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Chapter 4

Genetic Dissection of Blackleg Resistance Loci in Rapeseed (*Brassica napus* L.)

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1. Introduction

Blackleg disease caused by the heterothallic ascomycete fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. (anamorph: *Phoma lingam* Tode ex Fr.), is the major disease of Brassica crops such as turnip rape (*Brassica rapa* L. syn. *B. campestris;* 2n = 2x = 20, genome AA), cabbage (*B. oleracea* L.; 2n = 2x = 18, genome CC), rapeseed (syn. canola or oilseed rape *B. napus* L.; 2n = 4x = 38, genome AACC), and *B. juncea* L. (Indian or brown mustard; 2n = 4x = 36, genome AABB) grown in temperate regions of the world. It was recorded for the first time on stems of red cabbage [1]. *B. napus* originated as a result of natural interspecific hybridization and genome doubling between the monogenomic diploid species, *B. rapa* and *B. oleracea,* in southern Europe approximately 10,000–100,000 years ago [2, 3]. However, it was selected and grown as an oilseed crop only 300-500 years ago [4, 5]. *B. napus* originally evolved as a spring or semi-winter type under the Mediterranean climates, and spread rapidly from southern to northern Europe after the development of winter *B. napus* varieties [6]. Both spring and winter types are affected by blackleg disease, particularly in Australia, Europe and North America. Currently *B. napus* is the world's third most important oilseed crop, grown on an area of over 23 million hectares and produce almost 53.3 million tonnes annually [7]. Increase in *B. napus* production has been attributed to the development and release of high yielding superior varieties including hybrids having traits such as high oil content, improved protein quality and herbicide resistance for better crop management.

Among the bacterial, fungal, viral and phytoplasmic-like diseases, blackleg is the most important global disease of *B. napus* crops and causes annual yield losses of more than $900 million in Europe, North America and Australia [8-10]. *L. maculans* has an ability to kill plants even at the seedling stage, infecting cotyledons, leaves, stems, roots and pods. Under epiphytotic conditions, this disease can cause yield losses of up to 90 per cent [11 - 13]. Therefore, control of blackleg disease has been one of the major objectives of many *B. napus* breeding programs.

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2. Symptoms

Blackleg disease causes two distinct symptoms; leaf lesions and stem canker. Outbreak of the fungus is characterised by dirty-whitish spots on leaves with small dark fruiting bodies (pycnidia). Black lesions are generally also seen on the leaves and deep brown lesions with a dark margin can be seen on the base of stem [11]. In severe epidemic conditions fungus girdles the stem at the crown, leading to lodging of the plant and possible severance of the stem. Typical lesions of blackleg can also occur on pods. Pod infection may leads to premature pod shatter and seed infection.

3. Biology of the pathogen and epidemiology of the *L. maculans*

The pathogen can infect several crucifers, including cruciferous weeds. Up to 28 crucifer species have been reported as hosts [14]. During infection, the pathogen grows systemically down towards the tap root of the plant, producing severe disease symptoms at the adult plant stage characterised by stem cankers. *L. maculans* reproduces both asexually and sexually on host species and can complete several disease cycles during a single growing season. The fungus survives as mycelium, pycnidia and pseudothecia on crop residues, mainly on stubble [15, 16] subsisting from one season to the next. Sexual mating occurs on crop residues, resulting in the production of ascospores which can travel up to 8 km [17]. High humidity and moderate temperatures during vegetative growth promote disease development [18].

In Australia and most parts of Europe, *L. maculans* infection generally occurs during the seedling stage from infected seed and wind-dispersed ascospores (sexual spores), released from pseudothecia. In western Canada and Poland, asexual pycnidospores are the primary source of inoculum [19], dispersed largely by rain-splash. Under high humidity conditions, ascospores and pycnidiospores adhere to cotyledons or young leaves and germinate to produce hyphae which penetrate through stomata and wounds [9, 20, 21] and grow into sub-stomatal cavities without forming appressoria [22]. After entering into substomatal cavities, the fungus grows between the epidermis and palisade layer and then into intercellular spaces in the mesophyll of lamina. The fungus then reaches the vascular strands and grows within the plant asymptomatically, until eventually invading and killing cells of the stem cortex and causing the stem canker symptom [22-24]. Variability for virulence in *L. maculans* for the first time was reported in 1927 [25]. Australian populations of *L. maculans* have a high level of genetic variability as compared to European and North American isolates [26], along with a high diversity of avirulence genes [27]. Molecular analyses of populations of *L. maculans* have shown high gene flow within and between populations. Isolates of *L. maculans* are usually classified either on the basis of their aggressiveness or into pathogenicity groups [28].

4. Management of the *L. maculans*

Various practices such as crop rotation, stubble management, time of sowing, seed dressing and foliar application of fungicide, and deployment of genetic resistance have been employed

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to control this disease and subsequently reduce yield losses [9, 29]. Deployment of host resistance has been used as the most cost-effective and environmentally sound measure for disease control in various crops including in rapeseed. This strategy has been extensively used to manage blackleg disease especially in Australia, Canada, France, and Germany.

5. Evaluation of germplasm for *L. maculans* resistance

An efficient and reliable method for phenotyping resistance to *L. maculans* is required for germplasm evaluation and predictive breeding including molecular mapping and gene cloning research. Various criteria are used to assess disease severity, such as severity of cotyledon or stem canker lesions, which rely principally on scales or estimates of the percent of diseased leaf tissue at either seedling (intact and detached leaf) or at adult plant stages. Symptom expression can vary with the environmental conditions, test locations (glasshouse, environment chamber and field conditions), and the method of inoculations (cotyledon, leaf and stem).

Resistance of *B. napus* germplasm to *L. maculans* is tested on the basis of disease reaction under glasshouse and/or field conditions. Cotyledon inoculations, performed under controlled conditions in either a growth chamber or glasshouse, allow for large scale and efficient screening of germplasm. Various environmental conditions such as temperature, light intensity and humidity can be reliably controlled, expediting the development of suitable resistant cultivars [30] as selections can be performed at early stages of plant development. This method also overcomes some of the uncertainties inherent in field testing with its dependence upon growing environment and further reduce the genotype by environment (G x E) interactions. Growth conditions are typically maintained with at 18˚C to and 22˚C. For uniform infection, a spore suspension is used to inoculate wounded cotyledons of 7 to 15 day-old seedlings [31-33]. Alternatively, seedlings can be sprayed with a spore suspension at up to the third leaf stage and kept at 100 % humidity for 48-72 hr. Spore suspensions of *L. maculans* are generally raised from single-spore isolate cultures grown on different media such as V8-agar, malt-agar and rapeseed leaf extract-agar [21, 22, 34]. Published studies used spore concentrations in the range from 4×10^6 to 1×10^8 spores per ml [31 - 33].

Doubled haploid (DH) populations were screened for resistance to *L. maculans* in the glass-house at three plant growth stages: cotyledon, true leaf and adult plant, as well as under field conditions and reported a high correlation ($r \geq 0.82$) for disease severity between glasshouse and field grown lines [33]. Similar observations were also made by McNabb et al [35]. High correlation coefficient values suggest that the resistance to *L. maculans* can be evaluated at all three stages [33]. However among three stages, cotyledon stage was the most promising as inoculum-droplets can be kept at the inoculation site as compared to true leaves.

Assessment of adult plants for resistance to *L. maculans* populations under field conditions is considered very important for the selection of resistant germplasm by the rapeseed breeders. Inoculum is provided by either spreading infected stubble in a disease nursery or spraying plants with fungal spore suspension. Two measures; disease severity and disease incidence

are commonly used for evaluating resistance to *L. maculans*. However, disease severity is much more difficult to estimate than disease incidence, due to the G x E interactions and unreliable and inconsistent estimation of canker lesions, even within the same genotype, particularly when infection is not uniform. The use of increased sample size (25 to 50 plants/genotype) and reliable and congenial growing conditions for the disease development will allow better estimation of canker lesions.

Assessment of blackleg resistance under field conditions is usually performed by exposing the plants to a mixed population of *L. maculans* races, which can make the detection of race-specific *R*-genes difficult. No relationship between the degree of cotyledon-lesion development at the seedling stage and crown canker development in mature plants was observed in the intercross population derived from Maluka/Niklas [36]. This study concluded the limited value of the cotyledon test in screening for adult plant blackleg resistance. Similarly a lack of correlation between cotyledon (seedling) resistance and stem (adult plant) resistance in *B. napus* and B genome sources has also been reported [37]. Recently, a poor correlation between seedling and field reactions was reported in the DH from Skipton/Ag-Spectrum which could have been due to the prevalence of different pathotypes under field conditions as contrary to cotyledon test, where often a specific isolate is used for phenotyping [32]. In order to mimic field conditions and increase reliability of disease development, an ascospore shower test [38] has been used for germplasm evaluation and varietal release in Australia. In this test, stubble with mature pseudothecia is sprayed with distilled water until run-off, producing 'ascospore shower'. The infected plants can then be assessed for resistance at both the cotyledon and adult plant stages. This method has shown a high correlation with canker lesions scored under field conditions [39].

6. Natural genetic variation for resistance to *L. maculans*

The introgression of blackleg resistance (*R*) genes into *B. napus* germplasm for blackleg disease management is one of the major objectives of breeding programs aiming to release cultivars in disease-prone areas. Genetic variation for resistance to *L. maculans* exists within *B. napus* germplasm [39, 40, 41]. Some other Brassica species such as *B. rapa, B. juncea, B. nigra* (black mustard; 2n = 2x = 16, genome BB) and *B. carinata* (Abyssinian or Ethiopian mustard; 2n = 34, genome BBCC), as well as other crucifers such as *Sinapis arvensis* have been reported to carry resistance [42-53]. Some of these sources were utilised in transferring resistance into *B. napus* breeding lines and cultivars. A continuous variation for blackleg resistance in a world-wide collection of *B. rapa* genotypes was reported [54]. None of genotypes were completely susceptible or completely resistant to either *L. maculans* pathotypes used. However, some *B. rapa* accessions that were either highly resistant or completely susceptible were identified (Raman et al., unpublished) in a set of differential cultivars currently being used in Australia [39].

It has been reported that all B genome Brassica species; *B. nigra, B. carinata* and *B. juncea* carry complete resistance to *L. maculans* which remains effective throughout the life of the plant [40], however susceptible *B. juncea* cultivars have also been identified [55] demonstrating that complete resistance is not a feature of all B genomes. Some B genome resistance genes have been introgressed into *B. napus* lines. [47, 56-59]. Earlier studies have shown that C genome

species of the *Brassica* are susceptible to blackleg [50, 53, 60]. However, a recent study [61] evaluated three accessions of *B. oleracea* var. *virids*, collected from the USDA germplasm collection and found that the accession NSL6146 was moderately resistant to *L. maculans*.

Genetic resources for adult plant resistance are very limited and most of them are derived from the French cultivar Jet Neuf [62]. Efforts are currently being made to identify both qualitative and quantitative resistance in the Australian Brassica Germplasm Improvement Programs.

7. Inheritance of resistance to *L. maculans*

Genetic inheritance studies revealed that resistance to *L. maculans* is complex. Resistance is either described as qualitative (also referred as monogenic/seedling/race-specific resistance/ vertical resistance) or quantitative (also referred as polygenic/adult plant/race non-specific resistance/horizontal resistance) in *Brassica*.

7.1. Qualitative resistance

Monogenic inheritance was reported in several spring and winter cultivars of *B. napus* such as Cresor, Maluka, Dunkeld, Maluka, Skipton, and Major [32, 63-67]. Eighteen major genes for resistance to *L. maculans*; *Rlm1* to *Rlm11*, *RlmS*, *LepR1* to *LepR4*, *BLMR1* and *BLMR2*, have been identified in Brassica species; *B. rapa*, *B. napus*, *B. juncea* and *B. nigra* [31, 32, 40, 45, 68-73]. Six of them, *Rlm1*, *Rlm2*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9* were identified in *B. napus*, all of them except *Rlm2* were clustered genetically on chromosome A07 [74]. *Rlm2* was mapped on chromosome A10 [45]. The *Rlm5* and *Rlm6* were identified in *B. juncea*; *Rlm8* and *Rlm11* in *B. rapa*, and *Rlm10* was identified in *B. nigra*. Four resistance genes; *LepR1*, *LepR2*, *LepR3*, and *LepR4* were introgressed into *B. napus* from *B. rapa* subsp. *sylvestris* (Table 1).

Species	Locus	*Population	Phenotyping stage	Marker type	Mapping strategy	chromosome	Linked markers/interval	Reference
B. napus	*Rlm1*	Maxol/S006 (140 DH)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	T04.680 (14cM)	74
		Quinta/Score (110 F_2)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	C02.1375/O15.1360 (19cM)	57, 74
		Maxol/Westar-10 (96 DH)	Cotyledon inoculation and stem canker	SSR, DArT	Whole genome mapping	A7	Xna12a-02a/Xra2-a05b	82
		Columbus/Westar-10	Cotyledon inoculation and stem canker	SSR	A7 chromosome specific mapping	A7	Xol12-e03a/Xna12-a02a	82
B. napus	*Rlm2*	Glacier/Score (110 F_2)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	M08.1200, M08.600, P02.700	57, 74

Species	Locus	*Population	Phenotyping stage	Marker type	Mapping strategy	chromosome	Linked markers/interval	Reference
		Glacier/Yudal (BC189)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	M08.1200, M08.600, P02.700	74
		Darmor/Samourai (133 DH)	Cotyledon, Field	RAPD	Bulked segregant analysis	A7	M08.1200 (10cM),	74
B. napus	*Rlm3*	Maxol/S006 (140DH)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	Q12.750 (7cM)	74
B. napus	*Rlm4*	Quinta/Score (110F2)	Cotyledon inoculation	RAPD	Bulked segregant analysis	A7	C02.1375 (3.6 cM)/ O15.1360 (`33 cM)	74
		Skipton/Ag-Spectrum	Cotyledon and Stem canker	SSR	Whole genome mapping		BRMS075 (`0.7 cM)	32
B. juncea	*Rlm6*	Recombinant lines (*B. napus-B. juncea*)	Cotyledon and field test	RAPD/ RFLP	Bulked segregant analysis	B8	OPG02.800, OPT01	47
B. napus	*Rlm7*	2311.1/Darmor (221 F_2)	Cotyledon	RAPD	Bulked segregant analysis	A7	T12.650 (4cM)	74, 85
B. napus	*Rlm9*	Darmor-bzh/Yudal (132 DH)	Cotyledon	RAPD	Bulked segregant analysis	A7	T12.650/C02.1375	74
B. nigra	*Rlm10*	Addition lines (Darmor/Junius)	Cotyledon test	Isozyme RAPD	Whole genome mapping	B4	OPA11.1200, OPC19.3300	83, 84
B. rapa ssp. *sylvestris*	*LepR1*	6270/Springfield (DHP95)	Cotyledon inoculation and field resistance	RFLP	Whole genome analysis	A2 (N2)	pR4b, pO85h, pW180b, pN181a, pW207a	31
B. rapa ssp. *sylvestris*	*LepR2*	6279/3027 (DHP96)	Cotyledon inoculation and field resistance	RFLP	Whole genome analysis	A10 (N10)	pN21b, pR34b, pN53b	31
B. napus	*LepR3*	Surpass400/Westar (N-o-1)-BC	Cotyledon inoculation	SSR	A1 and A10 chromosome specific mapping		sR12281a (2.2 cM) sN2428Rb (0.7 cM)	69
		Topas (DH16516)/ Surpass400	Cotyledon inoculaton	SSR, SCAR	A10 chromosome specific mapping	A10 (N10)	Ind10-12	79
B. rapa ssp. *sylvestris*	*LepR4*	16S/PAS12//16S (BC_3S_2)	Cotyledon inoculation Disease nursery (field)	SSR	A genome specific marker analysis	A6	sN2189b (8.8cM) sR9571a (8.3 cM)	77
B. napus	*BLMR1*	Surpass400/Westar (1513 F_3BC_2)	Cotyledon inoculation	SRAP, SNP	Selective genotyping	A10 (N10)	80E24a (0.1 cM)	70
B. napus	*BLMR2*	Surpass400/Westar (1513 F_3BC_2)	Cotyledon inoculation	SRAP, SNP	Selective genotyping	A10 (N10)	R278 (1.2 cM)	70
B. napus	*LmFr1*	Cresor (resistant)/ Westar (susceptible)	Field/artificial inoculation	RFLP	Whole genome analysis	Linkage group 6 (A7)	cDNA011/cDNA110	64

Species	Locus	*Population	Phenotyping stage	Marker type	Mapping strategy	chromosome	Linked markers/interval	Reference
B. napus	*LEM1*	Major (resistant)/Stellar (susceptible)	Cotyledon Stem inoculation	RFLP	Whole genome analysis	A7	TG5D9b/WG5A1A	65
B. napus	*cLmR1*	Shiralee/90-3046 (153 DH lines)	Cotyledon incoulation	RFLP, RAPD	Bulked segregant analysis	A7	RAPD654 (~4.8cM)	66
B. napus	*cLmR1*	Shiralee/PSA12 (BC1 lines)	Cotyledon incoulation	RFLP, EST, SCAR	Bulked segregant analysis	A7	est126M9a/est149M9d	140
B. napus	*cLmR1*	DH12075/PSA12 (BC1 lines)	Cotyledon incoulation	RFLP, EST, SCAR	Bulked segregant analysis	A7	est126M9a/est149M9d	140
B. napus	*cLmR1*	Maluka/90-3046 (34 DH lines)	Cotyledon incoulation	RFLP, RAPD	Bulked segregant analysis	A7	RAPD654 (~4.8cM)	66
B. napus	*cRLM (cRLMm)*	Maluka/Westar	Cotyledon, Adult	RFLP, AFLP, RAPD	Bulked segregant analysis	A7	22-25	67
B. napus	*cRLM (cRLMrb)*	RB87-62/Westar	Cotyledon, adult plant	RFLP, AFLP, RAPD	Bulked segregant analysis	A7	22-25	67
B. napus	*cRLM (cRLMc)*	Cresor/Westar	Cotyledon, adult plant	RFLP, AFLP, RAPD	Bulked segregant analysis	A7	22-25	67
B. napus	*Rlm.wwai -A1*	Skipton/Ag-Spectrum (DH)	Cotyledon incoulation	SSR	Whole genome analysis	A1	Xpbcessrna16-Xbrms017b	32
B. napus	*QRlm.wwai-A10a*	Skipton/Ag-Spectrum (DH)	Cotyledon inoculation	SSR	Whole genome analysis	A10	Xcb10079d-Xcb10079c	32
B. napus	*Rpg_3Dun*	Westar/Dunkeld (F_2)	Cotyledon inoculation	SRAP	Bulked segregant analysis	A7	$NA_{12}A_{02}$-200/$NA_{12}A_{02}$-190, $BG_{20}SA_{12}$-480/$BG_{20}SA_{12}$-475/ BN204	81
B. juncea	*#Rlm5*	150-2-1, 151-2-1, Aurea, Picra	Cotyledon inoculation	-	-	-	-	71
B. rapa	*#Rlm8*	156-2-1	Cotyledon inoculation	-	-	-	-	71
B. rapa	*#Rlm11*	02-159-4-1	Cotyledon inoculation	-	-	-	-	72
B. napus	*#RlmS*	Surpass400	Cotyledon inoculation	-	-	-	-	73
B. juncea	*LMJR1*	AC Vulcan/UM3132 (F_2)	Cotyledon test	RFLP, SSR	Whole genome mapping	J13 (B3)	PN199RV (22.1 cM), sBb31143F (8.7 $_C$M)	43
B. juncea	*LMJR2*	AC Vulcan/UM3132 (F2)	Cotyledon test	RFLP, SSR	Whole genome mapping	J18 (B8)	PN120cRI, sB1534	43
B. juncea	*rjlm2*	B genome introgression lines	cotyledon	RAPD, RGA & SCAR	B genome-specific	not defined	B5-1520, C5-1000, RGALm	80

Table 1. Molecular mapping of qualitative genes for resistance to *Leptosphaeria maculans* in *Brassica.** BC: Backcross population, DH: Doubled haploid population. # loci not mapped with molecular markers to date.

Recently, two genes *BLMR1* and *BLMR2* in Surpass 400; an Australian cultivar developed from an interspecific cross between wild *B. rapa* subsp. *sylvestris* (resistant) from Sicily and *B. oleracea* subsp. *alboglabra* were identified [70, 76]. However, *LepR1* to *LepR4* genes are thought not be related with *Rlm* genes on the basis of their map locations, except for *Rlm2* and *LepR3*, which are phenotypically different [31, 69, 77]. It appears that loci *LepR3*, *BLMR1* and *BLMR2* localised on chromosome A10 control resistance to *L. maculans* in Surpass 400. However, Van de Wouw et al. [73] demonstrated that two independently segregating *L. maculans* avirulence (Avr) genes, *AvrLm1* corresponding to *Rlm1* (on chromosome A7) and *AvrLmS*, are responsible for inducing resistance in this cultivar. Subsequently, Larkan *et al.* [78] investigated the interaction of *AvLm1* and *AvLmS* isolates with *B. napus* populations segregating for the resistance genes *Rlm1* (from the French cultivar Quinta) and *LepR3* (from Surpass 400). This study reported that (i) *AvrLm1* interacts in a gene-for-gene manner with both *Rlm1* and *LepR3*, (ii) *AvrLmS* is not responsible for triggering the *LepR3* mediated defence response, (iii) Surpass 400 does not contain *Rlm1,* and (iv) *Rlm1* and *LepR3* may be the same genes located in two distinct loci or may have evolved as two functional genes. Recently, *LepR3* has become the first functional *B. napus* resistance gene to be cloned and was shown to encode a receptor-like protein. Additionally, *LepR3*-transgenic *B. napus* and *AvrLm1*-transgenic *L. maculans* were used to demonstrate that *AvrLm1* conveys avirulence to *LepR3*. The shared genomic location of *LepR3* and *BLMR1* also suggested that these were the same gene [79]. Several other genes such as *LmR1, ClmR1, LmFr1, cRLMm, cRLMrb, aRLMrb,* and *LEM1* have also been identified using uncharacterised isolates, which are thought to be allelic to known *R*-genes [45, 68, 74]. Qualitative resistance conferred by single major genes is usually dominant and expressed at the seedling growth stage. Qualitative *R*-genes explain majority of phenotypic variation for blackleg resistance at adult plant stage [32, 74]. However, digenic mode of inheritance has also been reported in *B. napus and B. juncea* populations [40, 80].

7.2. Quantitative resistance

Quantitative inheritance for field resistance has been reported in segregating populations derived from *B. napus*, *B. juncea* and their hybrid derivatives [30, 32, 65, 80, 86]. Some of the QTLs identified are given in Table 2. Quantitative genetic analysis revealed that significant non-additive genetic variance for all measures of disease severity indicated the presence of strong dominance/epistasis at loci controlling blackleg resistance [36]. In the literature, the term 'QTL' as a quantitative locus has been used even when a large percent of genotypic variation is explained by the major locus. In classical genetics, QTL refers to genes that have, low heritability, non-Mendelian and quantitative accumulative effects. The majority of genetic analyses have utilised doubled-haploid (DH) populations, which are not suitable to infer modes of inheritance. Advanced intercross populations are required to interpret such phenomena, as used in [74].

Mapping Population	Stubble, Location	Flanking markers	Chromosome	LOD# score	%Genetic variance (R^2)#	Additive effect	Reference
Av-Sapphire/ Westar10	*B. napus,* Lake Bolac, Australia	E34M15_S190/ E35M53_S416	A1	2.5-5.6	14-16	Not known (-)	86*

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Mapping Population	Stubble, Location	Flanking markers	Chromo-some	LOD# score	%Genetic variance (R^2)#	Additive effect	Reference
		E34M15_S218/ E35M53_S350	A2	1.3-3.8	4-26	-	
	Dahlen, Australia	CB10443_W258_ S269	C1	0.8-2.9	3-8	-	
	Lake Bolac	E36M47_W197/ E34M62_W127	LG1	1.0-3.6	4-10	-	
Caiman$_3$/Westar	*B. napus,* Lake Bolac	BRMS056/ E34M50_W140	A1	2.9-3.5	20-22.7	-	86*
	Dahlan	E35M53_C455/ E34M15_W271	A10	1.4-3.0	5-34	-	
		Not shown	C5	4.4-5.6	19-23	-	
Camberra$_4$/Westar	Lake Bolac	E36M55_C306/ E33M57_C306	A5	0.5-2.6	1.5-33	-	86*
	Dahlen	Na12D10_w203	A1/C1	2.9-5.1	17-18	-	
	Lake Bolac	E36M62_W414/0 1ju1fE07_cl_3b	A10	0.3-2.7	2-31	-	
		Not shown	C7-2	2.7-3.7	13-24	-	
		E33M59_W107/ E33M53_C75	LG2	2.1-2.8	14-28	-	
Darmor/Samourai	*B. napus*, Le Rheu	*BN483*	A1	2.3	6.7	Samourai	88
		BN239	A2	2.3-3.02	8.1-14.6	Darmor	
		BN182.1	A6	1.9-2.8	6.2-10.0	Samourai	
		At17	A10	2.7	11.0	Samourai	
		BN204	C2	2.0-2.2	8.0-8.4	Darmor	
		BN167	C4	2.4-3.2	6.7-12.2	Darmor	
		Vers6.9	C8	1.9	-	Samourai	
Darmor/Yudal	*B. napus*, Le Rheu, France	*OPE02.1200*	A2	2.4-5.5	3.8-8.5	Darmor	87 Delourme et al, 2008; comm. pers.)
		OPW08.1620	A4	3.3	4.8	Darmor	
		OPW05.750-Bzh	A6	5-12.2	7.2-20	Darmor	
		Bras023	A7	4.5	6.9	Darmor	
		CB10026b	A8	7.2	13.0	Darmor	
		OPW15.1470	A9	3.3	4.8	Darmor	
		Fad8	C2	5.5-6.6	8.3-13.3	Yudal	
		OPD08.1310	C4	4.7-9.5	6.7-15.2	Darmor	
		OPH06.CD1	C8	4.2	6.2	Darmor	
Rainbow/Av-Sapphire	Lake Bolac	E33M57_R105	A9-2	3.7	13	-	
Skipton/Ag-Spectrum	*B. napus* Mixed stubble,	*Xbras123/Xem1-bg11-237*	A2	7.0	11.5	Ag-Spectrum	32

Mapping Population	Stubble, Location	Flanking markers	Chromo-some	LOD# score	%Genetic variance (R^2)#	Additive effect	Reference
	Wagga, Australia	*Xbrms319-Xbrms176*	A9	2.9	5.0	Skipton	
		Xcb10172-BnFLC10	A10b	2.2	6.2	Skipton	
		Xbrms287a-Xcb10034	C1	4.2	11.5	Ag-Spectrum	
		Xol10-c10/Xna12-c03	C2a	6.8	16.6	Skipton	
		Xpbcessrna13/Xol13-d02a	C3	4.2	24.5	Skipton	
		Xem1-bg23-89/Xol12-e03	C6	6.1	14.5	Ag-Spectrum	
	B. napus, ATR Beacon stubble, Wagga, Australia	*Xol12-f11/Xpbcessrbr21*	A1a	6.1	26.1	Ag-Spectrum	

Table 2. Significant QTLs associated with blackleg resistance (scored as Internal infection due to canker development at adult plant stage) identified from mapping populations, * QTL with consistent effect, # range of LOD and R^2 varied with method of regression analysis (simple and composite interval mapping).* refers to predicted markers from supplementary figures ESM7-10 shown in Kaur et al [81]

8. Gene-for gene interactions

Host resistance genes (*R*-genes) interact in paired combination with pathogen avirulence (*Avr*) genes to condition resistance [89]. Two types of interactions may occur; compatible and incompatible. Compatible interaction occurs when there is an absence of an effective host defence response, due to a lack of a resistance allele in host (*r*) or an allele for virulence (*avr*) at the corresponding pathogen locus. An incompatible interaction occurs when there is no disease development due to the presence of both an effective host resistance allele (*R*) with an allele for *Avr* at the corresponding pathogen locus [90]. Biochemically, gene-for-gene interactions have been interpreted as the interaction of a race-specific pathogen elicitors with either cultivar-specific plant receptors or alternatively with a cultivar-specific signal transduction compounds [91]. Differential interaction between specific *R*-genes in the host (Brassica) and corresponding *Avr* genes of the pathogen *(L. maculans)* was first studied at the seedling stage using a cotyledon inoculation test in *B. napus* [92] and subsequently verified [57]. Qualitative and quantitative resistance differ with respect to host-pathogen interaction, as the latter does not appear to (but not proven) follow the gene-for gene hypothesis, being more effective against diverse pathogen populations (non-race specific). While quantitative resistance normally provides partial resistance to the pathogen and it is less likely to be rapidly overcome by shifting pathogen populations.

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At least ten *Avr* genes have been identified in *L. maculans*, many of which map to two gene clusters; *AvrLm1-AvrLm2-AvrLm6* and *AvrLm3-AvrLm4-AvrLm7-AvrLepR1* ([71, 72, 86, 87]. Four of the *Avr* genes; *AvrLm1, AvrLm6, AvrLm4-7* and *AvrLm11* have been cloned. It has shown that although *AvrLm1* and *AvrLm6* are physically clustered together in the *L. maculans* genome, they are not allelic forms of a single gene [85, 96]. However, *AvrLm4* and *AvrLm7* are allelic variants of a single *Avr* gene that corresponds to the two resistance genes; *Rlm4* and *Rlm7* [71, 85]. It has also been demonstrated that *AvrLm1* interacts with two distinct resistance loci; *Rlm1* and *LepR3*, though these loci are located on different chromosomes (A7 and A10, respectively) [78]. The cloning and characterisation of additional Brassica *R*-genes and *L. maculans* Avr genes will lead to a better understanding of how these functional redundancies developed. In the recent years, understanding of *L. maculans*/*Brassica* interactions has increased our ability to deploy appropriate *R*-genes in new cultivars and manage blackleg disease with the increased knowledge of the distribution of *Avr* alleles in *L. maculans* populations [27, 94, 98]. Currently, it seems that the genes involved in race-specific resistance and polygenic non-specific resistance are distinct. A better understanding of the mechanisms underlying quantitative resistance would help our understanding of the relationships between quantitative and major resistance genes [99].

9. Alien gene introgression for blackleg resistance

Deployment of *R*-genes has been used as the most cost-effective and environmentally sound measure for disease control in various crops since a century ago when first *R*-genes were identified [100]. Conventional plant breeding methodologies have played an important role in gene introgression for disease resistance, especially in easily-crossed genetic backgrounds. As a result several cultivars rated for resistance to *L. maculans* now dominate commercial cultivation worldwide. There has been a continuous threat of 'breakdown' of resistance, especially when a resistant cultivar is grown extensively on large acreages over long period of time. For example, 'breakdown' of resistance in cultivar Surpass 400 occurred within three years of its release [101, 102] due to the evolution and spread of more virulent strain of *L. maculans*. 'Breakdown' of resistance implies that the resistance has not changed rather the pathogen population has shifted/been selected for virulence. The effectiveness of *Rlm1* in France was also greatly reduced from 1997 to 2000 following wide deployment of *Rlm1* varieties, effectively selecting for enrichment of the virulent *avrLm1* allele in *L. maculans* populations [34]. Interestingly, a similar enrichment for the virulent *avrLm1* allele was documented after the 'breakdown' of *LepR3* resistance in Australia [103]. Due to the threat of current resistance being rendered ineffective by shifting *L. maculans* populations, new effective sources of resistance are constantly in demand. In order to enlarge genetic variation for resistance to *L. maculans*, interspecific and intergeneric donor sources have been utilised. This has been achieved by conventional sexual crossing [44, 52, 75, 104] or via laboratory tools such as somatic hybridization [105], and embryo culture. Roy [52] crossed *B. juncea* and *B. napus* to introgress genes for blackleg resistance but none of the interspecific hybrids

achieved the same level of *B. juncea* resistance as the donor parent. Wide hybrids (interspecific, intergeneric or intertribal) have also been produced either by sexual crossing followed by embryo culture or by somatic hybridisation as a result of protoplast fusion to transfer genes for blackleg resistance [106, 107]. Previous studies have reported hybrids between *B. napus* and *Arabidopsis thaliana*, belonging to different tribes; the Brassiceae and Sisymbrieae, respectively [108]. These hybrids were further utilized for identifying genetic regions associated with blackleg resistance [49]. Two regions localised on chromosome 3 of *A. thaliana* were shown to be linked with resistance to *L. maculans*.

Crouch *et al.* [75] transferred genes for resistance to *L. maculans* derived from *B. rapa* subsp. *sylvestris* into *B. napus*, using a resynthesised amphidiploid, as a result of hybridisation between *B. rapa* subsp. *sylvestris* and *B. oleracea* subsp. *alboglabra*. As a result, several cultivars derived from the re-synthesized *B. napus* lines were released for commercial cultivation in Australia such as Surpass 400, Surpass 404CL, Surpass 501TT, Surpass 603CL, Hyola 43, and Hyola 60. The *R*-genes *LepR1*, *LepR2* and *LepR4* have also been introgressed into *B. napus* via conventional interspecific crosses [75, 109]. Introgression of genes for resistance to *L. maculans* from *Sinapis arvensis*, *Coincya momensis* and *B. juncea* into *B. napus* was attempted [110]. Hybrid derivatives of *B. napus* and *S. arevensis*, and *B. napus* and *C. momensis* showed a high levels of resistance at the seedling (cotyledon) and/or adult plant stages. The offspring from asymmetric hybrids between *B. napus* and *B. nigra, B. juncea* and *B. carinata* were analysed for the presence of B genome markers and resistance to *L. maculans* [111]. This study revealed that resistance is conserved in one triplicate region in the B genome. Often, the majority of wide-hybrid derivatives exhibit unwanted traits and low frequencies of recombination between the different species which complicate the development of *B. napus* cultivars resistant to *L. maculans* by traditional breeding [43, 47]. Linkage drag due to suboptimal/undesired genes can be eliminated using the application of high density genome-wide molecular markers such as SNPs [112]. However, Rouxel and Balesdent [93] cautioned that before important breeding efforts are devoted to introgression of resistance genes from distant species into Brassica, there is a need thoroughly to evaluate their genetic control, putative redundancy and potential durability in the field.

Using transgenic technology, *R*-genes from other organisms can also be transferred irrespective of natural barriers to crossing. However, it is possible that transferred genes may not always contribute novel resistance specificities to the transgenic crop. Although several approaches have been used to induce host resistance in plants [113, 114] no major breakthrough has been made for an efficient management of blackleg disease. For example, Hennin *et al.* [115] demonstrated the expression of *Cf9* gene, which confers *Avr9*-dependant resistance to *Cladosporium fulvum* in tomato, along with co-expression of *Avr9* produced increased resistance to *L. maculans* in transgenic *B. napus* plants. Manipulation of plant defense responses is resource-expensive [116] and may be deleterious to the plant. Plants need to be selected for both appropriate expression of beneficial defense responses and avoidance of unnecessary ones [117], making artificially-induced constitutive expression of these responses an impractical solution to engineering resistance.

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10. Durability of resistance to *L. maculans*

Durable disease resistance can be achieved by utilisation of one or more single dominant *R*-genes [118]. However, the effectiveness of the specific *R*-genes depends on the *L. maculans* population structure, i.e. on the frequency of the corresponding *Avr* allele, which is known to differ according to regions/countries [27, 94] and the rapid evolution of virulent pathotypes. For example, the mean number of virulence alleles per isolates was reported to be higher in Australia (5.11 virulence alleles) than in Europe (4.33) and Canada (3.46) [27]. It has been suggested that there is a fitness cost associated with pathogen evolution from avirulence to virulence to overcome host resistance [38, 119].

Previous research has shown that different qualitative gene sources for resistance vary in providing effective durable resistance over period of time. For example, Light *et al.* [120] reported that the adult plant survival of French winter lines such as Doublol (*Rlm1*), Capitol (*Rlm1, Rlm3*), Columbus*1 (*Rlm1, Rlm3*), Carolus (*Rlm1, Rlm2, Rlm3*) and Rlm_EX (*Rlm7*) was higher than the Australian cultivar, AV-Sapphire and concluded that French winter canola cultivars have effective resistance under Australian conditions.

Single resistance genes do not always provide a durable resistance as has been shown in a field experiment using the *Jlm1*/*Rlm6* gene introgressed into *B. napus* from *B. juncea* [121]. Several incidences on the breakdown/ineffectiveness of race-specific resistance genes in Surpass 400 (*(LepR3, RlmS)*), in Vivol and Capitol (*Rlm1),* and *Rlm6* genes in *Brassica* have been reported in literature particularly when they were grown extensively [34, 94, 122]. As a consequence, breeders have to develop new cultivars and replace 'old' cultivars in order to change pathogen specificity of *R-gene* even without the knowledge of comprehensive distribution of *Avr* genes. The latter is now feasible and being used in order to monitor the pathogen population [123]. In order to avoid selection pressure against a particular *Avr* gene in the pathogen population, pyramiding of several host *R*-genes and deployment of quantitative resistance is being practiced in several crops such as in wheat, and barley. However, this strategy has not resulted in greater durability of resistance [124, 125]. In contrast, a recent study [121] demonstrated that a major *R*-gene (*Rlm6*) is more durable when expressed in a genetic background that also has quantitative resistance, indicating the need to identify and combine both qualitative and quantitative loci for blackleg resistance. Although the proposed strategy may be useful for blackleg disease management in areas where 'less' disease pressure and low variability with *L. maculans* populations exists, in Australia polygenic resistance derived from the French cultivar Jet Neuf [87], was reported to become less effective over time [37]. Additionally, several Australian cultivars which are reported to harbour both qualitative and quantitative loci for blackleg resistance are susceptible to natural populations of *L. maculans* Delourme et al [99]. It is difficult to know whether this evolution results from a change in virulence, or in aggressiveness in the pathogen populations since these polygenic-resistance cultivars may also carry specific *R*-genes [99]. In order to keep the frequency of isolates virulent towards any race–specific gene under a 'threshold' level, an integrated approach based upon best farm practices such as crop rotation, stubble management, application of fungicides and deployment of resistance genes including rotation of race-specific genes [126] needs to be implemented for

sustainable canola production, especially in areas where *L. maculans* populations are highly diverse and rapidly evolving.

11. Molecular dissection of qualitative and quantitative resistance loci

Molecular markers have been applied to identify loci associated with resistance to *L. maculans*, which relies on the availability of sequence variation among parental genotypes of mapping populations and diversity panels. Several genotyping methods based upon DNA hybridisation such as Restriction Fragment Length Polymorphism (RFLP) and Diversity Arrays Technology (DArT); PCR-based techniques such as Randomly Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP); Sequence-related amplified polymorphism (SRAP); and sequence-based analysis such as Single Nucleotide Polymorphism (SNP); Restriction site-associated DNA, (RAD) and Genotyping by Sequencing (GBS) have been developed for molecular analyses [81, 127 - 136]. RFLP, RAPD, SSR, SRAP and AFLP markers have all been used to map loci for resistance to *L. maculans* (Table 1). New marker technologies such as DArT, 60K SNP Infinium array, RAD and GBS are currently being developed and applied for mapping of blackleg resistance loci. These high-throughput approaches are expected to complement or replace low-throughput marker assays that were used previously to facilitate genetic and physical map-based cloning of resistance genes.

Loci for resistance to *L. maculans* have been mapped using linkage/QTL mapping and association mapping approaches [32, 74, 82, 86, 137] using structured (F_2, doubled-haploid (DH) and backcross) and unstructured (diversity sets/breeding lines) populations (Table 1). Bulked Segregant Analysis approach, used for the first time [138], is particularly useful when a limited number of traits are to be mapped and resources (money and time) required for extensive genotyping are limited [81]. Whole-genome analysis has been used to locate both qualitative and quantitative loci associated with resistance to *L. maculans* [32, 86]. Generally, it requires the framework linkage map of all 19 chromosomes (linkage groups) for linkage (QTL) analysis.

11.1. Qualitative resistance

The majority of genes for resistance to *L. maculans* have been genetically mapped with molecular markers (Table 1) on chromosomes A1, A2, A6, A7, A10, B3, B4 and B8 in *Brassica species: B. rapa, B. napus, B juncea* and *B. nigra* [31, 32, 45, 68-70, 99]. None of the race-specific genes have been mapped on the C genome yet. Previous linkage mapping studies revealed that at least five resistance genes (*Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9)* are localised in a cluster within a 35 cM genomic region on chromosome A7 [32, 45, 64-68, 74, 82]. This genomic region showed extensive inter- and intra-genomic duplications, as well as intra-chromosomal tandem duplications [140]. Whether some of these *R*-genes are allelic remains unknown. For example, it was concluded that at least four resistance genes *Rlm3*, *Rlm4*, *Rlm7*, and *Rlm9* could correspond to a cluster of tightly linked genes, to a unique gene with different alleles, or to a

combination of these two hypotheses. However, *Rlm1* has been shown to be linked with *Rlm3* but is not allelic [74].

A major gene named *LmFr1* controlling adult plant resistance to blackleg was tagged in the DH population from French cultivar Cresor (resistant to *L. maculans*) and Westar (susceptible to *L. maculans*) with RFLP markers [64]. Similar study [65] mapped loci for blackleg resistance in a DH population from Major/Stellar and found that genetic control of resistance vary with inoculation techniques. A major gene designated as *LEM1* was mapped to linkage group 6 based on qualitative/quantitative scores of the interaction phenotype on inoculated cotyledons with a single ascospore-derived PG2 isolate, PHW1245. However, four other putative QTL for resistance were also identified on linkage groups LG8, LG17 and pair 4. This study further showed that none of the QTL that were associated with resistance at the seedling and stem stage had a significant effect in conferring resistance in the field. This may be attributed due to use of different pathogen population (PHW 1245 in cotyledon and stem experiments and natural *L. maculans* population in field experiment). The *Rpg3Dun* gene was mapped in an F_2 population from Westar/Dunkeld and identified a suite of SCAR markers that showed cosegregation with resistance to *L. maculans* [81]. Recently, the whole genome average interval mapping approach was applied to localise both qualitative and quantitative trait loci controlling blackleg resistance [32] in a DH population derived from the Australian *B. napus* vernalisation responsive cultivars, Skipton and Ag-Spectrum. Marker regression analyses revealed that at least fourteen genomic regions were associated with blackleg resistance, explaining 19.5% to 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 BRMS075 (Table 1).

Genomic regions of chromosome A10 harbours *Rlm2*, which has been shown to be the most common *R*-gene in winter *B. napus* varieties, such as Samourai, Eurol, Bristol, Symbol, Andol, Kintol, Akamar, Colvert, Synergy, and Tapidor, [41]. Chromosome A10 also harbours *LepR2*, *LepR3* and *BLMR2* genes derived from *B. rapa* subsp. *sylvestris* sources [31, 69, 70]. *LepR1* and *LepR2* were mapped on chromosomes A2 and A10, respectively with RFLP markers [31]. Genetic analysis revealed that both genes confer resistance independently and therefore are additive. *LepR1* was a dominant nuclear gene while *LepR2* was an incompletely dominant gene. This study further showed that *LepR1* generally conferred a higher level of resistance than *LepR2*. Both genes exhibited race-specific interactions with pathogen isolates.

The blackleg resistance gene *Rlm6* has been identified on B genome chromosome 8 [47]. *Rlm6* has been successfully introgressed to *B. napus* (AACC) from *B. juncea* (AABB) [47, 141] and provides excellent resistance to *L. maculans* isolates [58], though this gene has not yet been deployed in commercial cultivars [47, 58].

11.2. Quantitative resistance

The genetic basis of quantitative resistance has been investigated only in limited *B. napus* cultivars such as in Darmor; a derivative of Jet Neuf [119, 137, 142]. However, a number of DH populations have recently been utilised for identification of loci for quantitative resistance under field conditions [32, 86, 143], and are currently being validated (Raman *et al*., unpublished, Larkan *et al*., unpublished). Thirteen quantitative trait loci (QTL) on 10 linkage groups

associated with quantitative field resistance to *L. maculans* were identified in a DH population from Darmor-*bzh*/Yudal [87]. Their detection was dependent upon phenotypic method used; seven QTL for mean disease index and six QTL for per cent survival (percentage of lost plants due to canker) and were also dependant on growing environment (year of evaluation). However, only four of the QTL were stable across experiments. These QTL accounted from 23% to 57% of the genotypic variation (Table 2). The unexplained variation was described due to non-detected additive QTL, G x E interaction and incomplete map coverage. This study further showed that resistance to *L. maculans* is influenced with growth habit. For example, one QTL, located close to a dwarf gene (*bzh*), was detected with a very strong effect, masking the detection of other QTL. This study further showed that these dwarfing genes also affect other traits such as earliness, and glucoinsolate content.

In order to validate the stability of QTL for field resistance to *L. maculans*, QTL were mapped and characterised in $F_{2:3}$ population from Darmor (resistant)/Samourai (susceptible) revealing only four QTL on LG3, LG11 and DY5 and DS6 that were consistent in Darmor/Yudal and Darmor/Samourai populations [143]. This study found that the genetic background and inoculum pressure are the major factors of the QTL instability and therefore suggested that QTL mapping must be carried out separately for each population. The genomic regions carrying the most consistent resistance QTL in Darmor do not correspond to the two regions on N7 (A7) and N10 (A10) identified as carrying race specific resistance genes to *L. maculans* [74]. The position of *Rlm2* on N10 (A10) corresponds to a QTL identified for adult plant resistance in the Darmor/Samourai DH population [88]. The cultivar Samourai carries both the resistance allele at this QTL and *Rlm2*. Since it has been reported that no French isolates of *L. maculans* carry *AvrLm2* [34], two hypotheses were proposed to explain this co-location; either the *Rlm2* gene has a residual effect at the adult plant stage, similar to that suggested in other pathosystems, or genes linked to *Rlm2* are responsible for part of variation for resistance at this QTL [99].

QTL for blackleg resistance were identified in four mapping populations derived from the crosses Caiman/$Westar_{10}$, Camberra/$Westar_{10}$, ^{AV}Sapphire/$Westar_{10}$ and Rainbow/^{AV}Sapphire [86]. Multiple QTLs were identified accounting for 13–33% of phenotypic variance. A recent study [32] identified seven significant QTL associated with blackleg resistance, scored on the basis of internal disease score, on chromosomes A2, A9, A10, C1, C2, C3 and C6 in a DH population derived from Skipton/Ag-Spectrum. The genotypic variation explained by the individual QTL ranged from 5% to 24.5%. Both parents contributed the alleles for blackleg resistance. This study showed poor correlation between canker lesion scores over the two years (2009, 2010). Some of the genomic regions for blackleg resistance may be the same as reported previously that have been identified using both classical QTL and association mapping approaches [31, 69, 87, 137, 144, 145]. The conservation of QTL between Australian and French studies is interesting and suggests the non-specificity of these QTL, irrespective of the environment, genetic background and G x E interactions [32]. However, it is possible that some of the original donor gene sources in French and Australian parental lines used for mapping resistance genes may be the same.

The majority of mapping populations used to map blackleg resistance genes in *B. napus* so far have been comparatively small (Table 1). The development of a high density map utilising larger populations, comprising several hundred to thousands lines, will allow for the precise mapping of resistance loci. Stability of QTL resistance needs to be tested in different environments. Although QTL mapping studies provide comprehensive information on the nature of inheritance, location, magnitude and allelic effects of QTL, much of the information tends to be 'population' specific. In biparental (structured) populations, generally two alleles at each locus are sampled and therefore trait-marker association may not be highly relevant to diverse genetic backgrounds. The validation of trait-marker association is necessary before their use for routine marker-assisted breeding (MAS). Association mapping can be utilised for investigating linkage disequilibrium close to loci of interest in a diverse germplasm [145-149] and therefore offers an alternative to linkage and QTL mapping. This approach has been applied in determining and confirming the markers located within the QTL associated with resistance to *L. maculans* previously identified in Darmor and established their usefulness in MAS [137]. A diverse set of an oilseed rape collection, comprised of 128 lines showing a large spectrum of responses to infection by *L. maculans*, was characterised using 72 SSR and other markers. At least 61 marker alleles were found to be associated with resistance to stem canker. Some of these markers were associated with previously identified QTL, which confirms their usefulness in MAS. Markers located in regions not harbouring previously identified QTL were also associated with resistance, suggesting that new QTL or allelic variants are present in the collection [137]. Genome-wide association based on 1513 markers enabled identification and validation of genomic loci associated with blackleg resistance. This study 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. Both studies suggested that association mapping is an efficient approach for identifying novel loci/alleles associated with blackleg resistance in diverse germplasm [137, 142]. Superior molecular marker allele(s) associated with resistance to *L. maculans* may be captured by canola breeding programs. Molecular markers associated with seedling and stem canker resistance will help identify accessions carrying desirable alleles and facilitate QTL introgression to develop elite germplasm having new gene/allele combinations for blackleg resistance [32].

12. Host *R*-gene cloning and candidate gene analysis

At least 20 *R*-genes and several allele variants and haplotypes of cloned *R*-genes have been identified in plants [151-158]. Molecular analyses revealed that these genes belong to large multiple gene families, which encode nucleotide binding site- leucine–rich repeats (NBS-LRRs), serine-threonine-kinases, leucine zipper and protein kinase domains, and toll/interleukin-1 receptor domains [159-164]. These genes are often clustered in many plant species including crops such as rice, maize and soybean and transduce the hypersensitive response to defend against pathogen attack [164-167]. At least 30 CC-NBS-LRR and TIR-NBS-LRR non-redundant genes have been identified in *B. rapa* [167]. Two major gene clusters for resistance to *L. maculans* exist on chromosomes A7 [74] and A10 [31, 69, 70], along with other genes

dispersed on different chromosomes. It is possible that some of these *R*-genes may represent to multiple copies of the same functional gene. A recent study has shown that at least eight functional copies of *FLOWERING TIME LOCUS C* (*FLC*) exist within *B. napus* [6] which may modulate flowering time and other functions in different cultivars [168].

In *B. napus*, only few studies aimed at characterizing the genes underlying the resistance to *L. maculans* have been attempted. The recent cloning of the first functional *B. napus* resistance gene *LepR3* revealed a receptor-like protein responsible for conferring resistance to *AvrLm1 L. maculans* isolates [79]. Resistance genes effective against *L. maculans* have also been cloned in *A. thaliana* [169-171], which encode Toll interleukin-1 receptor-nucleotide binding (TIR-NB) or TIR-NB-LRR class proteins. Based on the synteny between *B. napus* and *A. thaliana*, it was deduced that several *B. napus* resistance genes are localised in a region of A7 (N7) that corresponds to the chromosome segment on *Arabidopsis* chromosome 1 which harbours *RLM1*Col [139, 167]. However, a recent report detailing the gene responses to *L. maculans* infections suggests very different responses in *B. napus* and *A. thaliana* [172]. Both salicylic acid and ethylene signaling was triggered in *B. napus*, possibly due to the hemibiotrophic nature of the infection. This stands in contrast to the JA signaling observed in *A. thaliana*, suggesting *L. maculans* is acting as a necrotroph during infection of susceptible *A. thaliana* lines. Since many *R*-genes are conserved and share sequence similarity, degenerated primers based on conserved motifs of *R*-genes have also been used to localise potential resistance gene loci in Brassica species such as *B. oleracea* (on chromosomes C1 (O1), C4 (O4), C8 (O8) and C9 (O9) and *B. napus* on linkage groups LG1a, LG1b, LG2, LG5, LG8, LG12, LG13, LG14, LG15 and LG18 [173, 174]. However their association with loci controlling resistance to *L. maculans* have not yet been established/validated.

In order to clone genes controlling blackleg resistance in *B. napus* population, high resolution mapping of *LmR1* and *ClmR1* loci was performed using 2500 backcross lines from two crosses between PSA12 and Shiralee, and PSA12 and Cresor, respectively [140], and reported that both resistance loci are located in a highly duplicated genomic region on chromosome A7. This region contained several genes encoding protein kinases or LRR domains. It is reported that the SCAR marker (BN204) that showed cosegregation with *RpgDun* locus for resistance to *L. maculans* is derived from a region showing 92% amino acid identity with the defense-related gene serine threonine 20 (ste-20) protein kinase of *Arabidopsis thaliana* [81]. A proteomic approach has also been utilised to understand gene expression in response to *L. maculans* infection [176]. However, candidacy of any of these genes has not yet been reported.

Recently an alternative approach for identifying candidate *R*-genes has been employed based on genomics [177]. Next-generation massively parallel sequencing platforms such as the Roche 454 genome sequencer FLX instrument, the Illumina Genome Analyser (HiSeq), and the ABI SOLiD System have revolutionized genome sequencing by providing high throughput and cost-effective high coverage sequencing [179-182] and has enabled much quicker identification of candidate genes [178]. Molecular markers associated with *RlmSkipton* (*Rlm4*) locus in the DH population from Skipton/Ag-Spectrum were aligned with the complete genome sequence *B. rapa* as reported in [32]. Eighteen candidate genes, designated as *BLR1-18* with disease resistance characteristics, several of which were clustered around a region syntenic to *Rlm4*.

Among candidates, *BLR2* and *BLR11* were the promising candidates for *Rlm4*-mediated resistance [178]. High resolution mapping and gene sequencing of different sources of *L. maculans* resistance will allow for a better understanding of the structural organisation and function of *R*-genes. Recently, the reference genome of *B. rapa* has been published [182] and genomes of *B. oleracea*, *B. nigra* and *B. napus* are expected to be published in coming years. Re-sequencing of whole genomes of known blackleg-resistant genotypes will allow identification of genetic variation between individuals, which can provide molecular genetic markers and insights into gene function [183]. Sequencing of different *R*-genes and understanding their function will also enable us to manipulate resistance to *L. maculans*, as genes with different specificities can be created.

13. Predictive breeding for resistance to *L. maculans* using molecular markers

Success of new disease resistance genes relies heavily on the successful transfer of target genomic regions from donor sources and the development of rigorous selection methods. Molecular markers have been used to improve the effectiveness and efficiency of selection strategies in predictive breeding in several agricultural crops. However, the development of molecular markers in *B. napus* and their application in breeding is a challenging exercise due to the large genome size, amphidipliod (4X) nature, open-pollination and lower research funding as compared to other key crops such as wheat, barley, maize and soybean. The *B. napus* genome is highly complex and homologous recombination plays a major role in chromosome rearrangements such as duplications and reciprocal translocations. These arrangements further add to the complexity of molecular analysis and interpretation. *B. napus* chromosomes C6 and A7, which harbours *Rlm1, Rlm3, Rlm4, Rlm7* and *Rlm9* genes for resistance, produced a reciprocal translocation in some cultivars such as in Westar, Marnoo, Monty and Maluka [185, 186] which makes analysis of resistance genes difficult [142].

In most of the breeding programs, selection for blackleg is conducted once a year during the growing season, hampering selection efficiency. Several studies suggest a significant correlation between cotyledon test and canker lesion scores. Therefore, cotyledon tests can be used for selection for resistance to *L. maculans*. However, in many developed countries, it is costly and laborious to perform, particularly as compared to molecular marker analysis, when several tests need to be carried to screen large populations. Furthermore, analysis of different blackleg resistance genes in a canola breeding program using a differential set of *L. maculans* isolates at various stages of the breeding cycle is a very slow process [39]. Interpretation of *R*-gene content using a differential set of control *B. napus* varieties, especially of Australian origin, is a challenging exercise, as majority of cultivars used are heterozygous and/or heterogeneous [32, 41]. In addition, phenotypic tests are dependent upon the growing environment (microclimate conditions and other factors such as powdery and downy mildew), which can complicate scoring of inoculated seedlings. Molecular markers generally out-perform conventional seedling assays, in both efficiency and reliability. It is also possible to identify haplotypes using

molecular markers and then validate trait-marker associations, in conjunction with comprehensive phenotyping and conventional allelism tests.

The published literature suggests that little effort has been made to evaluate the allelic relationship among the known genes from different sources, to test stability of majority of QTL or qualitative genes identified over diverse growing environments, or to test their usefulness in achieving long term durable control of the disease. Table 1 also suggests that majority of markers are not very closely linked (<1cM) with resistance loci. Diagnostic or perfect markers for resistance genes are required for routine MAS and will assist allele enrichment strategies in breeding programs, although this is not always possible, even if the complete gene is cloned and characterised for its functionality [187]. The linkage between molecular markers and *Xbn204* flanking the *RlmSkipton* locus was verified in an F_2 population derived from Skipton/Ag-Spectrum [32]. The results showed that SSR markers linked to *RlmSkipton* are suitable for enrichment of favourable alleles for blackleg resistance in breeding programs. A separate study [82] validated the map location of *Rlm1* in the DH population derived from Maxol/Westar with SSR and DArT markers. Previously, *Rlm1* and *Rlm3* genes were mapped on chromosomes A7 in the Maxol (resistant to blackleg)/S006 (susceptible to blackleg) utilising RAPD markers and with single spore isolates with known *Avr* genotypes in the *B. napus* European cultivars, Columbus and Maxol [41, 71, 74]. RAPD markers are not amenable for high throughput marker analysis, as they are assayed on low-throughput agarose or polyacrylamide gel systems. Validation of a large array of genes for blackleg resistance in diverse segregating populations representing *B. napus* germplasm is a challenging exercise. However, an association mapping approach can be employed to test trait-marker associations in a large set of germplasm as demonstrated recently [137, 142].

14. Conclusions

It is now clear that major resistance genes will be overcome in time, as has been seen in many crop plants. Therefore, there is constant need to identify new sources of both qualitative and quantitative resistance loci and to properly utilise the resources available to us so that resistance can be deployed long term. Recent advances in molecular marker systems, such as the development of highly-parallel systems for genotyping and sequencing, have created new opportunities and strategies to select for qualitative and quantitative traits, including resistance to *L. maculans*. Strategies for deploying resistance in breeding programs will vary with individual breeding programs; monitoring introgression of specific loci, using whole-genome marker scans (genomic selection) or identifying individual plants that may offer the greatest opportunity for genetic gain. This is now becoming reality as several genome-wide signals associated with blackleg resistance have been identified (but need to be validated) and alleles at these loci can be selected efficiently and at a cheaper rate with new marker technologies. Development and validation of tightly-linked molecular markers amenable to high throughput marker screening with both qualitative and quantitative resistance and cost effective systems will enable the increased adoption in *B. napus* breeding programs. In addition to genetic resistance, deployment of agronomic practices such as use of rotation and stubble

management will remain key management tools for reducing pathogen inoculum for subsequent crops.

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