008 and ATR-Beacon in 2009 (Table 3) was scattered in the field nursery prior to sowing to increase pathogen pressure. Each line was sown into a 2-m row containing up to 30 individual plants. Irrigation was used to promote development of the epidemic. Up to ten mature plants from each DH line were cut with secateurs and scored for percentage of internal infection at physiological maturity (November/December).

The experimental design for the 2008 trial consisted of 177 DH lines, each parent, and Karoo, as a resistant variety, in a two-replicate design arranged in 30 rows by 12 columns of plots. In 2009, due to shortage of seed of some of the DH lines, only 154 DH lines of the population were screened for blackleg resistance, along with the parental lines. This was a partially replicated (p-rep) design (Cullis et al. 2006) with two replicates of 115 DH lines and one plot of 39 DH lines, with the parents replicated seven times. The control cultivars in 2009 were Karoo (resistant, 4 replicates), Trigold (susceptible, 3 replicates), and Hyola50 (resistant, 7 replicates).

Construction of framework map

DNA was isolated from approximately 10-week-old, shade house-grown seedlings using a standard phenol–chloroform method. Six hundred and eighty-four simple sequence repeat (SSR), sequence-related amplified polymorphism (SRAP), sequence characterised amplified region (SCAR), and candidate gene-based markers, originating from *B. rapa*, *B. oleracea*, *B. napus,* and *B. juncea* were collated from sources in the public domain (Cheng et al. 2009; Choi et al. 2007; Hopkins et al. 2007; http://ukcrop.net/perl/ace/search/BrassicaDB; Li and Quiros 2001; Long et al. 2007; Lowe et al. 2002, 2004; Piquemal et al. 2005; Sun et al. 2007; Suwabe et al. 2002, 2006; Tsuro et al. 2005) and investigated for polymorphism. The SSR primer pairs were synthesized by Sigma-Aldrich Australia Pty. Ltd. (Lismore, Australia). The forward primers of each primer pair were tagged with a 19-bp M13 sequence and PCR amplifications and allele sizing were carried out as described by Raman et al. (2005). A SCAR marker (BN204) derived from a region showing 92 % amino acid identity with the defence-related gene serine threonine 20 (ste-20) protein kinase, of *Arabidopsis thaliana*, was also used. This marker was completely linked with the *Rpg3Dun* gene in an F_2 population from Westar/Dunkeld (Dusabenyagasani and Fernando 2008).

The genetic linkage map was produced using Map Manager version QTL20b (Manly et al. 2001) using the Kosambi mapping function at a probability of 0.01, as described previously (Raman et al. 2009). Accuracy of the marker order within linkage groups was checked using the R/qtl statistical analysis package (Broman et al. 2003), RECORD computer package (van Os et al. 2005), and compared with previously published maps (Choi et al. 2007; Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2008; Suwabe et al. 2006). The linkage data were exported into the Map Chart package (Voorrips 2002) to display the trait-marker data graphically.

QTL detection and validation of *RlmSkipton* linkage

An integrated map consisting of 216 SSR, SRAP, SCAR, and EST-SSR markers covering 24 linkage groups, representing at least 17 chromosomes, was subsequently employed for the QTL analysis for blackleg resistance using the whole-genome average interval mapping approach (Verbyla et al. 2006), which simultaneously models genetic and environmental variation. Environmental variation was

accounted for by including terms relating to design factors such as replicates, columns, rows, and scorer effects (where applicable). Putative QTLs with a LOD score ≥2.0 have been reported. All QTL analyses were conducted using the ASREML-R package (Butler et al. 2007) using original disease scores (0–9) rather than using arbitrary thresholds.

Subsequently, leaf lesion scores based on the cotyledon reaction were classified into two groups in order to map blackleg resistance precisely onto a genetic map of the SASDH population. A disease score of <4 was rated as resistant and a score of >4 was rated as susceptible. Standard Chi-squared (χ^2) tests for 'goodness-of-fit' were used to test the validity of Mendelian ratios with observed data. Linkage between phenotypic and marker alleles was determined by Map Manager version QTX20b (Manly et al. 2001), at a threshold of $P = 0.001$. In order to confirm the location of the markers *Xbrms075* and *Xcb10439*, that were found to be linked with the *RlmSkipton* resistance locus on A7, we compared the sequence of 12 of the SSR markers (Fig. 2; Electronic supplementary material 2) with the assembled genome sequence of *B. rapa* using BLAST (Altschul et al. 1990).

Genetic control of blackleg resistance and *RlmSkipton*-SSR marker linkage was verified in an F_2 population comprising 101 plants derived from Skipton/Ag-Spectrum. Disease expression was tested by inoculating both lobes of cotyledons with the single-spore isolate 04MGPS021 under shade house conditions, as described earlier. After phenotyping, leaf tissue was harvested for DNA analysis. Five flanking markers to the *RlmSkipton* locus, *Xol09-a06*, *Xcb10278a*, *Xcb10439*, *Xbrms075,* and *Xbn204*, were verified to determine whether the markers discriminate between corresponding alleles associated with resistance and/or susceptibility. An integrated linkage map including *RlmSkipton* and molecular marker loci in an F_2 population was generated with the segregation data using Map Manager QTX017b (Manly et al. 2001). Linkage analysis and Chi-square tests were performed at a threshold of $P = 0.001$ as described previously. The *Xcb10278a* and *Xbn204* markers exhibited segregation distortion (Electronic supplementary materials 3a, 4) and, therefore, were not used in map construction.

To test the effectiveness of the SSR alleles in predicting blackleg resistance, the allele diversity of two validated markers (*Xcb10439* and *Xbrms075*), flanking the *RlmSkipton* locus in the DH and F_2 populations from Skipton/Ag-Spectrum, as well as the *Xbn204* marker linked with *Rpg3Dun* (Dusabenyagasani and Fernando 2008) were compared in a set of 15 canola genotypes (Table 4) used as parents in Australian canola breeding programs. These genotypes were also evaluated for cotyledon resistance to isolate 04MGPS021, as described previously. At least five seedlings (20 lobes) of each genotype were inoculated as described previously, and experiments were repeated twice.

Nomenclature of chromosomes, qualitative genes and QTL

Standard nomenclature endorsed by the Multinational *Brassica* Genome Project steering committee was adopted to name the linkage groups of *B. napus* (N1–N10 correspond to A1–A10, respectively, and N11–N19 correspond to C1–C9, respectively), as described previously (http://www.brassica.info/resource/maps/lg-assignments.php). We undertook a pragmatic approach to assign linkage groups to endorsed nomenclature; a linkage group with at least two markers that have been mapped previously in *B. rapa*, *B. oleracea,* and/or *B. napus* were designated accordingly. QTLs identified were named using a standard 'designation' system adopted by the international wheat community (Mcintosh et al. 2003). The '*Q*' indicates a QTL or a genomic region associated with the trait (in this case resistance to *L. maculans*) detected through QTL mapping, which is followed by an abbreviation of the laboratory designator (*wwai*), a hyphen (-) and the symbol for the chromosome in which the QTL is located. We used symbols '*ii*' and '*s*' for QTLs identified in field conditions using internal infection (canker development) and percent plant survival, respectively, as measures for blackleg resistance. For example, *QRlm(ii).wwai-A1* represents a QTL associated with resistance to *L. maculans* (*Rlm*) identified using internal infection that was mapped at Wagga Wagga on chromosome A1. An additional suffix (a, b, c, d, and e) was used if either more than one QTL affecting the trait was identified on the same chromosome or if multiple segregating loci were detected by a primer pair.

Results

Molecular marker polymorphism and linkage map construction

Two hundred and one PCR-based markers were polymorphic between Skipton and Ag-Spectrum and exhibited segregation in the DH population. Genotyping of these markers allowed mapping of 256 loci on different linkage groups/chromosomes (Electronic supplementary material 5, 6). The majority (60.8 %) of the markers exhibited a significantly distorted segregation ratio in the DH population at $P < 0.05$. Marker alleles of these loci were skewed towards the maternal parent Skipton.

A framework molecular map comprising 24 linkage groups, representing 17 chromosomes of *B. napus* was constructed from 185 SSRs (229 loci), 12 SRAP (22 loci), four candidate gene loci and one SCAR (BN204) based markers. Forty markers that were either unlinked or formed small linkage groups were excluded for estimation of the linkage map length. Each of the linkage groups was assigned to previously designated chromosomes (A1–A10, C1–C9) on the basis of the location of known markers to *Brassica* chromosomes (Lowe et al. 2004; Piquemal et al. 2005; Suwabe et al. 2006, 2008). No polymorphism was found between the parental lines for markers that have been mapped previously on chromosomes C4 and C7; therefore, linkage maps of these chromosomes could not be constructed. The genetic map covered approximately 2,672 cM of the genome (Electronic supplementary material 6). The distance between markers varied from 0 to 59.3 cM, with an average marker density of one marker per 12.4 cM. There were weak linkages between different linkage groups belonging to the same chromosome, for example A2, A8, and C6; therefore, we did not incorporate them into a single chromosome group. Clustering of markers was observed on certain chromosomes, for example on A5. Amplification of homeoalleles and marker loci duplication was also common (Electronic supplementary material 5).

Genetic analysis of blackleg resistance

Genetic variation for resistance to 11 different isolates of *L. maculans* was identified between the parental lines of the DH population: Skipton and Ag-Spectrum (Electronic supplementary material 1). The susceptible control, Canadian cultivar Q2 exhibited susceptibility to all isolates of *L. maculans* and had a mean disease score >5. We utilized two isolates 04MGPS021 and 06MGPP041 that exhibited contrasting phenotypes in the parents for evaluation of resistance to blackleg in the SASDH lines using mapped associated loci. Analysis of variance components indicated that the main source of variation under shade house conditions was genetic, ranging from 26.7 to 95.1 % of the total variance (Table 1). Histograms of the mean of cotyledon lesion scores in the DH population are presented in Fig. 1. There was evidence of transgressive segregation, as a number of DH displayed more resistance and susceptibility than parental lines. The number of resistant and susceptible DH lines to isolate 04MGPS021 was 106 and 51, respectively ($\chi^2_{(1:1)} = 19.3$, $P = 0.0001$). This segregation ratio was significantly different from 1:1, if a single gene confers resistance to isolate 04MGPS021 of *L. maculans* (Fig. 1a; Electronic supplementary material 3a). Cotyledon lesion scores with isolate 06MGPP041 showed continuos distribution (Fig. 1b) strongly suggesting that more than one gene controls this trait.

In order to assess dominance of blackleg resistance, we evaluated an F_2 population from Skipton/Ag-Spectrum and found that 77 lines were resistant, and 24 lines were susceptible (Fig. 1c, Electronic supplementary material 4). This observed segregation ratio fits the dominant monogenic segregation ratio ($\chi^2_{(3:1)} = 0.083$, $P = 0.77$), indicating that, a single locus, *RlmSkipton*, encodes the blackleg resistance difference between Skipton and Ag-Spectrum at the cotyledon stage.

Mapping of blackleg resistance genes under shade house conditions

We mapped loci for resistance to isolates 04MGPS021 and 06MGPP041 in the SASDH population (Table 2). A genome-wide average interval mapping approach detected a major locus *QRlm.wwai-A7* on chromosome A7 (N7) that was significantly ($P < 0.001$; LOD $\geq$ 50) associated with blackleg resistance scored at the cotyledon stage and explained approximately 89 % of genotypic variation for resistance against the 04MGPS021 (Table 2). Skipton contributed the allele for increased resistance to isolate 04MGPS021. *QRlm.wwai-A7* was delimited by the SSR markers *Xbrms075* and *Xcb10278a* (Table 2; Fig. 2). This region also showed significant association (LOD $\geq$ 50) with blackleg resistance at the adult plant stage (Table 2) and accounted for up to 67.8 % of genetic variation with the same flanking markers, *Xbrms075* and *Xcb10278a*, as for seedling resistance.

In order to map *QRlm.wwai-A7* precisely onto a genetic map of the SASDH population, we binned quantitative data from cotyledons inoculated with the 04MGPS021 isolate into two discrete categories as resistant (disease score $\leq$4) and susceptible (disease score >4). Lines from SASDH population exhibited a distorted segregation ratio for several markers representing 75.2 cM of chromosome A7 delimited with *Xbrms005b* and *Xbn204* (Fig. 2; Electronic supplementary material 3a) and for resistance and susceptibility to blackleg (106 resistant: 51 susceptible ($\chi^2_{(1:1)} = 19.3$). Linkage analysis revealed that one major locus designated as *RlmSkipton* is localized on chromosome A7 and was flanked by *Xbrms075* and *Xcb10439* SSR loci with a mapping distance of 0.8 and 12.3 cM, respectively (Fig. 2). The genomic region of the *RlmSkipton* locus was aligned with the *B. rapa* physical map. Of the 12 markers compared, 11 matched within a 13.5-Mbp region on chromosome A7 (Fig. 3). One marker (*XoI09-a06*) did not map within this region. Comparison of the position of these markers between the physical and genetic maps demonstrated good correspondence, with some minor variation for markers *Xbrms005* and *Xbrms075*, which may reflect genomic variation between *B. napus* and *B. rapa* within this syntenic region. This is further supported by the

Table 1 Component of variance for blackleg resistance evaluated under shade house and under field conditions

Condition	Plant development stage	Components of variance			Heritability, h^2 (%)
Shade house		Genotype	Run:column:row	Residual	
Isolate 04MGPS021	Cotyledon	95.1	0.2	4.8	93.1
	Adult plant	83.6	–	16.4	84.2
Isolate 06MGPP041	Cotyledon	26.7	20.1	53.2	31.4
	Adult plant	54.7	–	45.3	60.0
Field conditions-2008	Stem Canker	5.6	6.8	87.5	41.2
2009	Stem Canker	18.3	28.2	53.5	52.6
2009	Percent survival	34.3	–	65.7	51.1

Components include variables that account for spatial variability in the field and/or temporal variation in the shade house when they were found to be significant sources of non-genetic variation

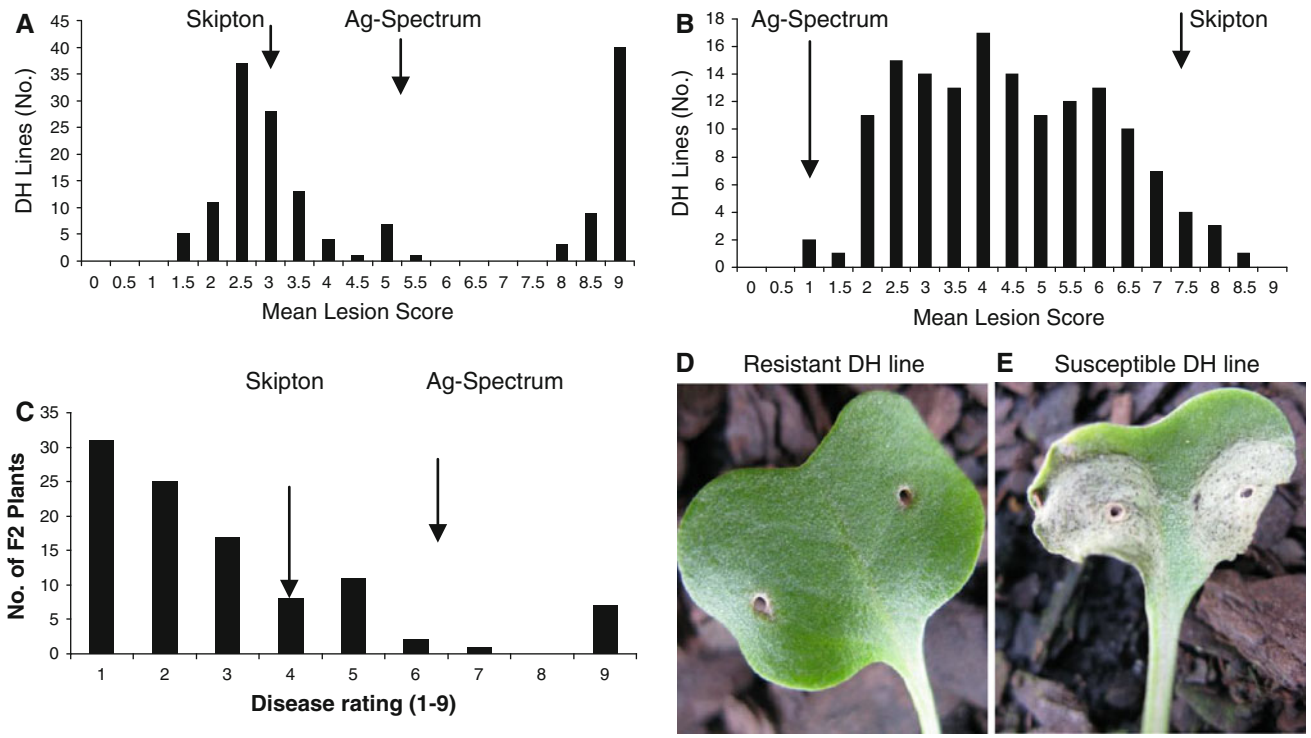

Fig. 1 Phenotypic distributions of mean lesion scores at the cotyledon stage of SASDH doubled-haploid (DH) and F_2 populations. DH lines were inoculated with the single-spore isolates: **a** 04MGPS021, and **b** 06MGPP041. F_2 plants derived from 'Skipton'/'Ag-Spectrum' inoculated with isolate 04MGPS021 (**c**). Mean lesion scores of parental lines are shown by *arrows*. **d** Resistant and **e** susceptible phenotypes of SASDH population upon inoculation with 04MGPS021 isolate (at the cotyledon stage). Photos were taken 21 days after inoculation

failure to locate marker *XoI09-a06* within this region of *B. rapa*.

We located two genomic regions *QRlm.wwai-A1*(*LG1*), and *QRlm.wwai-A10a* that were significantly associated with resistance to *L. maculans* isolate 06MGPP041(LOD score = 2.4) at the cotyledon stage (Table 2). These regions were located on chromosomes A1 and A10, respectively, and accounted for up to 22.8 % of genotypic variance. Ag-Spectrum contributed the alleles for increased blackleg resistance at both the loci. However, only *QRlm.wwai-A1a* showed significant association (LOD = 8.8, genotypic variation = 24.6 %) with resistance to isolate 06MGPP041 at the adult plant stage. This QTL was flanked by the markers *Xol12-f11* and *Xpbcessbr21* (Table 2).

Mapping of blackleg resistance under field conditions

We observed normal distribution of disease scores (Electronic supplementary material 7) indicating that blackleg resistance at the adult-plant stage is likely to be

Table 2 Significant QTLs associated with blackleg resistance identified from a doubled-haploid population derived from Skipton/Ag-Spectrum, grown under shade house conditions

Isolate	Plant development stage	Locus	Flanking markers	LOD score	Genetic variance, R^2 (%)	Additive effect
04MGPS021	Seedling (cotyledon test)	*QRlm.wwai-A7*	*Xbrms075–Xcb10278a*	>50	88.9	Skipton
	Adult plant (internal infection)	*QRlm.wwai-A7*	*Xbrms075–Xcb10278a*	>50	67.8	Skipton
06MGPP041	Seedling	*QRlm.wwai-A1 (LG1)*	*Xpbcessrna16–Xbrms017b*	3.0	22.8	Ag-Spectrum
		QRlm.wwai-A10a	*Xcb10079d–Xcb10079c*	2.4	19.5	Ag-Spectrum
	Adult plant	*QRlm.wwai-A1a*	*Xol12-f11–Xpbcessbr21*	8.8	24.6	Ag-Spectrum

Flanking markers that show the maximum LOD scores, additive effect refers to parental allele that showed an increased effect, and percentage of genotypic variation (r^2) explained. QTLs associated with blackleg resistance having LOD score ≥2 are only shown. Cotyledons of each plant were inoculated (four inoculation sites on each plant); disease was scored on cotyledons at 0–9 scale according the size of lesions and on stems at adult plant stage by assessing the percentage of internal necrosis. QTL analysis was carried out using whole-genome average interval mapping approach in R software

under the control of at least two genes. In order to identify QTLs for blackleg resistance at the adult plant stage and to compare genomic regions associated with resistance under shade house conditions, we evaluated DH lines in blackleg nurseries under field conditions over 2 years (2008 and 2009). Analysis of variance indicated that the main source of variation at the adult plant stage was 'residual', and the genetic component of the DH lines ranged from 5.6 to 34.3 % (Table 1). The general line heritability (h^2) for blackleg resistance under field conditions ranged from 41.2 to 52.6 %. Predicted means of internal infection scores over 2 years were poorly correlated (Pearson's $r = 0.25$, Electronic supplementary material 8).

In the 2008 field trial, we identified seven significant QTLs associated with blackleg resistance scored on the basis of internal disease score, designated *QRlm(ii).wwai-A2*, *QRlm(ii).wwai-A9*, *QRlm(ii).wwai-A10b*, *QRlm(ii).wwai-C1*, *QRlm(ii).wwai-C2a*, *QRlm(ii).wwai-C3,* and *QRlm(ii).wwai-C6*, on chromosomes A2, A9, A10, C1, C2, C3 and C6, respectively (Table 3). The genotypic variation explained by these individual QTL ranged from 5 to 24.5 %. Skipton contributed the alleles for blackleg resistance located on chromosomes A9, A10, C2, and C3. The resistance alleles on A2, C1, and C6 were contributed by Ag-Spectrum. In 2009, only one significant QTL for blackleg resistance scored on internal disease score, *QRlm(ii).wwa1-A1a* (LOD = 6.1, $R^2 = 26.1$ %), was identified on chromosome A1 (Table 3). However, five significant QTLs associated with percent plant survival were detected, accounting for a total of 52.2 % of genetic variation (Electronic supplementary material 3b).

Verification of the *RlmSkipton*-SSR linkage

Five markers, *Xol09-a06*, *Xcb10439*, *Xbrms0075*, *Xcb10278a*, and *Xbn204* flanking the *RlmSkipton* locus (Fig. 2) were analyzed in 101 plants from an F_2 population, derived from a single F_1 from Skipton/Ag-Spectrum to verify the linkage between these markers and the *Rlm-Skipton* locus. Among the markers tested, *Xcb10278a* and *Xbn204* showed distorted segregation towards the paternal parent Ag-Spectrum (Electronic supplementary material 4). The SSR markers *Xcb10439* and *Xbrms075* showed dominant Mendelian segregation ratios, whereas *Xcb10278a* and *Xbn204* displayed a co-dominant Mendelian segregation ratio. The *RlmSkipton* locus for blackleg resistance showed dominant inheritance and tight linkage with the SSR markers *Xbrms075* and *Xcb10439* in the F_2 population (Electronic supplementary material 9). The order of *RlmSkipton* and marker loci was *Xcb10439* (8.5 ± 2.2) –*Xbrms075* (5.2 ± 1.7)–*RlmSkipton* (47.5 ± 10.1)–*Xcb10278a* (22.3 ± 0.4)-*Xbn204.* (Electronic supplementary material 4). The BN204 marker, that showed complete segregation with the *Rpg3Dun* locus for blackleg resistance in a 'Dunkeld'/'Westar' population (Dusabenyagasani and Fernando 2008), was mapped at least 27.1 cM from the *Xcb10278a* locus in the F_2 population from Skipton Ag-Spectrum (Electronic supplementary material 10).

Allele diversity at *RlmSkipton* for blackleg resistance

We investigated allelic diversity at the *RlmSkipton* locus in 15 canola genotypes that are extensively used in Australian canola breeding programs and in the National Brassica Germplasm Improvement Program for gene discovery (Table 4). Cotyledon tests indicated that all rapeseed lines that are known to harbor the *Rlm4* resistance gene (Rouxel et al. 2003b) amplified the 164-, 200-, and 680-bp alleles at the *Xbrms075*, *Xcb10439,* and *Xbn204* loci, respectively, linked with the resistance marker alleles from Skipton, and susceptible lines Quinta, Westar, and Ag-Spectrum had null alleles. However,

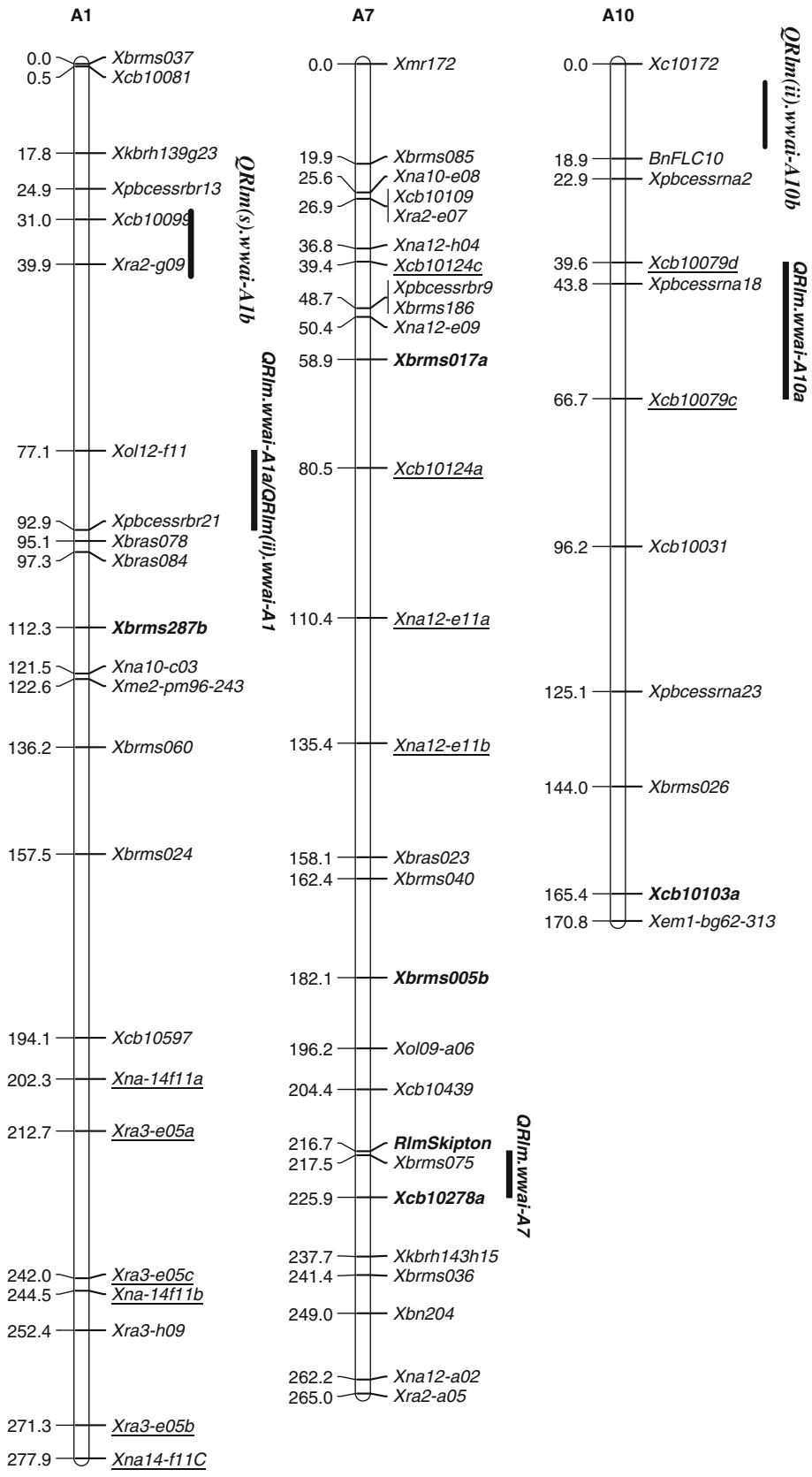

Fig. 2 Graphical representation of location of qualitative and quantitative loci for blackleg resistance on the partial linkage map of the SASDH breeding population (details are shown in ESM 2). Map distances are given in cM on the left of each linkage group. Intra- and inter-genomic duplicated loci identified by molecular markers are *underlined*. Duplicated loci within genomes (A or C) and homoallelic (across A and C genomes such as A1 and C1) loci are in *bold*. Locations of SSR markers and consistent loci associated with blackleg resistance were redrawn using MapChart (Voorrips 2002) using actual linkage distances calculated with Map Manager QTX20b

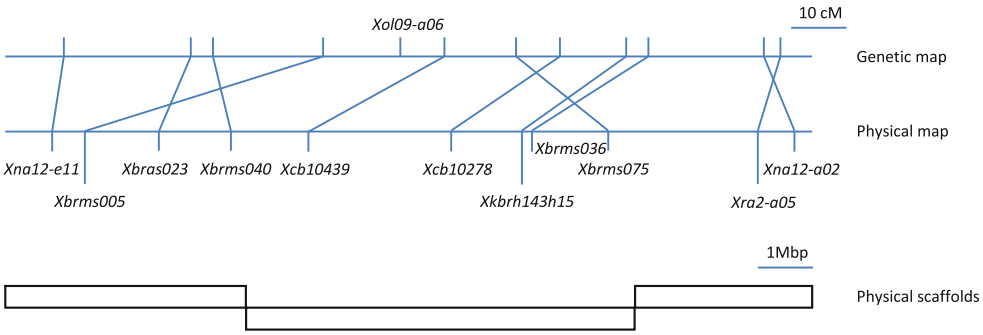

Fig. 3 Alignment of the partial genetic map of chromosome A7 of *Brassica napus* (derived from SASDH) against the physical map of *Brassica rapa* using common SSR primer pair sequences

Table 3 Significant QTLs associated with blackleg resistance (scored as internal infection due to canker development at adult plant stage) identified from a doubled-haploid population derived from Skipton/Ag-Spectrum, grown in blackleg nursery raised under field conditions at the Wagga Agricultural Institute in years 2008 and 2009

Year	Locus	Flanking markers	LOD score	Genetic variance, R^2 (%)	Additive effect
2008 (mixed stubble)	*QRlm(ii).wwai-A2*	*Xbras123/Xem1-bg11-237*	7.0	11.5	Ag-Spectrum
	QRlm(ii).wwai-A9	*Xbrms319-Xbrms176*	2.9	5.0	Skipton
	QRlm(ii).wwai-A10b	*Xcb10172-BnFLC10*	2.2	6.2	Skipton
	QRlm(ii).wwai-C1	*Xbrms287a-Xcb10034*	4.2	11.5	Ag-Spectrum
	QRlm(ii).wwai-C2a	*Xol10-c10/Xna12-c03*	6.8	16.6	Skipton
	QRlm(ii).wwai-C3	*Xpbcessrna13/Xol13-d02a*	4.2	24.5	Skipton
	QRlm(ii).wwai-C6	*Xem1-bg23-89/Xol12-e03*	6.1	14.5	Ag-Spectrum
2009 (ATR-Beacon stubble)	*QRlm(ii).wwai-A1a*	*Xol12-f11/Xpbcessrbr21*	6.1	26.1	Ag-Spectrum

QTL showing position of flanking markers on chromosome, LOD score and percentage of genotypic variation (r^2) explained. Additive effect refers to the parental QTL allele that showed an increased effect. QTLs associated with blackleg resistance having LOD score ≥2 are only shown

Table 4 Allele diversity (in base pairs) of SSR markers linked with the *RlmSkipton* locus for blackleg resistance in canola cultivars

Genotype	Reaction to isolate 04MGPS021	*Xbrms075*	*Xcb10439*	*Xbn204*
Av-Sapphire[b]	R	164	200	H (680, 500)
BLN3347[b]	H (67 % R, 33 % S)	164	200	H (680, 500)
Dunkeld[a,b]	H (73 % R, 27 % S)	164	200	H (680, 500)
Karoo[a,b]	R	164	200	680
Lantern[b]	R	164	200	680
Maluka[a]	S	164	200	680
Oscar[a,b]	H (17 % R, 83 % S)	164	200	H (680, 500)
Rainbow[a,b]	H (38 % R, 62 % S)	164	200	H (680, 500)
Shiralee	S	164	203	680
Skipton[b]	R	164	200	680
Tornado[b]	R	164	200	H (680, 500)
Westar	S	Null	Null	500
Ag-Spectrum	S	Null	Null	500
Major[a,b]	S	164	200	680
Quinta	S	Null	Null	H (680, 500)

Genotypes that were heterogeneous/heterozygous were indicated as H. The percent resistant plants within H category are given in parenthesis

[a] Cultivars having *Rlm4* gene (Rouxel et al. 2003a, b)

[b] Cultivars having *Rlm4* gene based upon reaction on differentials at cotyledon stage (Raman et al. unpublished data)

Maluka, Shiralee, and Major, having *cRLMm*, *LmR1,* and *LEM1* genes, respectively, for blackleg resistance on chromosome A7 (Ferreira et al. 1995; Mayerhofer et al. 1997), were susceptible to isolate 04MGPS021. Some of the genotypes also showed heterogeneity for resistance.

Discussion

Construction of the linkage map of 'SASDH'

We constructed a genetic linkage map covering approximately 2,672 cM. This map is comparable with previous *B. napus* maps based upon RFLP, AFLP, RAPD, and/or SSRs covering distances of 1,173–2,619 cM (Kaur et al. 2009; Lombard and Delourme 2001; Piquemal et al. 2005). Our results revealed good correspondence of chromosome locations of SSR markers between *B. rapa* (Suwabe et al. 2006) and *B. napus*, indicating their transferability across species. However, some SSRs did not show any correspondence for map positions between previous investigations (Suwabe et al. 2006, 2008). This discrepancy could be due to the amplification of multiple loci, which may not be polymorphic in *B. rapa* populations. Significant segregation distortions of many SSR markers were observed, including those linked with blackleg resistance (Electronic supplementary materials 3a, 4). Such features are known to skew genetic distances (Foisset and Delourme 1996; Kim et al. 2006; Lombard and Delourme 2001; Mayerhofer et al. 1997; Piquemal et al. 2005; Rimmer et al. 1999) and the size of linkage maps tend to increase as observed for linkage groups A1, A7, and C3 (Electronic supplementary material 5). Segregation distortion may also be due to misscoring of homeologous alleles as single-dose dominant markers (Kaur et al. 2009; Osborn et al. 2003). Co-amplification of homeologous marker alleles from the A and C genomes, as found in chromosomes A1/C1, A2/C2, and A3/C3 may be due to synteny and colinearity of genomic regions as reported previously (Parkin et al. 2003; Piquemal et al. 2005).

Molecular mapping of blackleg resistance loci

We localized loci that have large and small phenotypic effects underlying qualitative and quantitative resistance to blackleg, both at the seedling and adult plant stages, utilizing a DH breeding population from Skipton/Ag-Spectrum relevant to Australian canola breeding programs. Previously, many molecular mapping studies utilized contrasting mapping populations for identifying loci associated with blackleg resistance (Dusabenyagasani and Fernando 2008; Kaur et al. 2009; Yu et al. 2005).

Mapping of race-specific loci for blackleg resistance to 04MGPS021

We mapped *RlmSkipton* on chromosome A7 in the vicinity of other mapped major genes for blackleg resistance, see Electronic supplementary material 10. (Ansan-Melayah et al. 1998; Balesdent et al. 2001, 2002; Delourme et al. 2004; Delwiche 1980; Dion et al. 1995; Dusabenyagasani and Fernando 2008; Ferreira et al. 1995; Mayerhofer et al. 1997). This suggests that the *RlmSkipton* locus is located in a region carrying many *L. maculans* resistance genes.

Screening of different parental lines suggested that Skipton possesses *Rlm4* and *Rlm9*, and Ag-Spectrum showed heterogeneity for *Rlm9* (Raman et al. unpublished data). Characterization of the *L. maculans* isolate 04MGPS021 with PCR-based markers and whole-genome sequence data has indicated that this isolate has *AvrLm4*. Therefore, we predict that *Rlm4* (*RlmSkipton*) controls blackleg resistance to isolate 04MGPS021 at the cotyledon stage in the SASDH population.

In the present study, the *RlmSkipton* locus was mapped approximately 27 cM from the marker BN204. Previously, correspondence between *Rpg3Dun* (co-segregating with BN204) and *Rlm4* to the same locus has been suggested (Dusabenyagasani and Fernando 2008). Discrepancies for mapping distances between BN204 with *RlmSkipton* in the SASDH population and *Rpg3Dun* and BN204 may be attributed to the difference of genetic background, population structure (F_2 vs. DH) or to genotypic, and phenotypic errors. A high-resolution mapping population, comprising several thousand lines, is required to establish whether BN204 cosegregates with the *Rpg3Dun* locus for race-specific resistance to blackleg. Chromosomal abnormalities such as translocation and or deletion may also lead to mapping distance discrepancies in different populations. An interstitial reciprocal translocation in the Westar parent, that was used in mapping the *Rpg3Dun* locus (Dusabenyagasani and Fernando 2008), has been reported in the literature (Osborn et al. 2003; Sharpe et al. 1995) and this may have adversely affected the findings. Furthermore, reciprocal translocation in the Australian cultivars Marnoo and Maluka have also been reported (Kelly 1996; Osborn et al. 2003). Maluka is related to the cultivar Skipton which may therefore have a similar translocation. In the present study, we could not establish from our marker data whether Skipton and/or Ag-Spectrum has an interstitial reciprocal translocation between A7 and C6, as only one polymorphic SSR marker (*Xcb10278*) was common between these chromosomes (Electronic supplementary material 5).

Relationship between *RlmSkipton* and *Rlm4* containing cultivars

Correspondence of the genes, *LmR1* (Shiralee), *cRLMm* (Maluka), *CRLMrb,* and *LEM1* (Major) with *Rlm4* gene has been proposed (see Rimmer 2006). Ag-Spectrum was a single plant selection from Rainbow (http://pericles.ipaustralia.gov.au/pbr_db/docs/2003119.doc) that was derived from RZ6/Eureka. Skipton has Barossa (that lacks *Rlm4*) (Rouxel et al. 2003b) and Shiralee in its pedigree.

Eureka, and Maluka (Haya//Zephyr/Bronowski/3/Chisaya//Zephyr/Bronowski), Shiralee (Haya//Zephyr/Bronowski/5/Sv62.371/Zephyr//Norin20/3/Erglu/4/BJ168/Creus-o-Precose/Chisaya//Zephyr/Bronowski), and Dunkeld (BJ168/Ccreus-o-Precose//Norin20/Tower/Tower/6/Chikuzen*2///Zephyr/Bronowski/5/Sv62.371/Zephyr//Norin20/3/Erglu/4/BJ168/Creus-o-Precose) are known to carry the *Rlm4* gene (Rouxel et al. 2003b). In the present study, BN204 generated an amplicon of 680 bp in Dunkeld, Skipton, and several other Australian canola cultivars (Table 4) that harbored the *Rlm4* allele for blackleg resistance (Rouxel et al. 2003a). However, our results (Table 4) showed that *LmR1*, *cRLMm* and *LEM1*, and *RlmSkipton* are not allelic, as Maluka, Shiralee, and Major are susceptible to 04MGPS021. This discrepancy could also be attributed to heterogeneity within these cultivars, multiallelic nature of *Rlm4* and/or lack of complete linkage between the markers that we have mapped and *Rlm4* in different genetic backgrounds. Nevertheless, our validation in F_2 progeny and allelic diversity data suggest that SSR markers are useful in predicting blackleg resistance (to isolate 04MGPS021) derived from Skipton.

Mapping of race-specific loci for blackleg resistance to 06MGPP041

Unlike previous studies aimed at mapping loci for blackleg resistance genes using single-spore isolates (Dion et al. 1995; Ferreira et al. 1995; Mayerhofer et al. 1997), we identified continuous distribution for segregation of disease scores at the cotyledon stage using the 06MGPP041 isolate (Fig. 1b). It is possible that this isolate may have different *Avr* genes which may have influenced segregation distribution. This is supported by the presence of two QTLs for blackleg resistance at the cotyledon stage (Table 2). Second, the low genetic component of variance (26.7–54.7 %) for blackleg resistance evaluated using the single-spore isolate 06MGPP041 (Table 1) clearly indicated that phenotyping was more influenced by environmental conditions as compared with isolate 04MGPS021. Presence of different genes in parental lines having an array of resistance genes (such as *Rlm4* and *Rlm9* in Skipton) may also influence phenotypic distribution of disease scores. Previous interaction studies have also shown the implication of two genomic regions on A7 and A10 in *B. napus* plants with intermediate phenotype at the cotyledon stage (Delourme et al. 2004, 2006b).

Mapping of race non-specific loci for blackleg resistance

The low genetic component of variance and the high residual variance for blackleg resistance evaluated under field nursery conditions in 2008 and 2009 (Table 1) clearly indicated that this trait is highly dependent upon environmental conditions. This is further supported from the poor correlation coefficient ($r = 0.25$) between scores of internal infection over both years (Electronic supplementary materials 7, 8). Therefore, genetic studies for adult plant resistance using only field evaluation are problematic.

The two QTLs *QRlm.wwai-A1a*, and *QRlm.wwai-A10* were the only ones observed to have a consistent effect on blackleg resistance at both the seedling (Table 2) and adult plant stages (Table 3). All other QTLs were inconsistent in their effects. This could be due to the use of different sources of stubble having different race structure in the blackleg nurseries. *L. maculans* is well known for a large-scale obligate sexual recombination on plant debris and a high gene flow through large-scale dissemination of ascospores (Hall 1992). Dynamic shifts from avirulence to virulence in the populations of *L. maculans* may contribute to discrepancies in the phenotypic scores. Furthermore, we did not find any significant effect of the *Rlm4* (*RlmSkipton*) locus in providing resistance under field conditions. This may be due to the presence of *Rlm4* attacking isolate(s) in the blackleg nurseries that we used. Our findings are consistent with a recent study (Light et al. 2011), where the *Rlm4* varieties showed poor adult plant survival with stubble from polygenic varieties including ATR-Beacon.

Polygenic control of field resistance has been reported in Canadian and European breeding lines (Ferreira et al. 1995; Pilet et al. 1998; Sippell et al. 1991). *QRlm.wwai-A1a* may be the same QTL identified on A1 for blackleg resistance at the adult-plant stage in DH populations from Caiman3/Westar-10, Camberra4/Westar-10 and Av-Sapphire-5/Westar-10 (Kaur et al. 2009). Our results suggest that this consistent QTL, that confers blackleg resistance in different experimental sites in Australia: Lake Bolac and Dahlen (Kaur et al. 2009) and Wagga Wagga (this study), can be introgressed by the canola breeding programs to enhance blackleg resistance using marker-assisted selection. Other genomic regions that we have identified for blackleg resistance (Table 2) may be the same as reported previously (Delourme et al. 2008; Diederichsen et al. 2005; Pilet et al. 1998). For example, the *QRlm.wwai-A2* and *QRlm.wwai-A10* may correspond to race-specific major genes *LepR2* and *LepR1*/*LepR3* (Yu et al. 2005, 2008). However, it was difficult to compare loci conferring blackleg resistance due to the different marker systems used in the mapping studies (Kaur et al. 2009; Yu et al. 2005). Several other QTLs that we identified in this study correspond to genomic regions associated (based upon their linkage with a common set of markers) with blackleg resistance that have been identified using both classical QTL and association mapping approaches (Delourme et al. 2008; Jestin et al. 2011; Piquemal et al. 2005). The conservation of QTLs between Australian and French studies

is interesting and suggests the non-specificity of these QTLs, irrespective of the environment, genetic background, and G × E interactions.

In the present study, we found small to moderate QTL effects for blackleg resistance. It is well established that *L. maculans* is a genetically diverse pathogen due to frequent sexual recombination (West et al. 2001) and exhibits G × E and *Avr* gene interactions. Therefore, identification of QTLs accounting for low genotypic variance for blackleg resistance under natural field conditions was expected. Under field conditions, different races, with combinations of avirulence genes, are present; therefore, identifying and validating corresponding host resistance genes consistent over years/different environments is a challenging exercise and was not possible in the smaller F_2 validation population that we utilized in this present study. Multi-locational trials need to be conducted to test the robustness of markers and stability of the QTLs, and their additive effects for resistance to *L. maculans*. However, our results suggest that genomic regions identified in this study that are consistent with the previous Australian and French studies can be tracked using molecular markers to enrich favourable alleles for blackleg resistance in canola breeding programs.

Unlike RFLP, AFLP, and RAPD markers that have been used to tag loci for blackleg resistance previously, we identified SSR markers suitable for the high-throughput analysis required for marker-assisted selection programs. Generally SSR markers are co-dominant and are amenable for characterisation of heterozygotes in the segregating populations. In the present study, we observed that many SSR behave as dominant markers. Although these markers cannot be utilized to identify heterozygotes in early generation breeding programs, they can be used for culling homozygous undesirable lines (negative selection).

Conclusion

A good knowledge of *Rlm* genes present in the varieties, along with the *AvrLm* genes present in the fungus population, is necessary to reach firm conclusions on the involvement of specific *Rlm* genes and non-specific quantitative factors in field resistance from one environment to another. Our results suggested that different loci controlling seedling and adult plant resistance to *L. maculans* along with their allelic effects can be determined using a whole-genome mapping approach. Skipton contains both race-specific qualitative (*Rlm4*) and race non-specific quantitative resistance (*QRlm(ii).wwai-A9*, *QRlm(ii).wwai-A10*, *QRlm(ii).wwai-C2,* and *QRlm(ii).wwai-C3*) to *L. maculans*. However, Ag-Spectrum contained QTLs for race-specific (*QRlm(ii).wwai-A1 and QRlm(ii).wwai-A10*), as well as for race non-specific resistance (*QRlm(ii).wwai-A1, QRlm(ii).wwai-A2, QRlm(ii).wwai-C1* and *QRlm(ii).wwai-C6*). Loci controlling both qualitative and quantitative resistances from Skipton and Ag-Spectrum can be pyramided using the molecular markers that we have identified. Gene pyramids will provide more durable resistance to blackleg, compared with major gene effects alone, as this host specific gene will not exert selection pressure on a specific *L. maculans* race.

Acknowledgments This research was partly funded by the Grains Research and Development Corporation, Australia (DAN117). We thank Dr. Simon Diffey, Biometrician, Wagga Wagga Agricultural Institute, for providing statistical research support, Drs. Angela Van de Wouw and Barbara Howlett, The University of Melbourne, Victoria, for providing data on *AvrLm1*, *AvrLm4* and *AvrLm6* markers and differential isolates of *L. maculans* and Dr. Vicki Thomas for assisting in phenotyping. We thank Peter Heffernan, Ray Cowley, David Roberts and Peter Deane for technical assistance in field work.

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