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Brief Communication

Brassica napus genes *Rlm4* and *Rlm7*, conferring resistance to *Leptosphaeria maculans*, are alleles of the *Rlm9* wall-associated kinase-like resistance locus

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Plant cell surface receptors are at the forefront of defence against pathogens, involved in pathogen sensing through the detection of conserved molecules named pathogen-associated molecular patterns (PAMP) and highly variable pathogen virulence (effector) proteins. The recently reported *Brassica napus* (canola, oilseed rape) disease resistance gene *Rlm9* encodes a wall-associated kinase-like (WAKL) receptor which confers race-specific resistance against races of the blackleg pathogen *Leptosphaeria maculans* carrying the corresponding effector gene, *AvrLm5-9* (Larkan *et al*., 2020). *Rlm4* and *Rlm7* are located on *B. napus* chromosome A07 and genetically tightly linked to *Rlm9* (Larkan *et al*., 2016). The *L. maculans* effectors AvrLm4-7 and AvrLm7 are small, secreted cysteine-rich proteins encoded by a single locus, *AvrLm4-7*. A single amino acid change in AvrLm4-7 masks recognition by Rlm4 without affecting Rlm7 function. Here, we report the cloning of *Rlm4* and *Rlm7*, both alleles of the *Rlm9* WAKL locus.

Genomic sequencing data was generated for the *B. napus* introgression lines; Topas-*Rlm4* and Topas-*Rlm7* using Illumina HiSeq 2500. Close to 537 million reads were assembled using SOAPdenovo assembler. Contigs generated from each line were mapped to the *B. napus* reference genome 'Darmor-*bzh*' using Bowtie2. Based on ~1.3 billion RNA sequence reads (using Illumina HiSeq 2500 2 × 125 bp, 3 biological replicates) mapped to the *Rlm3-4-7-9* gene cluster (Figure 1a), *Rlm4* and *Rlm7* genes were determined to be allelic, with each being 8507 bp in length consisting of three exons (Figure 1b). To prove the function of the predicted genes, the entire gene including introns and 5′ intergenic region (1750 bp) for each allele was synthesized (GenScript, USA) and cloned into the plant transformation vector pMDC123, modified to contain the *nosT* terminator sequence downstream of the cloning site using Gateway cloning technology. *Rlm4* and *Rlm7* genomic constructs were transferred into the blackleg-susceptible *B. napus* line, Westar N-o-1. Regenerated transgenic (T_0) plants that survived herbicide selection were screened via droplet digital PCR (ddPCR) to identify lines carrying insertions, then selfed to produce the T_1 generation. The resulting transgenic lines (13 for *Rlm4*, 17 for *Rlm7*) were initially tested for resistance response using the *L. maculans* isolate v23.1.3 (*avrLm3*, *AvrLm4-7*, *avrLm9*) using a standard cotyledon assay (Larkan *et al*., 2013) with all lines displaying hypersensitive response at the point of infection except for one *Rlm4* line with poor germination. Additional ddPCR was conducted to identify homozygous, single insertion events in T_1 plants, and one plant for each construct was selected and selfed to produce homozygous T_2 lines for further characterization (hereafter referred to as Westar:*Rlm4* and Westar:*Rlm7*). Further confirmation was obtained by utilizing transgenic *L. maculans* to demonstrate effector-specific activation of resistance conferred by the *Rlm4* and *Rlm7* candidate genes. The *L. maculans* isolate 2367 (*avrLm3*, *avrLm4-7*, *avrLm9*) and the transgenic isolates 2367:*AvrLm4-7*, 2367:*AvrLm7* (Larkan *et al*., 2016), and 2367:*AvrLm5-9* (Ghanbarnia *et al*., 2018) were used to inoculate Westar N-o-1, Westar:*Rlm4*, Westar:*Rlm7* and Westar:*Rlm9* (Larkan *et al*., 2020). Four seedlings of each line were inoculated with each isolate (performed in triplicate). No resistance reaction was induced in either Westar:*Rlm4* or Westar:*Rlm7* in response to *AvrLm5-9*. A hypersensitive response was induced in Westar:*Rlm4* only in response to *AvrLm4-7*, while Westar:*Rlm7* responded to both *AvrLm4-7* and *AvrLm7*, as expected, confirming the cloned genomic constructs as *Rlm4* and *Rlm7*, respectively (Figure 1c).

Rlm4 and *Rlm7* open reading frames are 2379 bp encoding proteins of 792 amino acids (aa). Sequence polymorphism between the two genes is limited to a total of 13 single nucleotide substitutions resulting in 4 synonymous and 9 non-synonymous changes. InterPro predicts Rlm4 and Rlm7 as transmembrane proteins consisting of secretory signal peptide (SP), extracellular wall-associated receptor kinase galacturonan-binding (WAK_GUB), WAK, epithelial growth factor (EGF) like calcium binding domains and a cytoplasmic kinase domain (Figure 1d). There are a total of 7 amino acid differences between Rlm4 and Rlm7 (Figure 1d). A further 128 lines that had been phenotyped for the presence of *Rlm4* and *Rlm7*, using isolates with *AvrLm4-7* or *AvrLm7*, were investigated using whole genome sequencing leading to the identification of two additional resistant alleles (*Rlm4-2* and *Rlm7-2*). The coding sequences of the *Rlm4-1* and *Rlm4-2* alleles were identical, with any

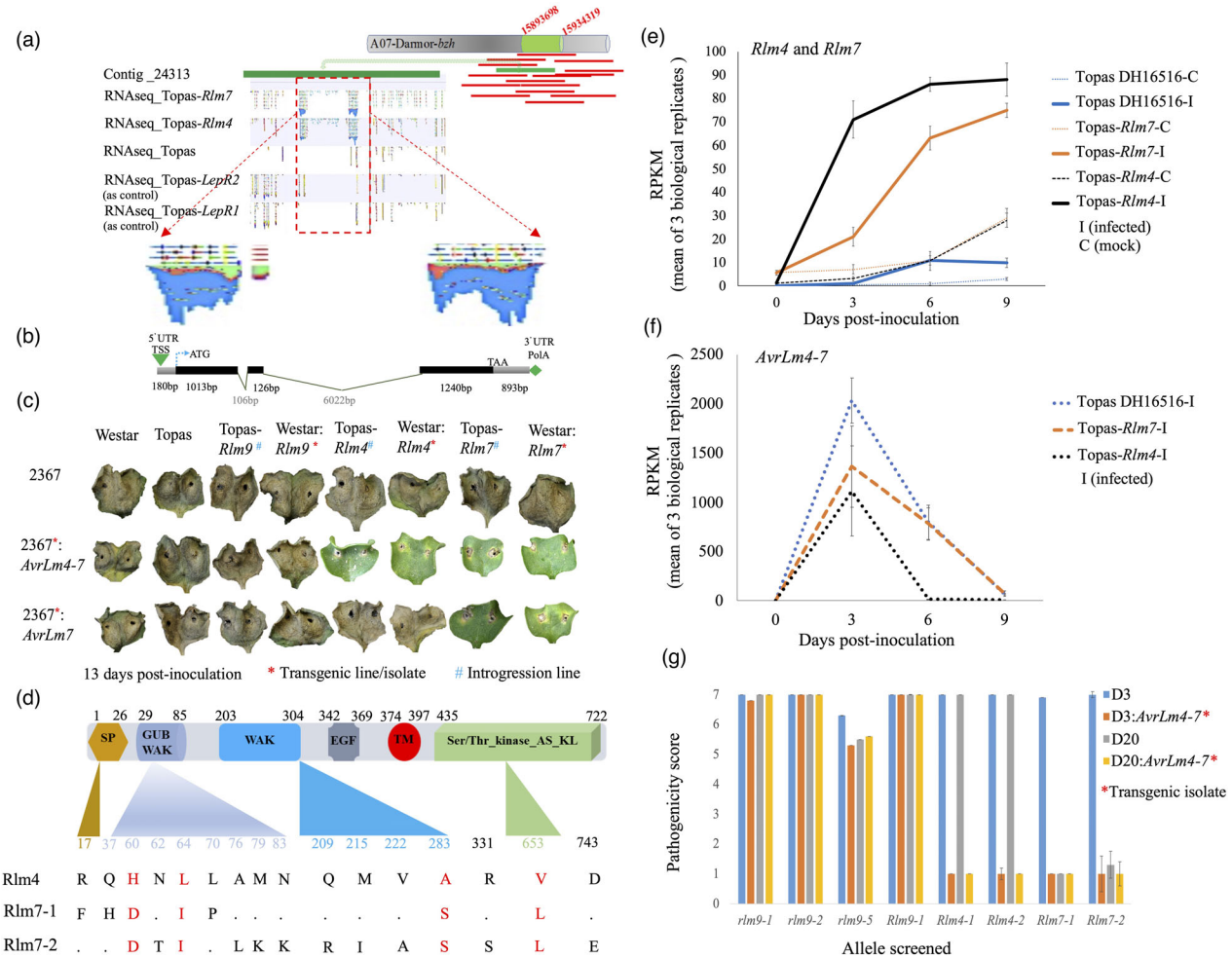

Figure 1 Cloning of *B. napus* genes; *Rlm4* and *Rlm7*. (a) Identification of *Rlm4* and *Rlm7* through contig walking. (b) *Rlm4* and *Rlm7* gene structures. Exons are shown as black bar, intron black line valley, and UTR as grey line. (c) Transgenic *B. napus* expressing *Rlm4* and *Rlm7*. Cotyledons of Westar, Topas, Topas-*Rlm9*, Westar:*Rlm9*, Topas-*Rlm4*, Topas-*Rlm7*, Westar:*Rlm4* and Westar:*Rlm7* inoculated with isolate 2367 (virulent towards *Rlm4, Rlm7 and Rlm9*), 2367:*AvrLm4-7* (avirulent towards *Rlm4* and *Rlm7*) and 2367:*AvrLm7* (avirulent towards *Rlm7*). (d) Protein domains of Rlm4 and Rlm7. Protein structure is represented by the signal peptide (yellow bar), extracellular GUB_WAK (dark blue), C-terminal WAK (blue bar), and EGF-like Ca^{2+} (grey bar), transmembrane (red bar) and an intracellular serine/threonine Protein Kinase (green bar) domains. (e, f) Expression profile of *Rlm4*, *Rlm7,* and *AvrLm4-7*. RNA-seq analysis showed a significant upregulation of *Rlm4*, *Rlm7* in the resistance lines and expression of *AvrLm4-7* peaked at 3 days post-inoculation in both susceptible and resistant lines. (g) Confirmation of *Rlm4* and *Rlm7* alleles through pathogenicity phenotyping. *B. napus* lines harbouring susceptible alleles, *Rlm9*, *Rlm4,* and *Rlm7* were screened using isolates D3, D20, D3:*AvrLm4-7* and D20:*AvrLm4-7*.

polymorphisms limited to the intronic regions of the gene. The *Rlm7-2* allele, identified in the *B. napus* variety Caiman, contained 13 non-synonymous SNPs; however, these amino acid changes do not affect the recognition of Rlm7. The Rlm7-1 and Rlm7-2 proteins differ from Rlm4 in conserved amino acids in the putative extracellular ligand-binding domains, at positions 60, 64 (WAK_-GUB), and 283 (WAK), which may explain the variation in recognition specificity between Rlm4 and Rlm7 towards AvrLm7. An additional conserved polymorphism is found in the kinase domain (at position 653), though this is unlikely to be involved in recognition of AvrLm4-7 (Figure 1d). While Rlm4 and Rlm7 proteins are highly similar, Rlm9 protein is more diverse, with numerous SNPs mainly at the ectodomain (Figure S1).

The Topas-*Rlm4* and Topas-*Rlm7* were utilized to monitor the expression of *Rlm4*, *Rlm7,* and the corresponding *Avr* gene, *AvrLm4-7*, during cotyledon infection by the reference isolate v23.1.3 (as previously described by Haddadi *et al.*, 2019). Expression of *Rlm4* and *Rlm7* increased substantially in both Topas-*Rlm4* and Topas-*Rlm7* in response to infection (Figure 1e). *AvrLm4-7* expression peaked at 3 days post-inoculation, both in susceptible and resistant lines, but rapidly declined at later time points (Figure 1f).

Additional pathology tests using lines harbouring both *Rlm4* and *Rlm7* alleles, as well as the *Rlm9* and four of the susceptible alleles, were performed in triplicate with isolates transformed with *AvrLm4-7*. Progenitor isolate D3 is virulent towards both *Rlm4* and *Rlm7* whilst D20 is only virulent towards *Rlm4*. Isolate D3:*AvrLm4-7* resulted in an avirulent reaction on lines harbouring each of the *Rlm4* and *Rlm7* alleles but remained virulent on all other lines as expected. Isolate D20:*AvrLm4-7* resulted in an avirulent reaction on lines harbouring both *Rlm4* alleles but remained virulent on the lines harbouring the susceptible alleles or *Rlm9* and remained avirulent on the *Rlm7* lines as expected (Figure 1g).

The cloning of *Rlm4* and *Rlm7* will provide valuable information for canola breeding programmes worldwide and expands the toolbox for further study of the newly emerging WAKL class of plant *R* genes.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

NJL and HB conceived and designed the study, PH and HB extracted the *Rlm4-1* and *Rlm7-1* ORFs and designed the transgenic constructs, NJL and EB analysed transformants, AVdW characterized additional Rlm4 and Rlm7 lines, YZ and TXN characterized additional alleles, PH, PB, and DE provided bioinformatic analysis. PH, NJL, AVdW, JB, and HB prepared the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Alignment of Rlm4, Rlm7, and Rlm9 proteins.