The *Brassica napus* wall-associated kinase-like (WAKL) gene *Rlm9* provides race-specific blackleg resistance

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SUMMARY

In plants, race-specific defence against microbial pathogens is facilitated by resistance (*R*) genes which correspond to specific pathogen avirulence genes. This study reports the cloning of a blackleg *R* gene from *Brassica napus* (canola), *Rlm9*, which encodes a wall-associated kinase-like (WAKL) protein, a newly discovered class of race-specific plant RLK resistance genes. *Rlm9* provides race-specific resistance against isolates of *Leptosphaeria maculans* carrying the corresponding avirulence gene *AvrLm5-9*, representing only the second WAKL-type *R* gene described to date. The *Rlm9* protein is predicted to be cell membrane-bound and while not conclusive, our work did not indicate direct interaction with *AvrLm5-9*. *Rlm9* forms part of a distinct evolutionary family of RLK proteins in *B. napus*, and while little is yet known about WAKL function, the *Brassica–Leptosphaeria* pathosystem may prove to be a model system by which the mechanism of fungal avirulence protein recognition by WAKL-type *R* genes can be determined.

Keywords: *Leptosphaeria maculans*, blackleg, *Brassica napus*, *Rlm9*, *AvrLm5-9*, disease resistance, wall-associated kinase-like.

INTRODUCTION

Plants detect invading microbial pathogens through the perception of ‘danger signals’ (Gust *et al*., 2017). Perception of microbe-associated molecular patterns by extracellular pattern recognition receptors (PRRs), consisting of various receptor-like kinases (RLKs) and receptor-like proteins (RLPs), initiates host pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), the first layer of defence against pathogens. PRRs also respond to damage-associated molecular patterns (DAMPs), such as host peptides and oligosaccharide fragments, including pectin-derived oligogalacturonides, released during pathogen breaching of the plant cell wall (Yang *et al*., 2012; Zipfel, 2014; Boutrot and Zipfel, 2017). Pathogens have evolved to overcome PTI by secreting small proteins called effectors, which often target components of the plant’s defence pathways. In turn plants are armed with an array of highly diverse resistance (*R*) genes which encode both cytoplasmic and extracellular receptors to perceive pathogen effectors and trigger a rapid and robust immune response called effector-triggered immunity (ETI) (Jones and Dangl, 2006; Bent and Mackey, 2008; Stotz *et al*., 2014). However, PTI and ETI share common signaling pathways (Katagiri and Tsuda, 2010) and the distinction between the two responses is blurred (Thomma *et al*., 2011; Rodriguez-Moreno *et al*., 2018). In light of the ambiguities and commonalities observed, a more general model encompassing all plant immune responses and based on ‘immunogenic patterns’ instead separates responses into extracellular- and intercellular-triggered immunity, based on the point of recognition by the host cell (van der Burgh and Joosten, 2019).

Plant RLK proteins are encoded by a large and diverse gene family which underwent massive expansion after the divergence of the plant and animal lineages (Shiu and Bleecker, 2001). RLKs are defined by a common set of domains; a signal peptide, an extracellular receptor domain, a single transmembrane domain and a cytoplasmic kinase domain. The extracellular regions of the proteins vary, and are adapted to the recognition of diverse signals. Based on their conserved intracellular kinase domains, plant RLKs form a monophyletic group distinct from other eukaryotic kinases (Shiu and Bleecker, 2001). Membrane-bound RLPs, which feature extracellular leucine-rich repeat domains involved in protein recognition but lack the intracellular kinase domain of RLKs, constitute...
a class of R proteins that confer resistance upon perception of apoplastic pathogen effectors (Stotz et al., 2014; Jamieson et al., 2018). We previously reported the cloning of two RLP-type resistance genes, LepR3 and Rlm2, residing in the A genome of the allotetraploid *Brassica napus* (canola, rapeseed) (Larkan et al., 2013; Larkan et al., 2015), conferring resistance against races of the fungal pathogen *Leptosphaeria maculans* (Lm) with the matching effectors AvrLm1 and AvrLm2, respectively (Gout et al., 2006; Ghanbarnia et al., 2015). Both LepR3 and Rlm2 pair with the RLK SOBIR1 (Ma and Borhan, 2015; Larkan et al., 2015), as RLPs, lacking any intracellular kinase, require a partner to transmit a signal across the plasma membrane and to activate cytoplasmic signal transduction cascades (Liebrand et al., 2014).

The *Brassica* R genes Rlm3, Rlm4, Rlm7 and Rlm9 confer race-specific resistance against blackleg disease caused by *L. maculans*. They form a tight genetic cluster on chromosome A07 and may possibly be allelic variants of the same *R* locus (Larkan et al., 2016). The corresponding avirulence (Avr) genes AvrLm3, AvrLm4-7 and AvrLm5-9 have been cloned from *L. maculans* and all encode small cysteine-rich secreted proteins (Parlange et al., 2009; Plissonneau et al., 2016; Ghanbarnia et al., 2018). Recognition of both AvrLm3 and AvrLm5-9 by Rlm3 and Rlm9, respectively, is masked in the presence of AvrLm4-7. However, AvrLm4-7 neither interferes with the expression of, nor interacts directly with, AvrLm3 or AvrLm5-9, nor do AvrLm3 and AvrLm5-9 interact (Ghanbarnia et al., 2018). To investigate this complex system of pathogen recognition we have pursued the cloning of *Brassica* R genes from the Rlm3/4/7/9 gene cluster. Here we report cloning of Rlm9 from the *B. napus* cultivar Darmor and show that it encodes a wall-associated kinase-like (WAKL) protein, a newly emerging class of race-specific plant RLK *R* genes (Keller and Krattinger, 2018).

**RESULTS**

**Rlm9 encodes a wall-associated kinase-like protein**

Through molecular mapping, the physical interval of the Rlm9 locus had previously been defined as approximately 4.3 Mb of chromosome A07 (Larkan et al., 2016) of the *B. napus* reference genome Darmor-bzh (Chalhoub et al., 2014), an Rlm9 variety. Rlm9 is genetically clustered with the other blackleg *R* genes Rlm3, Rlm4 and Rlm7, all of which were shown to co-segregate with the microsatellite marker sR7018 positioned at approximately 16 Mb on chromosome A07 (Larkan et al., 2016). Using this information along with previously generated genomic information for the Rlm3 locus (Mayerhofer et al., 2005) we searched the consensus physical interval of the overlapping Rlm3-4-7-9 maps (230 genes; Larkan et al, 2016) on the *B. napus* Darmor-bzh (Rlm9) genome for genes with similarity to *R* genes and expression in response to pathogen infection. BnaA07g20220D, a WAKL protein encoding gene, was identified as the best candidate for Rlm9. No other defence-related candidates were identified within the interval (Table S1). Rlm9 is predicted to encode a 794-amino acid protein with the typical features of the WAKL family of *Arabidopsis thaliana* (Verica and He, 2002), showing the highest homology to *A. thaliana* WAKL10 (At1g79680, 69% amino acid identity). The gene contains three exons encoding a transmembrane receptor protein, which contains predicted extracellular domains for pectin- and calcium-binding (wall-associated receptor kinase galacturonan-binding [GUB_WAK] and epithelial growth factor [EGF]-like Ca\(^2\+\) domains, respectively), a C-terminal WAK domain and an intracellular serine/threonine protein kinase domain with a guanylyl cyclase (GC) motif (Figure 1(ai)).

BnaA07g20220D, including native promoter and terminator regions, was isolated from Darmor and transferred to the susceptible *B. napus* cultivar Westar N-0-1. After transgenic events were analysed in the T\(_0\) generation by droplet digital polymerase chain reaction (ddPCR), four independent transgenic lines, carrying between 1 and 9 copies of the transgene, were selected for phenotypic analysis. Selfed seeds of each line (T\(_1\)) were inoculated with the *L. maculans* isolate 2367 (avr9; virulent towards Rlm9) and the transgenic *L. maculans* isolate 2367:AvrLm5-9 (Avr9; avirulent towards Rlm9; Ghanbarnia et al., 2018) using standard cotyledon infection protocols (Larkan et al., 2013). The race-specific resistance response was activated in all Westar:BnaA07g20220D transgenic lines, confirming that the WAKL gene is indeed Rlm9 (Figure S1). Further ddPCR analysis of the T\(_1\) plants derived from the transgenic line NLA68 (one heterozygous transgene insertion at T\(_0\); Table S2) allowed for selection of a T\(_1\) plant carrying a single homozygous insertion which was self-fertilised to produce homozygous T\(_2\) seed (hereafter referred to as Westar: Rlm9; Figure 2). Further testing of Westar:Rlm9 with additional transgenic isolates carrying avirulence genes matching other A07 blackleg *R* genes (2367:AvrLm1, 2367:AvrLm3, 2367:AvrLm4-7, 2367:AvrLm7) produced only compatible interactions (i.e., host was susceptible to infection; Table S3) as previously demonstrated (Ghanbarnia et al., 2018). Stable expression of the Rlm9 resistance phenotype (hypersensitive response [HR] indicated by blackening around the point of inoculation) was observed in all cases (four inoculations per seedling, four seedlings per test; Figure 2). This reconfirmed both the identity of BnaA07g20220D as Rlm9 and the specificity of the Rlm9–AvrLm5-9 interaction.

An identical Rlm9 allele was identified from the genome sequence of *B. napus* var. Tapidor (Bayer et al., 2017), which also harbours Rlm9 (Larkan et al., 2016) (Table S4). A susceptible allele (rlm9; Table S4) was obtained from the genome sequence (v2.0) of *B. napus* var. ZS11 (He et al., 2020).
The Brassica rapa var. Chiifu homologue (Bra003598–unknown Rlm9 phenotype) (Wang et al., 2011) was also included for comparison studies. Comparison of the Darmor (resistant) Rlm9 and ZS11 (susceptible) rlm9 proteins (95.72% identity overall, Figure S2) revealed that most of the variation appeared concentrated in the extracellular region.

Figure 1. (a) Domain organisation of the Rlm9 protein. The protein consists of three exons (introns denoted by ‘V’) and contains a predicted signal peptide (hashed box), extracellular GUB_WAK pectin-binding (light red), C-terminal WAK (blue) and EGF-like Ca^{2+} (green) domains, a transmembrane motif (red) and an intracellular serine/threonine protein kinase domain (light orange) with a guanylyl cyclase centre (dark orange). (b,c) Expression profile of Rlm9 and AvrLm5-9 alleles during infection by L. maculans. (b) Mean reads per kilobase of transcript per million mapped reads (RPKM) for mock (m) and L. maculans-infected (i) cotyledon lesions from B. napus lines Topas DH18518 (T–rlm9) and Darmor (D–Rlm9), showing significant upregulation of Rlm9 between both Dm and Di and between Di and Ti, at all timepoints after zero. (c) Mean RPKM values for fungal AvrLm5-9 during the same experiment, showing no significant difference in expression between Di and Ti.

Figure 2. Transgenic complementation of the Rlm9 phenotype in B. napus. Cotyledons of Westar (no R gene) and a Westar:Rlm9 transgenic line infected with L. maculans, 14 days post-infection: isolate 2367 (phenotype a9–virulent towards Rlm9) and the transgenic isolate 2367:AvrLm5-9 (phenotype A9–avirulent toward Rlm9).
predicted pectin-binding (GUB_WAK) domains (15 substitutions within the 119-amino acid domain; 87.39% identity), while the C-terminal WAK and EGF-like domains were well conserved (94.69% and 100% identity, respectively).

RNA-Seq time course analysis revealed a significant upregulation of Rlm9 during L. maculans infection (isolate 00-100; A2-3-5-6-(8)-9-10-L1-L2-L4) with an approximately 5-fold increase early in the infection (3 days post-inoculation, FDR < 0.03) and a nearly 8-fold increase in transcript abundance detected at 6 days post-infection (FDR < 0.001) in the Rlm9 variety Darmor when compared to a susceptible (rim9) variety Topas DH16516 or the mock (water)-inoculated control. No significant difference was detected in the expression of the fungal AvrLm5-9 between the inoculated susceptible and resistant lines over the same time course (Figure 1(b)). Expression of the B. napus SOBIR1 and BAK1 genes, previously shown to interact with the B. napus RLP-type R genes Rlm2 and LepR3 (Larkan et al., 2013; Larkan et al., 2015), was upregulated (up to 15-fold) during infection of the Rlm2 plants. However, in the infected Rlm9 plants, both the SOBIR1 and BAK1 homologues (six copies each in B. napus) showed little upregulation (1–2 fold), suggesting they may not be involved in the same manner during the WAKL R gene response (Figure S3).

Confirmation of Rlm9 in B. napus varieties

A selection of 22 B. napus cultivars, including many either previously identified as Rlm9 lines or suspected to harbour Rlm9 based on previous differential pathology, and the introgression line Topas-Rlm9 were tested for the presence of the Rlm9 allele. The presence of Rlm9 was first confirmed via infection with the transgenic L. maculans isolate 2367:AvrLm5-9, which induced an HR in all 13 Rlm9 lines (Table S4, Figure S4). All lines were susceptible to the non-transgenic 2367 isolate. The allele was successfully amplified from each of the 13 Rlm9 lines (Table S4), while only weak non-specific amplicons were produced from non-Rlm9 lines, including cultivars containing other A07 blackleg R genes (Rlm1, Rlm3, Rlm4 and Rlm7).

No direct physical interaction detected between Rlm9 and either AvrLm5-9 or AvrLm4-7

Recently, we reported the cloning of the L. maculans effector AvrLm5-9 (Ghanbarnia et al., 2018). As previously reported, recognition of AvrLm5-9 and AvrLm3 by their cognate R proteins, Rlm9 and Rlm3, is masked in the presence of AvrLm4-7 and this masking effect is neither due to direct interaction between these effector proteins nor due to the suppression of their transcription (Plissonneau et al., 2016; Ghanbarnia et al., 2018). To examine whether AvrLm5-9 directly interacts with Rlm9, we cloned the extracellular region of Rlm9 in the prey vector pGADT7 and AvrLm5-9 lacking the signal peptide sequences (ΔspAvrLm5-9) in the bait vector pGBK7T for yeast two-hybrid assay. The assay was performed by co-transforming the bait and prey constructs into the yeast strain Y2HGold. The combination of the L. maculans effector ΔspAvrLm1 (bait) and its B. napus host-interacting protein BnMPK9 (Ma et al., 2018) (prey) was used as a positive control. No interaction could be detected between ΔspAvrLm5-9 and the extracellular region of Rlm9 (Figure S5). To assess whether AvrLm4-7, which masks the recognition of AvrLm5-9 by Rlm9, directly interacts with either the extracellular region or the kinase domain of Rlm9 to suppress Rlm9-mediated resistance, we co-transferred the bait vector pGBK7T:ΔspAvrLm4-7 and either prey vector pGADT7: Rlm9-EX or pGADT7:Rlm9-KD to yeast. As shown in Figure S5 there was no interaction between ΔspAvrLm4-7 and Rlm9-EX or Rlm9-KD, indicating that the masking of Rlm9-mediated resistance by AvrLm4-7 is not due to the direct interaction of AvrLm4-7 and Rlm9.

Evolution of the WAKL gene family in B. napus

To compare the evolution of the WAKL gene family between A. thaliana and B. napus, predicted WAKL-encoding genes, those encoding proteins with homology to the external domain of Rlm9, were extracted from the Darmor-bzh reference B. napus genome. After annotation, 19 additional genes encoding potential functional RLKs (predicted proteins containing Signal Peptide (SP), Trans-Membrane (TM) and Protein Kinase (PK) domains) were identified. All predicted proteins contained GUB_WAK domains, 18 also contained C-terminal WAK domains, while 16 of the 19 contained EGF-like domains. WAKLs were distinguished from WAKs based on homology to A. thaliana WAKL-encoding WAKL genes clustered on two chromosomes in each of the A (A08 and A09) and C (C06 and C08) genomes (Figure 3). Genomic alignment between syntenic A. thaliana blocks containing 18 of the previously characterised 22 AtWAKLs (Verica and He, 2002) which also encode intact RLKs, and the two genomes of B. napus revealed that almost all of the predicted WAKLs in B. napus are syntenic to the WAKL genes clustered on A. thaliana chromosome 1 (Figure 3). Comparison of WAKL GUB_WAK domains to AtWAK1 showed that none of the four amino acid residues previously identified as contributing to homogalacturonan binding within the AtWAK1 GUB_WAK domain (Decreux et al., 2006) are conserved in either the resistant or susceptible alleles of Rlm9, nor in AtWAKL10, and show generally poor conservation in both WAKL and WAK predicted proteins from B. napus (Figure S6(a)). Phylogenetic analysis for the predicted GUB_WAK domains also suggests that the WAKL proteins are a distinct evolutionary group from WAKs (Figure S6(b)).
DISCUSSION

The number of characterised race-specific resistance (R) genes has significantly expanded since the cloning of the first R gene in 1992, with the majority of described R genes encoding intracellular nucleotide-binding domain leucine-rich repeat/nucleotide binding and oligomerisation domain-like receptors (Zhang et al., 2017; Kourelis and Van Der Hoorn, 2018). However, a number of cell surface receptor proteins, collectively referred to as plant PRRs, which are involved in the recognition of extracellular plant pathogens, both through PAMPs and specific effectors, have also been identified (Boutrot and Zipfel, 2017). Rlm9 and the recently cloned wheat (Triticum aestivum) Stb6 are the only examples of race-specific WAKL-type R genes to be reported to date. Stb6 confers resistance to races of the apoplastic fungal pathogen Zymoseptoria tritici which produce the matching effector AvrStb6, though resistance is semi-dominant and conferred without a typical HR (Zhong et al., 2017; Saintenac et al., 2018). In contrast, Rlm9 induces a clear, dominant HR at the site of infection, responding to the presence of the L. maculans avirulence protein AvrLm5-9 (Ghanbarnia et al., 2018). The emergence of WAKL proteins as new players in race-specific resistance brings with it many fundamental questions. Using yeast two-hybrid we did not detect a direct interaction between AvrLm5-9 and Rlm9, which was also reported to be the case between Stb6 and AvrStb6 (Saintenac et al., 2018).

Figure 3. Syntenic alignment of A. thaliana and B. napus WAKL genomic regions. Genomic alignment between A. thaliana genomic blocks containing WAKL genes ('AT...') labels) and their syntenic matches in the B. napus A and C genomes. B. napus genes predicted to encode intact WAKL proteins are labelled 'Bna...'. Syntenic links between A. thaliana and B. napus WAKLs are indicated by blue (A genome) and red (C genome) ribbons. Yellow ribbons indicate syntenic matches where no corresponding B. napus WAKL was found.
Although yeast two-hybrid is not an optimal test to detect direct interaction of membrane proteins, it is possible that Rlm9 recognition of AvrLm5-9 is indirect and mediated by a host target molecule. One such molecule could be a DAMP, for example, a pectin monomer. However, the mechanism by which these predicted pectin-binding proteins function as mediators of race specificity has yet to be determined. The Stb6 protein contains a predicted extracellular GUB_WAK domain but does not contain either the C-terminal WAK or EGF-like Ca\textsuperscript{2+} domains found in Rlm9 and most other WAKL proteins (Saintenac et al., 2018), which suggests that these domains could be dispensable for the WAKL-mediated effector-triggered immune response. The concentration of variation in the GUB_WAK domain between the resistant Rlm9 and susceptible rlm9 proteins in comparison to the other well-conserved extracellular domains (C-terminal WAK and EGF-like domains) also suggests that the GUB_WAK domain may play a pivotal role in recognition of AvrLm5-9. While the GUB_WAK domain of the A. thaliana WAK1 protein has been demonstrated to bind cell-wall pectins (Decreux and Messi-aen, 2005) and WAKL proteins have been suggested to be associated with the cell wall (Verica and He, 2002; Hou et al., 2005), the same pectin-binding activity has yet to be shown for the predicted GUB_WAK domains of the WAKL proteins, and much research needs to be undertaken before we can determine how these RLKs function. However, at present it should not be assumed that these proteins retain the ability to bind pectin. It may be that the protein originally functioned as a general DAMP receptor, as for AtWAK1 (Brutus et al., 2010), and that these proteins later evolved into a more specialised role in the detection of proteinaceous ligands, like this case AvrLm5-9. As the conservation between WAKs and WAKLs appears in the EGF and kinase domains, rather than the putative pectin-binding regions, it may be more appropriate to consider WAKs and WAKLs as subsets of the EGF protein superfamily, rather than grouping both WAKs and WAKLs together as ‘wall-associated’ proteins (Kohorn, 2016).

In A. thaliana, Rlm9 shares the highest protein homology with WAKL10 (At1g79680.1). AtWAKL10 is co-expressed with several pathogen response genes during biotic interactions. The protein kinase domain has been shown to be a twin-domain, also having GC activity (Meier et al., 2010). Rlm9 contains an identical GC motif (SFGVVLAEILITGEK) within the PK domain. GCs convert guanosine 5'-triphosphate (GTP) into guanosine 3',5'-cyclic monophosphate (cGMP), an important signaling molecule during biotic interactions (Durner et al., 1998; Gehring and Turek, 2017). Plant cGMP-binding proteins include several actors in defence response pathways, including hydrogen peroxide production (Donaldson et al., 2016). The potential GC activity of Rlm9 could be a key component of the HR to L. maculans infection. Interestingly, the wheat Stb6 protein, which does not trigger an HR (Saintenac et al., 2018), appears to lack a GC centre in its kinase domain.

A search for WAKL homologues within the B. napus genome showed far fewer intact genes than was expected. B. napus is an amphidiploid hybrid of B. rapa (A genome) and Brassica oleracea (C genome), with each diploid genome having evolved from a hexaploid ancestor, with some gene loss occurring over time (Parkin et al., 2005; Ziólkowski et al., 2006). Therefore, for each single A. thaliana gene there are generally expected to be six homologues within B. napus (Grant et al., 1998). Despite there being 22 WAKLs characterised in A. thaliana (Verica and He, 2002), we were only able to identify 19 intact WAKL genes predicted within the B. napus Darmor sequence which are predicted to retain the SP, TM and PK domains required to function as an RLK (Table S5). This suggests a disproportionate evolution of the genes since the Arabidopsis–Brassica split 20–24 million years ago (Ziólkowski et al., 2006). This may be due to functional redundancy, making many of the WAKL homologues within the amphidiploid genome of B. napus dispensable. The relative abundance of WAKL genes in A. thaliana may also be due to a higher rate of gene expansion, as evidenced by the dense clusters of WAKL homologues and abundant tandem duplications found on A. thaliana chromosome 1 (12 genes total; Verica and He, 2002) which are only partially represented within each of the B. napus A and C genomes as homologues, while several AtWAKLs found on other chromosomes do not appear to be represented by intact genes in B. napus, despite syntenic links between the genomes (Figure 3).

As we recently reported, the recognition of AvrLm5-9 by Rlm9 is masked in the presence of AvrLm4-7 (Ghanbarnia et al., 2018). Similarly, AvrLm3 recognition by Rlm9 is also masked in the presence of AvrLm4-7 (Plissonneau et al., 2016). With the cloning of Rlm9 and the characterisation of the gene as a WAKL we now have the basis for possibly identifying the other three blackleg R genes within the Rlm3/4/7/9 cluster, co-located on chromosome A07 (Larkan et al., 2016). Though Rlm9 is the only A07 blackleg R gene carried by any of the published B. napus genomes (we identified the same Rlm9 allele in both Darmor (Chalhoub et al., 2014) and Tapi-dor (Bayer et al., 2017), and showed a lack of specific R genes in ZS11 (He et al., 2015) during this study), previous investigations into the blackleg resistance carried by DH12075, a doubled-haploid F\textsubscript{1}-derived line from Westar (no R gene) and Cresor (Rlm3; Larkan et al., 2016) parents, identified three WAKL genes in the same location. We are currently investigating the allelic variation of the Rlm9 locus in multiple B. napus accessions carrying Rlm3, Rlm4 and Rlm7 through parental-specific genome resequencing. If these genes prove to be variants or duplications of the same WAKL locus we can potentially gain further insight as to the evolution of WAKL-type R genes, and the Brassica–Leptosphaeria pathosystem may prove to be a model system by which the mechanism of fungal avirulence protein recognition by WAKLs can be determined.
EXPERIMENTAL PROCEDURES

Candidate identification and transformation

The BnaA07g20220D gene, including 1000 bp upstream and 500 bp downstream of the predicted coding sequence (4141 bp total), was PCR-amplified (Q5 High-Fidelity 2× Master Mix, New England Biolabs, Ipswich, MA, USA) from Darmor DNA, verified by Sanger sequencing and transferred to the Gateway-compatible transformation vector pMDDC123 (Curtis and Grossniklaus, 2003). The same primers [GW-DarWAKL F + R; Table S6] were used to survey a selection of B. napus cultivars to confirm the presence of the target allele in multiple Rlm9 sources. The genomic candidate construct was transformed via Agrobacterium into the susceptible B. napus cultivar Westar N-o-1 as previously described (Larkan et al., 2013). Homozygous, single-insertion transgenic plants were selected in the T1 generation using ddPCR (Larkan et al., 2015). Final confirmation of phenotype was performed using the transgenic L. maculans isolate 2367:AvlRm5-9 (Ghanbarnia et al., 2018) using standard cotyledon infection protocols (Larkan et al., 2013). Briefly, 7-day-old cotyledons were wounded once per lobe (four wounds per seedling, four seedlings per test) and inoculated with L. maculans pycnidiospore suspension. The resistance response was rated 14 days post-inoculation on a 0 (no infection) to 9 (complete necrotic collapse) scale.

Transcript analysis

Transcription time course profiles were generated by RNA-Seq (Illuminia, San Diego, CA, USA) for the B. napus cultivars Topas DH18516 (no R gene) and Darmor (Rlm9) during infection with the L. maculans isolate 00-100 (avirulence profile A2-3-5-6-(8)-9-(10)-L1-L2-L4 (Larkan et al., 2016)) with sampling at 0, 3, 6 and 9 days after inoculation. Additional data for the lines Topas-Rlm2 and Topas-Rlm3, growth conditions, tissue sampling, RNA processing and read mapping protocols were as previously described (Becker et al., 2019; Haddadi et al., 2019). Confirmation of the predicted coding region and protein sequence was obtained by aligning merged RNA sequencing reads to the Darmor genome sequence using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml) and CLC Genomics Workbench v11 (https://www.clcgenomics.com/products/clc-genomics-workbench/).

Yeast two-hybrid assay

For the yeast two-hybrid assay, AvrLM5-9 lacking the signal peptide sequence was cloned into the pGBK7 bait vector with primer set ΔAspAvrLM5-9-NcoI/ΔAspAvrLM5-9-EcoRI, and AvrLM4-7 lacking the signal peptide sequence was cloned into pGBK7 with primer set ΔAspAvrLM4-7Ndel/ΔAspAvrLM4-7-PstI. The intracellular kinase domain of Rlm9 was cloned into the pGADT7 prey vector with primer set Rlm9-9KD-NdeI/Rlm9-9KD-EcoRI and the extracellular region of Rlm9 was cloned into pGADT7 with primer set Rlm9-EX-NdeI/Rlm9-EX-EcoRI (Clontech, Mountain View, CA, USA). All primers are listed in Table S6. We used the matchmaker GL44 two-hybrid system and yeast strain Y2HGold (Clontech). The yeast strain Y2HGGold was co-transformed with bait and prey plasmid combinations using lithium-acetate and polyethylene glycol 3350 following the manufacturer’s manual. Transformants harboring both bait and prey plasmids were selected on plates containing minimal medium lacking Leu and Trp (SD-WL). Empty prey vector pGBK7 or pGADT7 used as bait or prey served as controls. pGBK7::ΔAspAvrLM1 and pGADT7::BnMPK9 were used as positive control (Ma et al., 2018). One colony per combination was picked from SD-WL plates to inoculate 1 ml SD-WL culture. After 36 h of growth, cells were collected by centrifugation and resuspended in 25 μl 0.9% NaCl from OD600 = 1 to OD600 = 0.00001 and spotted on SD-WL and SD-AHVL plates supplemented with 40 μg ml⁻¹ X-gal (Clontech) and 200 ng ml⁻¹ Aureobasidin A (Clontech). After 3 days of incubation, the plates were checked for growth and photographed.

Genomic and phylogenetic analyses

Predicted B. napus WAKL protein sequences, matching the extracellular domain of Rlm9, were retrieved from the Darmor-bzh reference annotation (Chalhoub et al., 2014) using the blastp function (default values) in CLC Genomics Workbench v12. Protein sequences with >20% identity to Rlm9 were annotated using InterPro (http://www.ebi.ac.uk/interpro/). Only those proteins containing predicted domains required for RLK function (ISP, TM and PK) were included as potential functional WAKLs (Table S5). An additional tblastn search comparing all predicted Darmor genes to the annotated A. thaliana proteins was also performed and all genes with best matches to ‘wall-associated’ proteins were retrieved and analysed as described above. The homology between A. thaliana TAIr10 (Wensel et al., 2011) and Brassica napus Darmor-bzh (Chalhoub et al., 2014) was visualised using Circos (Krzywinski et al., 2009). Orthologous gene pairs identified from synteny analysis were used to determine regions of homology and intersections with the defined B. napus WAKL genes using BEDTools intersect (Quinlan and Hall, 2010). Multiple sequence alignments and dendrograms were produced using CLC Workbench v7.5.1 software.

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AUTHOR CONTRIBUTIONS

NJL and MHB conceived and planned the study. NJL, LM and MD conducted the experiments. PH and MB performed the bioinformatic analysis. IAPP provided the genome sequence and contributed to the data analysis. NJL and MHB prepared the manuscript draft and all authors helped with the revision and editing.

CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phenotypic reaction of B. napus lines and T1 transgenics to AvrLM5-9 transgenic L. maculans isolate.

Figure S2. Protein sequence alignment for resistant and susceptible B. napus cultivar Westar N-o-1 as previously described (Larkan et al., 2013).
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