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RESEARCH REPORT

In-silico identification and differential expression of putative disease resistance-related genes within the collinear region of *Brassica napus* blackleg resistance locus *LepR2'* in *Brassica oleracea*

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Abstract
Blackleg disease, caused by *Leptosphaeria maculans,* greatly affects the production of cabbage (*Brassica oleracea*). However, definitive R-gene(s) are yet to be identified in this crop. In contrast, a number of R-loci have been identified in A- or B-genome crops. Identification of few resistant cabbage genotypes indicates the presence of R-genes in this C-genome crop. High ancestral synteny between *Brassica* genomes suggests that the collinear regions of known A- or B-genome R-loci may also contain functional R-genes in the C-genome. Strong resistance was observed in the cotyledons of cabbage inbred line SCNU-98 against two *L. maculans* isolates, 03–02 s and 00–100 s. We investigated the collinear region of the *Brassica napus* blackleg resistance locus *LepR2'* in *B. oleracea* since both isolates of *L. maculans* contain corresponding avirulence genes. The locus was collinear to a 5.8 Mbp genomic segment of *B. oleracea* chromosome C09 containing 13 genes that have putative disease resistance-related domains. High expression of genes Bo9g117290 and Bo9g111510 against isolate 00–100 s, and high expression of genes Bo9g126150 and Bo9g111490 against both isolates in the resistant-line SCNU-98 indicate their putative roles in blackleg resistance, which remained to be functionally verified. This work enhances our understanding of R-gene-mediated resistance to blackleg in cabbage.

Keywords Blackleg · Cabbage · *LepR2'* · *Leptosphaeria maculans* · *LRR-RLK* · qRT-PCR · R-gene · Synteny

1 Introduction

Blackleg, a disease particularly devastating to canola, is also known to cause substantial economic damage to cabbage (Dilmaghani et al. 2010; Humpherson-Jones 1985; Piliponytė-Dzikienė et al. 2015). The disease is caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*) around the world and by *Leptosphaeria biglobosa*, a comparatively less damaging species, in Asian countries (Zhang et al. 2014; Liu et al. 2014; Hong et al. 2009; Hao et al. 2015; Mendes-Pereira et al. 2003). Cabbage is an essential ingredient of the daily diet, either in fresh or processed form, in East Asian countries such as Korea, Japan, and China, where 37.8% of the world's total cabbage is produced (FAO Statistics Database 2017).

The possibility of invasion by the more aggressive *L. maculans* is a growing concern for the East Asian cabbage industry, since *L. maculans* was previously reported to spread in Canada and Poland, where only *L. biglobosa* was predominant (Liu 2008; Fitt et al. 2008). Further, both pathogenic species inhabit similar ecological niches and preference for the agro-climatic conditions of this region may be conducive to the establishment of *L. maculans* (Fitt et al. 2008; West et al. 2001). The complicated life cycle of the pathogen, its ability to reproduce both sexually and asexually

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(Rouxel and Balesdent 2005), multiple disease cycles in a single growing season (Li et al. 2007), longer resting period (several years) in crop residues (West et al. 2001; Li et al. 2007), and substantial global diversity in the pathogenicity of *L. maculans* strains (Kutcher et al. 2011) make it difficult to control the disease via chemical and agronomic practices alone. Preventing the spread of *L. maculans* and the development of resistant cultivars are thus prioritized in these countries to safeguard both canola and cabbage industries (Zhang et al. 2014; van den Burg et al. 2008; Zhang and Fernando 2018).

Unlike rapeseed and canola, resistance to blackleg has not been extensively investigated in cabbage. Hence, sources of resistance to the disease are scarce in cabbage. Most of the R-loci against the disease have been identified in the A- or B- genomes of *Brassica* family crops (Balesdent et al. 2002; Bohman et al. 2002; Rimmer and van den Berg 1992; Chèvre et al. 1997; Christianson et al. 2006; Delourme et al. 2006; Delourme et al. 2004; Leflon et al. 2007; Marcroft et al. 2002; Pang and Halloran 1996; Plieske et al. 1998), while definitive R-loci/genes in C- genome crops such as cabbage are yet to be identified (Larkan et al. 2013; Robin et al. 2017). However, a few cabbage genotypes have been reported to show moderate resistance against the disease (Ananga et al. 2006; Badawy et al. 1991; Ferreira et al. 1992). Very recently, two Korean cabbage lines were found to be resistant at the cotyledon stage against two *L. maculans* isolates, 03–02 s and 00–100 s, which contain multiple avirulent genes (Robin et al. 2017). These findings suggest the presence of R-gene(s) in cabbage.

Functional analysis indicated that most plant R-genes usually contain several domains such as leucine-rich repeat (LRR), nucleotide-binding site (NBS), coiled-coil (CC), Toll/Interleukin-1 Receptor (TIR), receptor like protein kinase (RLK), F-box domain (FBD), and mitogen-activated protein kinase (MAPK), which have distinct roles in plant defence against phytopathogens (Larkan et al. 2013; Ellis et al. 2000; Liu et al. 2007; Meng and Zhang 2013; Meyers et al. 1999; Sekhwal et al. 2015; van den Burg et al. 2008). Since A-, B-, and C-genomes of Brassica family crops share common evolutionary history and high ancestral synteny, we hypothesized that functional disease resistance-related domain-containing genes may also be present within the collinear regions of known A- or B-genome R-loci in the C-genome (Cheng et al. 2014; Franzke et al. 2011; Chalhoub et al. 2014; Liu et al. 2014). Such approach has been used to identify candidate orthologous genes for Sclerotinia stem rot resistance, seed colour, seed oil content, yield, and efficiency traits in oilseed rape (*B. napus*) using the corresponding known loci of *Arabidopsis thaliana* (Ding et al. 2012; Stein et al. 2013; Wu et al. 2013; Zhao et al. 2012). In the case of blackleg resistance, Yu et al. (2013) tracked the collinear region of *B. napus* blackleg resistance locus *LepR4* in *B. rapa* and identified four putative disease resistance-related NBS-encoding genes. We have investigated the *B. rapa* R-loci *Rlm1*, *Rlm2*/*LepR3*, *LepR1*, and *LepR4* from the A-genome against the C-genome (Nou IS, unpublished data). Here, we report disease resistance-related functional domain-containing genes within the collinear region of *B. napus* blackleg resistance locus *LepR2'* in the C-genome of *B. oleracea* along with their putative roles in blackleg resistance in cabbage, determined via differential expression analysis in contrastingly resistant cabbage lines against two *L. maculans* isolates.

2 Materials and methods

2.1 In-silico analysis of the collinear region of *B. napus* blackleg resistance locus *LepR2'* in *B. oleracea*

The collinear region of *B. napus* blackleg resistance locus *LepR2'* was identified in the *B. oleracea* genome (ensembl database) using the homologous segments of *B. napus* clones that contain the *LepR2'* flanking markers *sN3888Fa* and *sR6903a* (Yu et al. 2012). Sequences of all the genes within this collinear region in *B. oleracea* were retrieved. The genes which contained putative functional disease resistance-related domains such as NBS, LRR, CC, TIR, MAPK, FBD, RLK, and RLP were identified using the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and the Conserved Domain Database Tool (https://www.ncbi.nlm.nih.gov/cdd/). MEME suite version 5.0.5 (http://meme-suite.org/tools/meme) was used to analyse the conserved motifs in the proteins encoded by these genes. Distribution of exons and introns of these genes were analyzed using the Gene Structure Display Server (GSDS2.0) web tool (http://gsds.cbi.pku.edu.cn/). The microsynteny relationship of these identified putative disease resistance-related genes with *B. rapa, B. nigra*, and *A. thaliana* were visualized via Circos v0.69.

2.2 *L. maculans* isolates and preparation of inoculum

L. maculans isolates 00–100 s and 03–02 s, possessing multiple avirulence genes, were cultured on V8 agar media (20%) at 22 °C and 16 h day length under fluorescent light for several weeks. Fungal spores were suspended in 10 mL sterile distilled water by scrapping the spores off culture plates with a sterile glass slide prior to collecting the spores by filtering the suspension with sterile Miracloth (EMD Millipore Corporation, USA). The spore concentration was adjusted to 2.25×10^7 spores mL^{-1} using sterile distilled water prior to inoculation.

2.3 Plant materials: inoculation and assessment of disease resistance

Seeds of two Korean cabbage inbred lines, SCNU-72 and SCNU-98, were germinated in multi-pot trays using cocopeat soil in a growth chamber at 24 ± 2 °C, 65% relative humidity, and a 16/8 h (light/dark) photoperiod under 420 µmol photons m^{-2} s^{-1} light intensity at bench level. After 12 days of germination, the center of each cotyledon lobe was punctured with a sterile needle prior to inoculation with 10 µL of the spore suspension. The disease responses were evaluated at 10 days post-inoculation (dpi) using a disease rating scale of 0–9 following the procedures described in Robin et al. (2017).

2.4 RNA Extraction and cDNA synthesis

Total RNA was extracted from control (0 h), mock-, and pathogen-inoculated cotyledons of resistant and susceptible cabbage lines at 6 h, 24 h, and 48 h using an 'RNeasy mini kit' (Qiagen, CA, USA). RNA purity and concentration were determined using a Nanodrop-2000 (Nanodrop Technologies, Wilmington, DE, USA). First-strand cDNA was synthesized from the extracted total RNA using 'SuperScript-III First-Strand Synthesis SuperMix' (Invitrogen, CA, USA) as per the manufacturer's guideline.

2.5 Expression analysis by qRT-PCR

Expression of the identified putative disease resistance-related domain (e.g., LRR, CC, FBD, CC, MAPK and RLK) containing genes within the *LepR2'* collinear region in *B. oleracea* were analyzed by qRT-PCR in a Roche LightCycler® 96 System (Roche Applied Science, Penzberg, Germany). Gene-specific primers were designed using 'Primer3Plus'. For each gene, the qRT-PCR reaction was performed with 5 µL of 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, London, UK), 1 µL of forward and reverse primers each (10 pmol), 2 µL of ultra-pure water, and 1 µL of template cDNA (60 ng μL^{-1}) in a final reaction volume of 10 µL. The reaction condition was as follows: initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at specific temperatures for 10 s, and amplification and signal acquisition at 72 °C for 30 s. Each biological replicate was tested with three technical replicates. Relative expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method using the mean of three actin genes as the internal control.

2.6 Statistical analysis

Statistical significance was tested using analysis of variance (ANOVA) and mean separation was performed using Tukey's pairwise comparison in Minitab (v18) (Minitab Inc., State College, PA, USA).

3 Results

3.1 Disease responses of cabbage lines to *L. maculans* isolates

Significantly different responses to the two *L. maculans* isolates, 03–02 s and 00–100 s, were observed in the inoculated cotyledons of cabbage inbred lines SCNU-72 and SCNU-98 (Fig. 1a, b). At 10 dpi, the disease scores against both 00–100 s and 03–02 s isolates ranged from 7 to 8 in the line SCNU-72, having 70% and 80% diseased cotyledon areas, respectively. In contrast, the disease scores in the line SCNU-98 ranged between 2–3 and 2–4 against these isolates, respectively, while having less than 30% diseased cotyledon area. This indicates that line SCNU-98 is resistant against both *L. maculans* isolates, 00–100 s and 03–02 s. At 30 dpi, overall blackening of the stems of the susceptible line SCNU-72 was observed, whereas the stems of the resistant line SCNU-98 appeared healthy (Fig. 1c).

3.2 Collinear region of *B. napus* blackleg resistance locus *LepR2'* in *B. oleracea*

BLAST search of the sequences of the clones containing the flanking markers *sN3888Fa* and *sR6903a* of *B. napus* blackleg resistance locus *LepR2'* identified a 5.8 Mbp genomic segment of chromosome C09 (C9:36168200-41971165) as the corresponding collinear region in the *B. oleracea* genome (Fig. 2a, b). This region was flanked by genes Bo9g111470 and Bo9g135890. Mining this collinear region identified a total of 661 genes. The complete list of these 661 genes and their functional annotations are shown in Supplemental Table S1.

3.3 Identification of genes containing disease resistance-related functional domains

Functional domain analysis of the 661 genes identified 13 genes as having putative disease resistance-related domains (Table 1; Table S2). Five different putative disease resistance-related domains, namely leucine rich repeat (LRR), coiled-coil (CC), F-box domain (FBD), mitogen-activated protein kinase (MAPK), and receptor-like kinase (RLK) were identified within these 13 genes. Among these, 11 genes contained two domains, an LRR domain and either a FBD, MAPK, or a RLK domain; two genes contained only one domain, including Bo9g120720 (LRR domain) and Bo9g135700 (CC domain). A domain-wise list of the selected 13 genes is shown in Table 2 and the domain

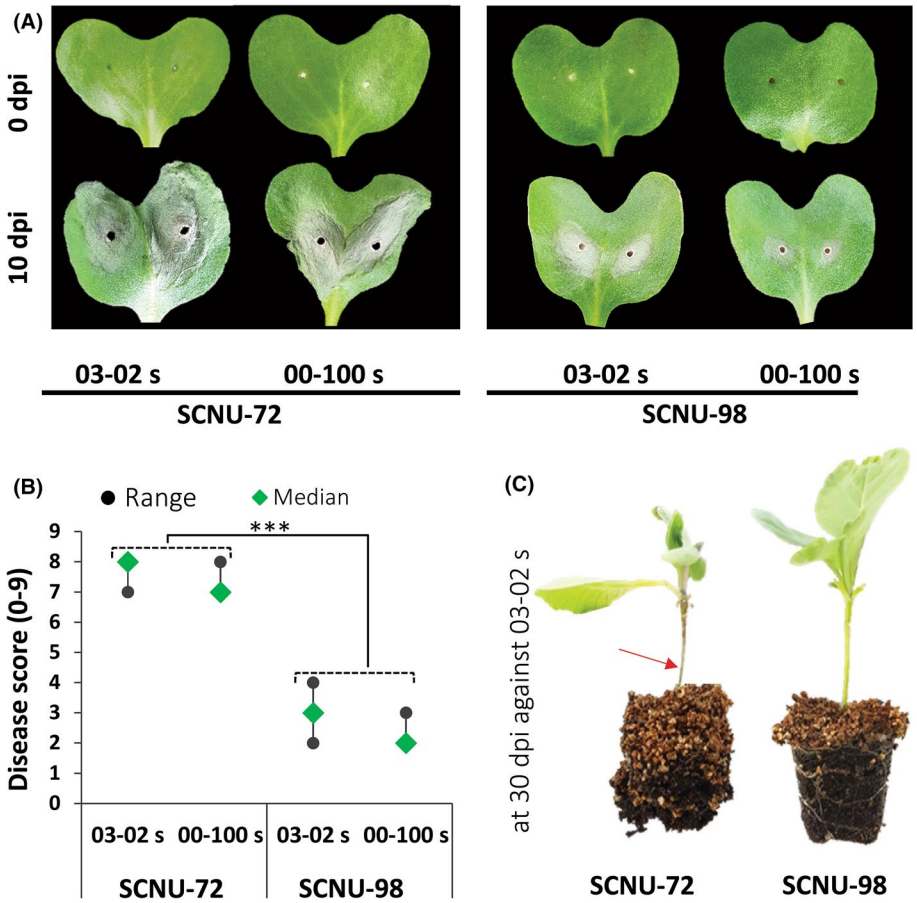

Fig. 1 Disease symptoms (**a**) and scores (**b**) at 10 days post inoculation (dpi) against *L. maculans* isolates 03–02 s and 00–100 s at the cotyledon stage and disease (blackened stems) symptoms (**c**) at 30 dpi against isolate 03–02 s in the stems of the seedlings of cabbage inbred lines SCNU-72 and SCNU-98. Cotyledons of 12-day-old seedlings were inoculated and disease scores were recorded at 10 dpi. Data of five replicates are presented as a range, with green diamond shape indicating median values. ***$p < 0.001$ (one-way ANOVA with Tukey's multiple comparison test). Blackened stem is indicated by red arrow

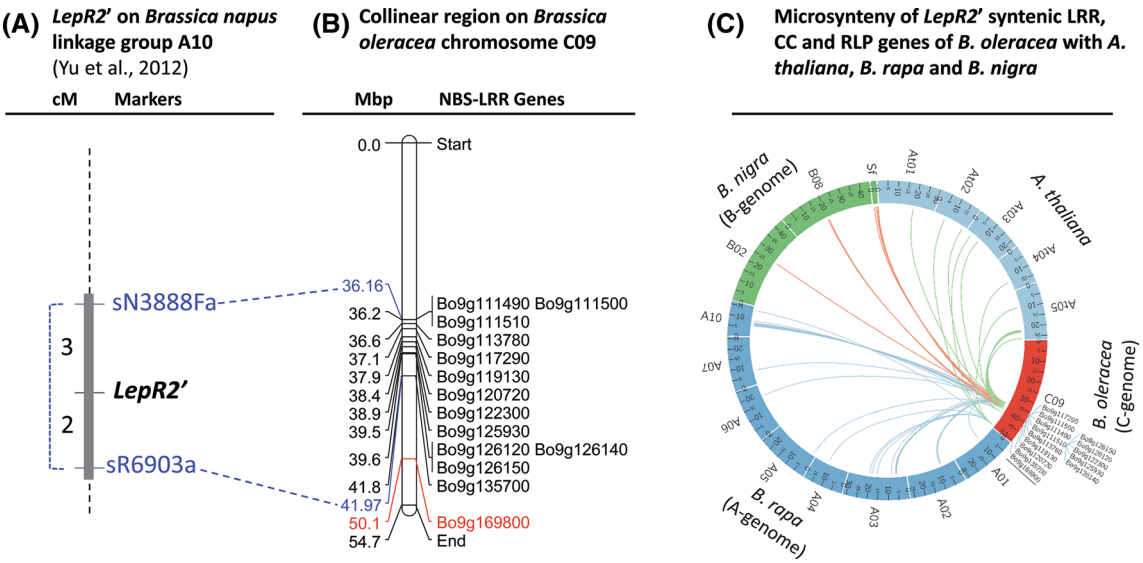

Fig. 2 Collinear region of *B. napus* blackleg resistance gene *LepR2'* (**a**) on *B. oleracea* chromosome C09 (**b**) and microsynteny relationship of the LRR, CC, FBD, MAPK, and RLK genes within the *LepR2'* collinear region of *B. oleracea* with *A. thaliana, B. rapa*, and *B. nigra* (**c**). Broken lines between the *B. napus* markers (**a**) and *B. oleracea* genes (**b**) indicate homologous genomic sequences. cM, centimorgan; Mbp, mega base pair; sf. *B. nigra* scaffold. The gene Bo9g169800 (indicated by red text in figure B and by an underline in figure C) is not within the *LepR2'* collinear region (located 8.15 Mbp apart). This gene is included for expression analysis, since it is orthologous to the cloned gene JX880110 (of *B. napus* blackleg resistance locus *LepR3*). The chromosomal location and microsynteny relations of this gene are shown, but not discussed in the results section

Table 1 List of LRR, CC, FBD, MAPK, and RLK domain-containing genes identified within the collinear region of *B. napus* blackleg resistance gene *LepR2'* in *B. oleracea*

SL.	Gene ID	Chromosomal location (strand)	CDS (bp)	Protein (AA)	Bolbase ID	Arabidopsis hit/e-value	Trembl ID	Description
1	Bo9g111490	C9:36205987-36206796 (+)	612	203	Bol012300	AT1G51370/3.2e−48	A5BU14	F-box/FBD/LRR-repeat protein
2	Bo9g111500	C9:36207783-36209300 (+)	1350	449	Bol038356	AT5G25850/1.3e−133	A0MEF3	Putative F-box/FBD/LRR-repeat protein
3	Bo9g111510	C9:36225550-36226914 (+)	1095	364	Bol045667	AT5G53840/1.9e−94	Q9M371	F-box/FBD/LRR-repeat protein
4	Bo9g113780	C9:36563098-36566127 (+)	3030	1009	Bol038795	AT5G53890/0	C0LGV8	Leucine-rich repeat receptor-like protein kinase
5	Bo9g120720	C9:38409782-38411047 (−)	1266	421	Bol018194	AT5G66330/0	Q9FH56	Leucine-rich repeat (LRR) family protein
6	Bo9g122300	C9:38921557-38924901 (+)	3264	1087	Bol006253	AT5G56040/0	Q2V2Y1	Leucine-rich receptor-like protein kinase family protein
7	Bo9g125930	C9:39471679-39472702 (−)	843	280	Bol007650	AT3G56780/1.1e−51	Q9XEG1	FBD, F-box & LRR domains containing protein
8	Bo9g126120	C9:39616439-39618336 (+)	1641	546	Bol009927	AT5G56560/4.4e−99	A0MFP8	FBD, F-box & LRR domains containing protein
9	Bo9g126140	C9:39646902-39648088 (+)	768	255	Bol009927	AT5G56560/3.5e−71	A0MFP8	FBD, F-box and LRR domains containing protein
10	Bo9g119130	C9:37855517-37856887 (−)	1371	456	Bol039693	AT5G55090/0	–	Mitogen-activated protein kinase kinase kinase 15
11	Bo9g126150	C9:39648887-39649535 (−)	339	112	Bol009929	AT5G56580/1.7e−70	B9RKG0	Mitogen-activated protein kinase kinase 6 (MAPK6)
12	Bo9g135700	C9:41809830-41811526 (−)	1353	450	Bol036595	AT2G42480/1.8e−79	Q9SLB4	MATH & coiled-coil (CC) domain-containing protein
13	Bo9g117290	C9:37066960-37069527 (−)	2568	855	Bol038854	AT5G54380/0	Q9LK35	Receptor-like protein kinase THESEUS 1

FBD, F-box domain; LRR, Leucine Rich Repeat; CC, coiled-coil domain; MAPK, Mitogen-activated protein kinase domain

Table 2 Functional domain-wise classification of the LRR, CC, FBD, MAPK, and RLK genes within the collinear region of *B. napus* blackleg resistance locus *LepR2'* in *B. oleracea*

SL	Domain	Gene ID
1	LRR	Bo9g120720
2	LRR-FBD	Bo9g111490, Bo9g111500, Bo9g111510, Bo9g125930, Bo9g126120, Bo9g126140
3	LRR-RLK	Bo9g117290, Bo9g113780, Bo9g122300
4	LRR-MAPK	Bo9g119130, Bo9g126150
5	CC	Bo9g135700

LRR, Leucine Rich Repeat; FBD, F-box domain; CC, coiled-coil domain; RLK, Receptor like kinase; MAPK, Mitogen-activated protein kinase domain

distribution along the length of these genes is shown in Fig. 3.

Conserved motif analysis of the selected 13 genes identified 15 statistically significant conserved motifs consisting of 18–50 amino acids (Fig. 4a; Table S3). Among these 15 motifs, 10 of the motifs were associated with LRR domains, while three (namely, motifs 2, 8, and 12) and two (namely, motifs 1 and 4) were associated with MAPK and FBD domains, respectively (Table S3).

Gene structure analysis identified the distribution of exons and introns in the selected 13 genes. Among these genes, a maximum of four exons were found in genes Bo9g111510, Bo9g126150, and Bo9g135700, while four genes (Bo9g113780, Bo9g120720, Bo9g119130, and Bo9g117290) contained only one exon (Fig. 4b).

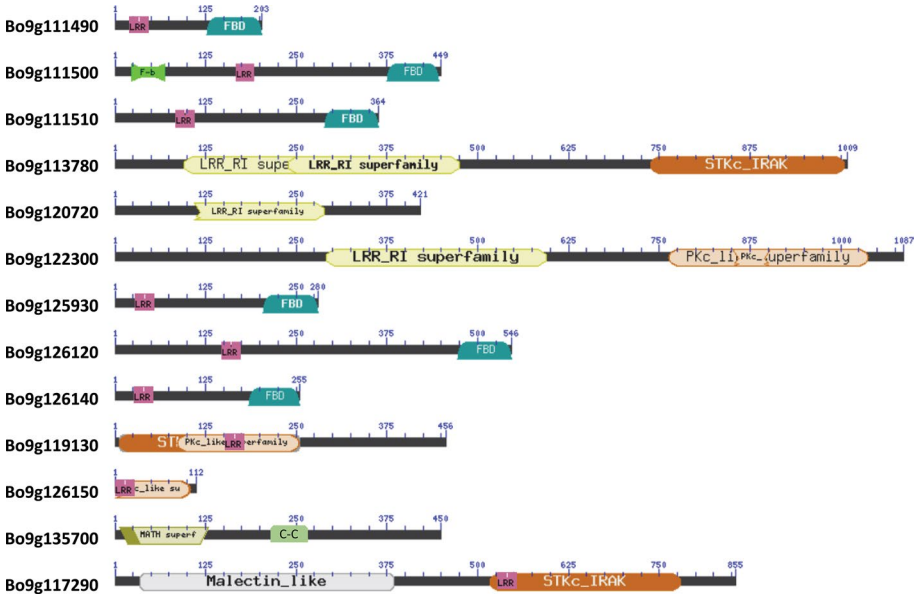

Fig. 3 Domain organization of the LRR, CC, FBD, MAPK, and RLK genes within the collinear region of *B. napus* blackleg resistance gene *LepR2'* in *B. oleracea*. FBD = F-box domain; LRR = Leucine Rich Repeat; CC, coiled-coil domain; STKc-IRAK, Serine/Threonine kinases, Interleukin-1 Receptor Associated Kinases; MAPK, Mitogen-activated protein kinase domain

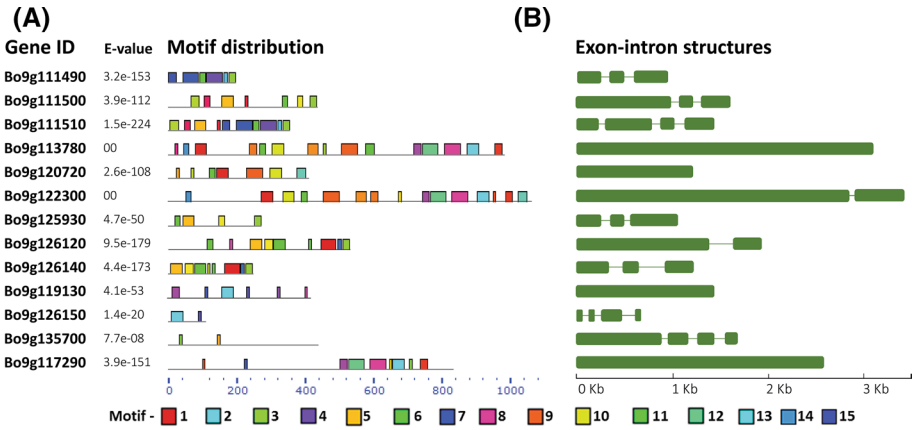

Fig. 4 Occurrence and distribution of protein motifs **(a)** and exon–intron structures **(b)** of the LRR, CC, FBD, MAPK, and RLK genes within the collinear region of *B. napus* blackleg resistance gene *LepR2'* in *B. oleracea*. The length and position of motifs in the protein sequences are indicated using coloured blocks. Exons and introns are represented by green boxes and green lines, respectively, and the genomic length is indicated at the bottom. Details of the motif sequences are shown in Table S3

The mono-exonic gene Bo9g113780 had the longest exon (3030 bp) followed by exon-1 (2800 bp) of the gene Bo9g122300, and the single exon (2568 bp) of gene Bo9g117290 (Fig. 4b).

Along with these 13 genes, another gene, Bo9g169800, encoding a receptor-like protein was also included for differential expression analysis against *L. maculans* isolates. This gene is orthologous to JX880110 (sequence similarity 92.5%, e-value = 0.0), the first ever cloned *B. napus* blackleg resistance gene *LepR3* on chromosome A10 (Larkan et al. 2013). This gene is included for expression analysis, since it is known to confer resistance in *B. napus* and is located 8.15 Mbp downstream of the *LepR2'* collinear region in *B. oleracea.*

3.4 Chromosomal location and microsynteny relationship of the identified LRR, CC, FBD, MAPK, and RLK domain-containing genes

The 13 selected LRR, CC, FBD, MAPK, and RLK domain-containing genes were evenly distributed throughout the *LepR2'* collinear region in *B. oleracea* (Fig. 2b). No distinct clustering of the genes was observed, except groups of three genes were found to be closely located: genes Bo9g111490, Bo9g111500 and Bo9g111510 located at 36.2 Mbp (at the flanking end of the collinear region) and genes Bo9g126120, Bo9g126140, and Bo9g126150 located at 39.6 Mbp (at approximately the center of the collinear region).

Microsynteny relationship analysis of the identified 13 genes of *B. oleracea* (a C-genome crop) with *B. rapa* (an A-genome crop), *B. nigra* (a B-genome crop), and *A. thaliana* identified seven orthologous gene pairs with *A. thaliana*, distributed across all five chromosomes of *A. thaliana* (Fig. 2c). For the A-genome (*B. rapa*), fifteen orthologous gene pairs were identified: a maximum of six pairs with chromosome A10, followed by three and two pairs with A03 and A02, respectively, and one pair with each of chromosomes A01, A05, A06, and A07. With *B. nigra* (a B-genome crop), a total of seven orthologous gene pairs were observed. These results suggest a high microsyntenic relationship for the 13 identified LRR, CC, FBD, MAPK, and RLK genes among A-, B-, and C-genome crops.

3.5 Expression profiling of the identified LRR, CC, TIR, FBD, MAPK, and RLK domain-containing genes

Expression of the identified LRR, CC, FBD, MAPK, and RLK domain-containing genes was determined in the resistant and susceptible cabbage genotypes at different time points after inoculation with *L. maculans* isolates 00–100 s and 03–02 s using gene-specific primers (Table S4). Hierarchical clustering of the expressions of these genes in a heatmap representation identified a cluster of seven genes (Bo9g126150, Bo9g117290, Bo9g111490, Bo9g126120, Bo9g111510, Bo9g111500, and Bo9g126140) that showed significant differential expression in the resistant line SCNU-98 comparted to that in the susceptible line SCNU-72 (Fig. 5). Among these seven genes, only two genes, Bo9g126150 and Bo9g111490, were induced in the resistant line against both isolates, whereas the remaining five genes were only induced against the isolate 00–100 s (Fig. 5, 6).

A common feature of these seven genes is that they were significantly induced in the resistant line at 6 h following inoculation with *L. maculans* isolate 00–100 s. Against this isolate, only the gene Bo9g126150 was consistently highly expressed in the resistant line at later time points as well (i.e., at 24 h and 48 h following inoculation) (Fig. 6). The gene Bo9g117290 also showed higher expression at 24 h, but it did not show significantly higher expression at 48 h following inoculation. The remaining five genes did not show distinctively increased expressions after 6 h of inoculation. Against the isolate 03–02 s, genes Bo9g126150 and Bo9g111490 were only induced at 24 h following inoculation.

Among the significantly expressed genes, the highest expression levels in the resistant line SCNU-98 were observed for the gene Bo9g117290 at 6 h (~ 11-fold) and

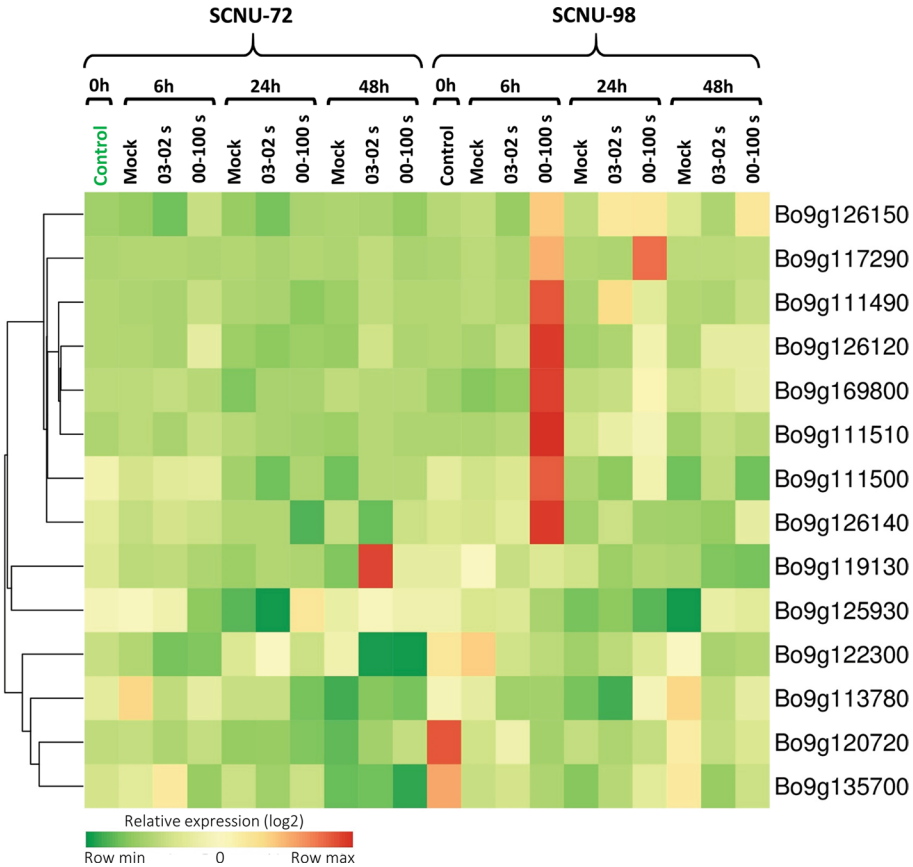

Fig. 5 Expression patterns of the LRR, CC, FBD, MAPK, and RLK domain-containing genes within the collinear region of *B. napus* blackleg resistance gene *LepR2'* in *B. oleracea* at different time points following inoculation with *L. maculans* isolates 03–02 s and 00–100 s in the cotyledons of resistant (SCNU-98) and susceptible (SCNU-72) cabbage lines, as determined by qRT-PCR. Gene expression values were log2-transformed and hierarchically clustered. Colour figure available online. The gene Bo9g169800 is located 8.15 Mbp downstream of the *LepR2'* collinear region. This is included for expression analysis since it is orthologous to JX880110, the cloned gene of *B. napus* blackleg resistance locus *LepR3* (Larkan et al. 2013)

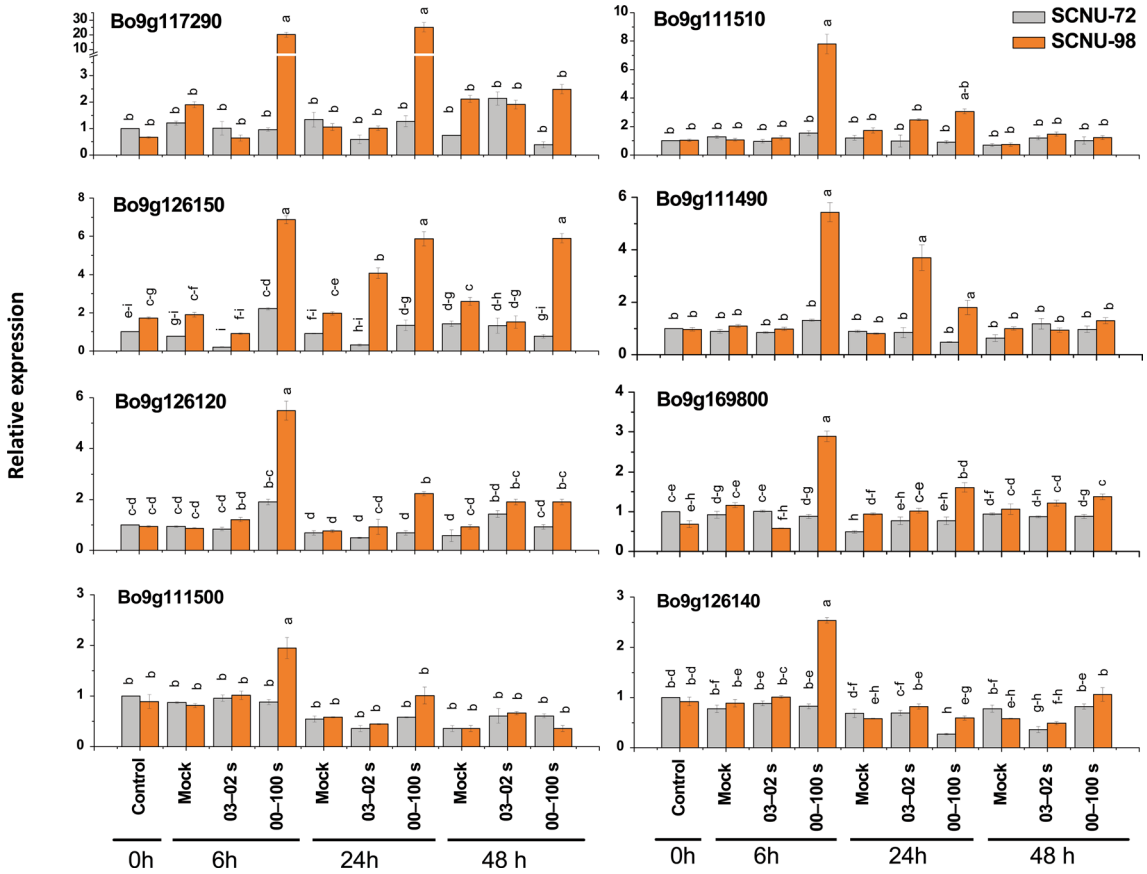

Fig. 6 Differential expression of the LRR, CC, FBD, MAPK, and RLK domain-containing genes within the collinear region of *B. napus* blackleg resistance gene *LepR2'* in *B. oleracea* at different time points following inoculation with *L. maculans* isolates 03–02 s and 00–100 s in the cotyledons of resistant (SCNU-98) and susceptible (SCNU-72) cabbage lines. Error bars represent standard deviation of the means of three independent replicates. Different letters above the bars indicate statistically significant differences based on Tukey's pairwise comparisons. The gene Bo9g169800 is located 8.15 Mbp downstream of the *LepR2'* collinear region. This is included for expression analysis since it is orthologous to JX880110, the cloned gene of *B. napus* blackleg resistance locus *LepR3* (Larkan et al. 2013)

24 h (~ 24-fold) followed by the gene Bo9g111510 (~ sevenfold) at 6 h after inoculation by the isolate 00–100 s (Fig. 6).

Among the genes induced by both isolates, gene Bo9g126150 showed ~ three–fourfold higher expression at 6, 24, and 48 h against isolate 00–100 s and ~ twofold higher expression against isolate 03–02 s at 24 h. On the other hand, the gene Bo9g111490 was highly expressed against isolate 00–100 s only at 6 h (~ fivefold) and against isolate 03–02 s at 24 h (~ fivefold) (Fig. 6).

4 Discussion

This study reports the identification of putative disease resistance-related LRR, CC, FBD, MAPK, and RLK-domain containing genes within the collinear region of *B. napus* blackleg resistance locus *LepR2'* in *B. oleracea*, while also determining the potential association of those genes with blackleg resistance in cabbage.

R-gene-mediated resistance to *L. maculans* in *Brassica* family crops is largely governed by compatible 'gene-for-gene' interactions, where a specific avirulence gene (*Avr*) of the pathogen is recognized by corresponding R-gene of the host (Ansan-Melayah et al. 1998; Flor 1971; Williams and Delwiche 1980). The cabbage inbred line SCNU-98 displayed a high resistance response against both *L. maculans* isolates, 03–02 s and 00–100 s, at the cotyledon stage. However, the specific R-locus that may confer resistance in this Korean cabbage inbred line could not be determined due to the lack of differential set of *L. maculans* isolates having different *Avr* gene profiles (owing to strict import restrictions on *L. maculans* strains). Mapping the R-genes in segregating populations could be an effective approach, but this is also time consuming, resource demanding, and expensive (Delourme et al. 2018; Miles and Wayne 2008).

A comprehensive meta-analysis of 314 cloned plant R-genes indicated nine different molecular mechanisms of resistance, which were mainly manifested by NBS, LRR,

TIR, CC, and RLP/RLKs domains (Kourelis and van der Hoorn 2018). Genome-wide, genes containing such disease resistance-related domains are known for major plant species including rice, potato, soybean, cucumber, melon, peach, grape, apple, and Arabidopsis [reviewed in 57–59]. Several studies have identified genome-wide NBS-LRR genes in major Brassicaceae crops (Alamery et al. 2018; Fu et al. 2019; Golicz et al. 2016; Yu et al. 2014), of which Alamery et al. 2018(Alamery et al. 2018) reported the maximum number of NBS-LRR genes in *B. rapa* (249), *B. oleracea* (443), and *B. napus* (641). The recent pan-genome of *B. oleracea* used an improved pipeline for genome assembly and resistance gene analog (RGA) candidate prediction, identifying a total of 213 RLP, 556 NBS-LRR, and 901 RLK genes (Bayer et al. 2019). A total of 97 NBS-encoding genes were reported to be orthologous between *B. oleracea* and *B. napus* (Fu et al. 2019). We reasoned that mining such disease resistance-related, domain-containing genes within the collinear regions of known *B. napus* R-loci in a C-genome and profiling their differential expressions in the resistant versus susceptible cabbage genotypes might lead to the identification of putative candidate genes for blackleg resistance, which upon validation by functional analysis, could be useful for improving blackleg resistance in elite cabbage cultivars. In this study, we focused on *B. napus* blackleg resistance locus *LepR2',* since both isolates (against which the cabbage inbred line SCNU-98 were resistant) contain corresponding avirulence gene (Robin et al. 2017). This suggests the existence of the corresponding R-gene in this line. The locus *LepR2'*, flanked by markers sR8548a and sN2551b on *B. napus* linkage group A10, was originally introgressed from *B. rapa* subsp. *sylvestris* via interspecific hybridization, and its complete expression in *B. napus* has been speculated to require some chromosomes and genes from the C-genome (Yu et al. 2012). This locus is allelic to the previously identified locus *LepR2* and is an incompletely dominant gene that provides strong resistance against a range of *L. maculans* isolates in *B. napus* (Yu et al. 2005, 2012).

A total of 13 genes were identified within the collinear region of *LepR2'* in *B. oleracea* which contain at least one of the LRR, CC, FBD, MAPK, and RLK domains. Among these genes, the receptor like protein kinase gene Bo9g117290 showed the highest expression level in the resistant line SCNU-98 against the isolate 00–100 s (~ 11- and ~ 24-fold induction at 6 h and 24 h following inoculation, respectively). RLKs are major component of membrane-localized pattern-recognition receptors (PRRs) that mediate the first line of plant defence by recognizing the pathogen-associated molecular patterns (PAMPs) and activating PAMP-triggered immunity (PTI) (Jones and Dangl 2006). Sixty out of 314 cloned plant's R-genes have been reported to be RLKs/RLPs (Kourelis and van der Hoorn 2018). RLKs were found to be involved in both broad-spectrum, elicitor-initiated defence responses such as *FLS2* (*FLAGELLIN SENSITIVE 2*) in Arabidopsis and pathogen-specific, dominant R-gene-mediated defence responses, such as *Xa21* in rice (Sekhwal et al. 2015; Goff and Ramonell 2007; Liu et al. 2017). The gene *FLS2* was the first RLK gene found to be involved in perception of the bacterial elicitor *Flagellin* in *Arabidopsis* (Gómez-Gómez and Boller 2000), and the gene *Xa21* conferred resistance to race 6 of *Xanthomonas oryzae* pv. *oryzae*, which causes bacterial blight in rice (Song et al. 1995). Comparison of functional domains revealed that our putative candidate gene Bo9g117290 also contain a cytoplasmic serine/threonine kinase domain along with an LRR domain.

Among known blackleg resistance loci in Brassica genomes, only two R-loci, namely *LepR3* and *Rlm2,* have been cloned so far. These two loci are confirmed to be allelic and to encode LRR-RLP genes (Larkan et al. 2013; Larkan et al. 2015). The corresponding orthologue of this LRR-RLP gene in C-genome is the gene Bo9g169800, which is located approximately 8.15 Mbp downstream of the *LepR2'* collinear region in *B. oleracea* (Fig. 2b). We have investigated its expression and found that it is only induced by ~ twofold in the resistant line SCNU-98 against the isolate 00–100 s at 6 h, which is much less than the 11–24-fold higher expression of the RLK domain-containing gene Bo9g117290 against the isolate at 6–24 h after inoculation (Fig. 6).

The two most well-known RLK genes, *FLS2* and *BAK1* (BRI1-associated receptor kinase 1) are known to initiate the MAP kinase cascade upon recognition of the bacterial PAMP flagellin, *flg22* (Deslandes and Rivas 2012; Chinchilla et al. 2007; Kim et al. 2013). Two MAP kinase genes, Bo9g119130 and Bo9g126150, were found within the *LepR2'* collinear region in *B. oleracea.* In particular, the gene Bo9g126150 was highly expressed against both isolates: ~ three–fourfold against isolate 00–100 s at all time points following inoculation and ~ twofold against isolate 03–02 s at 6 h, indicating a putative role in blackleg resistance.

Among the 13 selected genes within the collinear region of *LepR2'* in *B. oleracea*, six genes had an F-box domain (FBD) along with an LRR domain (Fig. 3). Of these, two genes were highly expressed in the resistant line SCNU-98, Bo9g111510 only against isolate 00–100 s at 6 h following inoculation (~ sevenfold; the second highest expression among the 13 genes) and Bo9g111490 against both isolates (~ fivefold, against isolate 00–100 s at 6 h and against isolate 03–02 s at 24 h following inoculation). Several F-box proteins are known to play roles in plant defence against phytopathogens. For example, *MAX2* contributed to resistance against *Pectobacterium carotovorum* and *Pseudomonas syringae* in Arabidopsis (Piisilä et al. 2015), *CPR1* was involved in controlling the stability of plant NBS-LRR proteins in Arabidopsis (Cheng et al. 2011), and *ACRE189/*

ACIF1 was involved in activating defence responses and regulating cell death during recognition of *Pseudomonas syringae* pv. *tabaci* and *Cladosporium fulvum* in tobacco and tomato (van den Burg et al. 2008).

5 Conclusion

In this study, genes containing putative disease resistance-related domains were identified in the collinear region of *B. napus* blackleg resistance locus *LepR2'* in *B. oleracea*. Very high expression of the LRR-RLK gene Bo9g117290 and the LRR-FBD gene Bo9g111510 against the *L. maculans* isolate 00–100 s, and high expression of the LRR-MAP kinase gene Bo9g126150 and the LRR-FBD gene Bo9g111510 against both isolates in the resistant cabbage line suggest putative roles of these genes in conferring resistance to blackleg in *B. oleracea.* A mapping-based approach will be necessary to identify if more than one locus is involved. In addition, functional analyses of these genes will be helpful in identifying the key gene(s) conferring resistance to blackleg in cabbage.

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Author contribution Conceptualization and supervision: ISN, JIP and HTK; in silico analysis: MJF, SN and MRH; assisting in sample preparation and RNA extraction: HJJ and AHKR; all experiments: MJF; data analysis, interpretation, manuscript editing and review: MJF and MRH; project administration and funding acquisition: ISN. All authors read the article and approved the manuscript.

Data availability The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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