The *Brassica napus* blackleg resistance gene *LepR3* encodes a receptor-like protein triggered by the *Leptosphaeria maculans* effector AVRLM1

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Summary

- *LepR3*, found in the *Brassica napus* cv 'Surpass 400', provides race-specific resistance to the fungal pathogen *Leptosphaeria maculans*, which was overcome after great devastation in Australia in 2004. We investigated the *LepR3* locus to identify the genetic basis of this resistance interaction.
- We employed a map-based cloning strategy, exploiting collinearity with the *Arabidopsis thaliana* and *Brassica rapa* genomes to enrich the map and locate a candidate gene. We also investigated the interaction of *LepR3* with the *L. maculans* avirulence gene *AvrLm1* using transgenics.
- *LepR3* was found to encode a receptor-like protein (RLP). We also demonstrated that avirulence towards *LepR3* is conferred by *AvrLm1*, which is responsible for both the *Rlm1* and *LepR3*-dependent resistance responses in *B. napus*.
- *LepR3* is the first functional *B. napus* disease resistance gene to be cloned. *AvrLm1*'s interaction with two independent resistance loci, *Rlm1* and *LepR3*, highlights the need to consider redundant phenotypes in 'gene-for-gene' interactions and offers an explanation as to why *LepR3* was overcome so rapidly in parts of Australia.

Introduction

Brassica oilseed crops (canola/oilseed rape) are the world's third leading source of vegetable oil with *Brassica napus* L. (AACC, $n = 19$), *Brassica rapa* (AA, $n = 10$) and *Brassica juncea* (AABB, $n = 18$) being the main *Brassica* oilseed species cultivated worldwide (Singh, 2006). Over the past four decades blackleg disease (stem canker) has become a major limitation for canola production (Fitt *et al.*, 2006). The Dothideomycete fungus *Leptosphaeria maculans* is the causal agent of the disease. The disease cycle initiates with spores that are released from infected plant debris. The spores germinate on cotyledons and leaves of young plants producing germ tubes that enter through stomata, leading to the subsequent intercellular growth of the hyphae. Except for the formation of lesions on the host leaves, *L. maculans* remains asymptomatic through most of its life cycle within the adult plant until it forms a canker at the crown of the stem, which results in lodging and significant yield loss (Howlett *et al.*, 2001). The most efficient approach for controlling blackleg disease is deployment of plant resistance genes (Delourme *et al.*, 2006).

There have been many genetically characterized *Brassica* genes described for resistance to *L. maculans*, though, as yet, none of these genes have been cloned. The majority of these genes have been mapped to the *Brassica* A genome, including *Rlm1* on chromosome A07 and *LepR3* on A10 (Ansan-Melayah *et al.*, 1998; Balesdent *et al.*, 2001, 2002; Delourme *et al.*, 2004, 2006; Mayerhofer *et al.*, 2005; Yu *et al.*, 2005, 2008; Long *et al.*, 2011; Raman *et al.*, 2012). At least three *R*-genes are carried in the B genome (Chèvre *et al.*, 1996, 1997; Balesdent *et al.*, 2002; Christianson *et al.*, 2006; Kutcher *et al.*, 2010) and none have yet been reported for the C genome. These genes convey resistance to *L. maculans* isolates in a race-specific manner. The corresponding avirulence (Avr) genes are often located in genetic clusters in the *L. maculans* genome (Balesdent *et al.*, 2002) and several have been mapped or cloned (Cozijnsen *et al.*, 2000; Gout *et al.*, 2006; Fudal *et al.*, 2007; Parlange *et al.*, 2009; Ghanbarnia *et al.*, 2012). The recent release of both the *B. rapa* (AA) genome (Wang *et al.*, 2011) and the *L. maculans* genome (Rouxel *et al.*, 2011) sequences should help facilitate a greater understanding of this host-pathogen system.

The *L. maculans* resistance (*LepR*) genes, *LepR1*, *LepR2* and *LepR3* have been introgressed into *B. napus* through a resynthesis of *B. napus* from a *B. rapa* ssp. *sylvestris* × *B. oleracea* var. *alboglabra* interspecies cross (Crouch *et al.*, 1994; Buzza & Easton, 2002). *LepR1* and *LepR2* were identified and mapped to linkage groups A02 and A10, respectively (Yu *et al.*, 2005). The third major blackleg resistance gene, *LepR3*, is present in the *B. napus* cultivar 'Surpass 400' and has been mapped to linkage group

A10, at a distance of 11.7 cM from the *LepR2* locus (Yu *et al.*, 2008). *LepR3*-initiated resistance in 'Surpass 400' is associated with a hypersensitive response (Li *et al.*, 2004, 2007), a feature of many, although not all, plant resistance gene (*R*-gene)-mediated defense responses (Hammond-Kosack & Jones, 1996). *LepR3* was first described as a single, dominant gene from field-based studies (Li & Cowling, 2003), although several recent reports suggest the presence of two independent *R*-genes in 'Surpass 400' (Van de Wouw *et al.*, 2009; Long *et al.*, 2011). It was demonstrated that two independent *L. maculans* Avr genes, *AvrLm1* and *AvrLmS*, trigger defence responses in 'Surpass 400', thus it was inferred that 'Surpass 400' contained both *Rlm1* and a second gene referred to as '*RlmS*' (Van de Wouw *et al.*, 2009).

Plant resistance responses to pathogens fall under two general categories based on the pathogen molecules that trigger the responses; 'pattern-triggered immunity' (PTI) where slowly-evolving pathogen-associated molecular patterns (PAMPS) trigger basal defense responses, or 'effector-triggered immunity' (ETI), in which specific pathogen effectors, targeted to disrupt PTI, either directly or indirectly trigger specific *R*-genes (Jones & Dangl, 2006). Though PTI and ETI are often described as separate pathways, the responses likely function as a coordinated network (Katagiri & Tsuda, 2010). A common feature of most plant *R*-genes is the presence of leucine rich repeat (LRR) motifs that play a major role in recognition of pathogen effectors by facilitating protein–protein interactions (McDowell & Woffenden, 2003).

The majority of the plant *R*-proteins are predicted to be located intracellularly; however, there are several examples of extra-cytoplasmic LRR (eLRR)-containing *R*-proteins that are anchored to the plasma membrane via a transmembrane (TM) domain (Kruijt *et al.*, 2005; Yang *et al.*, 2012). One group of well-characterized eLRR *R*-genes are the tomato *Cf* genes that confer resistance against *Cladosporium fulvum*, the causal agent of tomato leaf mold disease. *Cf* genes encode a group of receptor-like proteins with recognition specificity for different avirulence proteins encoded by *C. fulvum* (Wulff *et al.*, 2009). Hyphae of *C. fulvum* enter the intercellular space through stomata and are confined to the intercellular space (Thomma *et al.*, 2005), which is analogous to the infection and growth of *L. maculans* hyphae within the plant host tissues. While three of the *L. maculans* effectors – *AvrLm1*, *AvrLm4-7* and *AvrLm6* – have been cloned (Gout *et al.*, 2006; Fudal *et al.*, 2007; Parlange *et al.*, 2009) their function and subcellular location in the host remains to be determined. This paper provides insight into the molecular recognition of *L. maculans* by its host. Here we report high-resolution mapping of the *LepR3* locus on linkage group A10 of *B. napus,* the investigation of collinearity between the *LepR3* region of *B. napus, B. rapa* and *Arabidopsis thaliana*, and the cloning of the *LepR3* gene, which encodes a receptor-like protein. We also demonstrate that the *L. maculans* avirulence gene *AvrLm1* confers avirulence to both *LepR3* and *Rlm1*. We believe this to be the first published report of the cloning of an *R*-gene from the important oilseed crop species *Brassica napus*.

Materials and Methods

L. maculans isolates and inoculum

Two *L. maculans* isolates characterized for their virulence on 'Surpass 400'; 'S005' (*avrLm1*, *avrLmS*) and 'P042' (*AvrLm1*, *AvrLmS*) (Van de Wouw *et al.*, 2009) and progeny of a cross between the two; '3R5' (*AvrLm1*, *avrLmS*), '3C3' (*AvrLm1, AvrLmS*) and '3R11' (*avrLm1, avrLmS*) were kindly provided by Angela van de Wouw, University of Melbourne (Australia). Additional '*AvrLm1*' isolates 'WA74', '87-41' and '2354', as well as '*avrLm1*' isolate '99-53' were sourced from the Rimmer Collection, AAFC Saskatoon. Inoculum was produced at a concentration of 2×10^7 pycnidiospores ml^{-1} as described in Yu *et al.* (2005). To confirm *AvrLm1* genotypes of the isolates 9 µl of inoculum was added to 1 µl 10× TE (100mM Tris-Cl pH8, 10mM EDTA pH8), 2.5% Tween20, incubated at 95°C for 5 min then used as template for PCR with *AvrLm1* F & R primers (see the Supporting Information, Table S1).

B. napus lines and populations

First backcross (BC_1) and third backcross (BC_3) generations of *B. napus,* segregating for *LepR3*, produced via crosses between the susceptible spring-type double-haploid line 'Topas DH16516' and the resistant variety 'Surpass 400' backcrossed to the susceptible parent were used for the mapping study. A total of 446 plants (97 BC_1 and 349 BC_3) were screened for resistance to the isolate '3R5' (*AvrLm1*, *avrLmS*). All screening included 'Topas DH16516' (no resistance) and 'Surpass 400' (*LepR3*, *RlmS*) controls. The resistant *B. napus* varieties 'Columbus' (*Rlm1*, *Rlm3*) (Balesdent *et al.*, 2006), 'Quinta DH24288' (*Rlm1*, *Rlm3*) (Kutcher *et al.*, 2010) and 'Quantum' (*Rlm3*) (R. Kutcher, pers. comm.) were also used for pathotyping of isolates.

Determination of resistance phenotype

Seedlings were germinated in 96-cell trays in a controlled growth chamber (20°C, 16 h days (light intensity *c.* 450 µmol m^{-2} s^{-1} at bench level) and 18°C, 8 h nights). After 7 d the cotyledons were inoculated with a spore suspension of *L. maculans* (2×10^5 pycnidiospores per wound site, eight plants per test) and were rated 14 d postinfection. The resistance phenotype of the seedlings was rated using a continuous 0–9 scale modified after (Koch *et al.*, 1991) as shown in Fig. S1. A score of 0–4 was classified as 'resistant', 5 as 'intermediate' and 6–9 as 'susceptible'.

Molecular marker characterization of mapping populations

The approximate position of *LepR3* was first determined using 87 BC_3 individuals phenotyped for resistance using the *L. maculans* isolate '3R5' (*AvrLm1*, *avrLmS*) and surveyed using several microsatellite markers from the region of chromosome A10 identified previously as containing the *LepR3* locus (Yu *et al.*, 2008). All microsatellite markers used in this work (prefixed with 'sN' or 'sR') were developed by and obtained from

A. G. Sharpe and D. J. Lydiate, AAFC Saskatoon, Canada. After determining a wide interval for the gene the remaining BC_1 and BC_3 seedlings used in the fine mapping population were surveyed with the microsatellite markers sN8502 and sR9355, flanking the *LepR3* region. Individuals displaying a recombination event between the two microsatellite markers were reserved for the mapping study. After rating for disease at 14 d post-inoculation, the infected cotyledons were removed to prevent spread of the disease. Four leaf discs totaling *c.* 20 mg (fresh weight) were collected from young leaves and freeze-dried for DNA extraction by cetyltrimethylammonium bromide (CTAB) method scaled down from (Murray & Thompson, 1980) for 96-well format. The initial genotypes for these 'recombinants' and their resistance phenotypes were confirmed by examining the segregation of resistance from BC_1S_1 that is self-progeny from the recombinant BC_1 individuals. The PCR reactions for all markers were performed as described by Hughes *et al.* (2003). Genotyping was performed using a MegaBACE capillary sequencer (GE Health, Mississauga, ON, Canada).

Targeted marker creation

Further resolution was added to both maps by the targeted creation of markers that took advantage of small insertion/deletion events ('indels') between the parental lines. These markers were produced after first identifying the syntenic position of the microsatellite markers relative to the *A. thaliana* genome (Arabidopsis Genome Initiative, 2000; http://www.arabidopsis.org) by BLASTN (Altschul *et al.*, 1997). After determining the collinear intervals spanning the target genes, the *Brassica rapa* Genome Project database was searched via BLASTN (http://www.brassica-rapa.org) for bacterial artificial chromosomes (BACs) matching the collinear intervals, using either microsatellite clone sequence or *A. thaliana* genes that fell within the collinear spans. Primers were then designed using these BAC sequences and used to amplify 1300–1400 bp fragments from both 'Topas DH16516' and 'Surpass 400' parents. These fragments were cloned using TOPO TA Cloning Kits (Invitrogen) and screened for correct-sized inserts via PCR. For each fragment three or four plasmids were purified (QIAprep Spin Miniprep Kit; Qiagen), sequenced (Sharpe Lab, PBI, Saskatoon, SK, Canada), aligned using Vector NTI software (Invitrogen) and polymorphic indels identified. New fluorescently-labeled primers were designed flanking the polymorphic sequence and used for further genotypic characterization of the mapping population. All indel markers (prefixed with 'Ind') designed during this study are listed in Table S1. The PCR reactions and genotyping were performed as for the microsatellite markers (above) except for the marker 'Ind10-12' for which the following program was used: 95°C, 3 min; (95°C, 30 s; 54°C, 30 s; 72°C, 45 s) × 35; 72°C, 5 min; 12°C hold.

Linkage analysis

Interval spacing was determined manually from recombination frequencies in the recombinant subpopulation ($cM_{BC} = (x/n)$ *100, where x = recombination events and n = total population size). Linkage between markers and the *LepR3* locus was verified using JOINMAP 4 software (Stam, 1993) using a LOD threshold > 3 and the Kosambi mapping function (Kosambi, 1944). The marker positions were later aligned to the complete *B. rapa* genome sequence (www.brassicadb.org) following its public release.

Identification, cloning and transformation of candidate gene

A *B. rapa* BAC spanning the *LepR3* interval was annotated for gene content using FGENESH software (www.softberry.com) trained to 'Dicot plants (Arabidopsis)' to predict gene content. The protein translations were then used to search for homologous *A. thaliana* genes via BLASTP, allowing a candidate gene to be identified. The candidate gene was cloned by first using Phusion DNA Polymerase (New England Biolabs, Whitby, ON, Canada) and primers anchored within the flanking genes to amplify a fragment of *c.* 7 kb spanning the candidate gene locus from both parental lines. After subcloning and partial sequencing, primers 'GW-BnRLP F and R' were designed with Gateway *attB1* and *attB2* recombination tags, respectively (Table S1), and used to amplify a nested fragment of 4.25 kb, containing the full candidate gene locus from 'Surpass 400', including an additional 1144 bp upstream and 535 bp downstream of the coding region to capture the native promoter and termination sequences, respectively. A corresponding fragment was also amplified from 'Topas DH16516' using primers 'GW-BnRLP F and Rb' (Table S1). The fragments from both parents were cloned into the Gateway (Invitrogen) entry vector pDONR-Zeo and sequenced. The resistant 'Surpass 400' parent allele was transferred to the Gateway-compatible plant transformation vector pMDC123, which contains the $Basta^R$ gene for selection *in planta* (Curtis & Grossniklaus, 2003). The construct was transferred to *Agrobacterium tumefaciens* strain 'GV3101 pMP90' and used to transform 'Topas DH16516' essentially as described by De Block *et al.* (1989).

Sequences of the 'Topas DH16516' *lepR3* and 'Surpass 400' *LepR3* alleles were deposited in GenBank (JX880109 and JX880110, respectively).

Analysis of *B. napus* transformants and *LepR3* transcript

Transformants that tested positive for insertion of the candidate sequence via PCR were self-pollinated to produce T_1 seeds, which were tested for segregation of the resistance phenotype using the *L. maculans* isolate '3R5' as described above. A T_1 seed lot showing 3R : 1S segregation consistent with a single insertion event was selected for further phenotypic analysis and production of homozygous T_2 seed. Total RNA was isolated from cotyledons using a PureLink RNA Mini Kit (Ambion) and a near-full length copy of the *LepR3* transcript was amplified from both 'Surpass 400' and a resistant T_1 plant by reverse transcription using Superscript III One-Step RT-PCR System (Invitrogen) and the gene-specific primers 'RTR3C F & R', while 'RT-Top Fc & Rc' primers were used to amplify the 'Topas DH16516' transcript (Table S1). A GeneRacer kit (Invitrogen) was used for 5′ RACE

analysis, using the gene-specific primer 'RTR3-5′b' (Table S1). Identification of functional domains within the LEPR3 protein was performed using InterProScan (http://www.ebi.ac.uk/Tools).

Analysis of *LepR3*-transgenic *B. napus* with *AvrLm1*-transgenic *L. maculans*

A Gateway-compatible fungal transformation vector was created by modifying the vector pPK2 (Covert *et al.*, 2001) using the Gateway Vector Conversion Reagent System (Invitrogen). A 2221 bp genomic fragment spanning the *L. maculans AvrLm1* locus, including 1131 bp upstream and 423 bp downstream of the coding region, was amplified from the *AvrLm1* isolate 'WA74' using Phusion DNA polymerase (New England Biolabs) and the Gateway *attB*-tagged primers 'AvrLm1G F and R' (Table S1), transferred to the Gateway entry vector pDONR-Zeo and then to the GW-pPK2 fungal transformation vector. After confirmation of the insert by sequencing, the GW-pPK2-*AvrLm1*G construct was transferred into the *A. tumefaciens* strain 'AGL1 pTiBo542' and used for transformation of pycnidospores from the *L. maculans* isolate '3R11' (*avrLm1*), as described by (Utermark & Karlovsky, 2008). Selection of transformed colonies was achieved after 7 d incubation on Czapek Dox medium supplemented with 120 μg ml^{-1} Hygromycin B. A pycnidiospore suspension produced from the resulting '3R11 : *AvrLm1*G' transformant, along with the untransformed '3R11', were used for inoculation of 'Topas DH16516', 'Columbus', 'Quinta DH24288' (*Rlm1, Rlm3*), 'Quantum' and 'Surpass 400' controls as well as three homozygous T_2 lines produced from *LepR3*-transgenic 'NLA8' T_1 plants, as described above.

Results

Mapping of the *LepR3* resistance locus

Mapping of the *LepR3* locus was conducted using the isolate '3R5' which had previously been pathotyped as '*AvrLm1, avrLmS*' (A. Van de Wouw, pers. comm.). '3R5' was virulent on the susceptible *B. napus* parental line 'Topas DH16516' and avirulent on the resistant parent 'Surpass 400' (Table 1). The initial mapping of resistance using the first 87 'Topas DH16516' × 'Surpass 400' BC_3 plants correlated strongly, though not perfectly, with the original map position described for *LepR3* on chromosome A10 (Yu *et al.*, 2008). The presence of one 'recombinant' individual displaying a recombination event between the microsatellite marker sN2428R and *LepR3* suggested that the gene was located outside of the genetic interval previously described. Assaying the initial population with additional microsatellite markers located in the *LepR3* region of A10 identified four markers (sR2428R, sN1982, sR1443 and sR0685) that mapped inside the interval defined by the flanking markers sN8502 and sR9355. Mapping of the locus with the full population of 446 individuals gave a 1 : 1 segregation ratio (212R : 234S; χ^2 (1 : 1) = 1.09, P = 0.297), indicating that resistance to '3R5' is conferred by a single dominant gene. All six microsatellite markers spanning the map interval also conformed to the expected 1 : 1 ratio (χ^2 (1 : 1) = 0.002–0.56, P = 0.453–0.962). Sixteen individuals carrying recombination events between the flanking microsatellites (sN8502 and sR9355) were detected. This resulted in a genetic distance of 3.4 cM for the target region and a revised position for the *LepR3* locus in a 0.8 cM interval between the microsatellite markers sR1443 and sR0685 (Fig. 1), *c.* 2.1 cM below the previously described location (Yu *et al.*, 2008).

Investigation of collinearity and targeted marker creation

The six microsatellite markers and their respective genomic clones were used for the analysis of collinearity between the *LepR3* interval of *B. napus* and the *A. thaliana* genome. The sequences of five of the six microsatellite clones (except sR9355, which has no significant homology to *A. thaliana*) matched homologous sequences in a 695 kb region of chromosome 5 of *A. thaliana* (At5). The five matching markers were collinear in

Table 1 Phenotypic reaction of *Leptosphaeria maculans* isolates on *Brassica napus* controls

Isolates[a]	*B. napus* controls[b]				
	Topas DH16516 (–)	Surpass 400 (*LepR3,RlmS*)	Columbus (*Rlm1,Rlm3*)	Quantum (*Rlm3*)	NLA8 T_1 (*LepR3* seg.)
3R5 (*AvrLm1,avrLmS*)	S (8.6)	R (3.0)	R (3.2)	S (8.1)	38R : 10S
WA74 (*AvrLm1, ?*)	S (7.4)	R (3.0)	R (2.5)	S (7.0)	6R : 2S
87-41 (*AvrLm1, ?*)	S (7.2)	R (2.9)	R (3.0)	S (7.2)	7R : 1S
2354 (*AvrLm1, ?*)	S (8.8)	R (2.9)	R (3.0)	S (7.6)	6R : 2S
P042 (*AvrLm1,AvrLmS*)	S (9.0)	R (3.1)	R (3.2)	S (8.8)	6R : 2S
3C3 (*AvrLm1,AvrLmS*)	S (8.8)	R (2.9)	R (3.0)	S (8.6)	5R : 3S
S005 (*avrLm1,avrLmS*)	S (9.0)	S (8.6)	S (8.4)	S (9.0)	0R : 8S
3R11 (*avrLm1,avrLmS*)	S (9.0)	S (8.3)	S (8.0)	S (8.5)	0R : 8S
99-53 (*avrLm1,AvrLmS*)	S (9.0)	R (4.3)	S (8.5)	S (8.5)	0R : 8S

Isolates that were either avirulent (*AvrLm1*) or virulent (*avrLm1*) towards the *B. napus R*-gene *Rlm1* were characterized for the reaction induced on the *LepR3*-carrying line 'Surpass 400' and *LepR3*-transgenic 'NLA8' T_1 plants (segregating). R, resistant; S, susceptible. Mean phenotypic score (0–9 scale, 0 most resistant, eight plants per test) for controls given in brackets after phenotype. *Leptosphaeria maculans* Avr gene alleles (a) and *B. napus* genes (b) given in brackets after name. Resistant reactions, shaded green; susceptible reactions, pink.

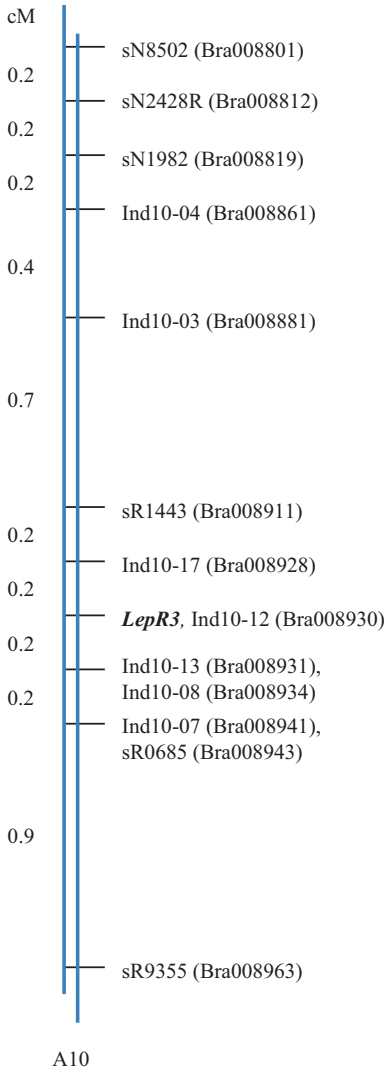

Fig. 1 Genetic map of the- *LepR3* locus on *Brassica napus* chromosome A10. Position of *LepR3* relative to microsatellite (prefixed 'sN' or 'sR') and Indel (prefixed 'Ind') markers. Nearest *Brassica rapa* homologue to each marker given in brackets. cM, centiMorgans.

the two species and the relative spacing was conserved (not shown). The specific interval of *B. napus* chromosome A10 in which *LepR3* resided was found to be syntenic to At5, between the genes At5g12150 (sR1443) and At5g11700 (sR0685). Further definition of the *LepR3* region was obtained by developing genetic markers from *B. rapa* BACs that had homologous sequences in the syntenic region of At5. Several sequenced *B. rapa* BACs within or adjacent to the *LepR3* interval were identified based on their homology to collinear *A. thaliana* genes. These BACs were used to produce seven targeted indel markers (prefixed 'Ind10', Table S1) that were placed within the 3.4 cM *LepR3* interval and again demonstrated a conserved order with respect to the corresponding homologous sequences in *A. thaliana* (not shown). These new markers were used to refine the *LepR3* map and define the locus to an interval of 0.4 cM (Fig. 1), syntenic to the At5g11880 – At5g11900 interval of At5 (Fig. 2).

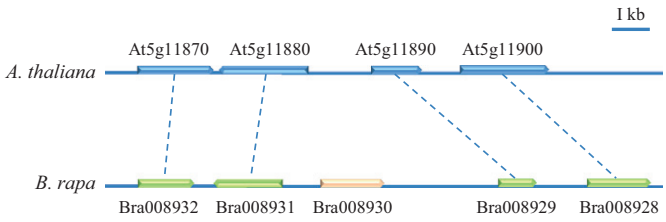

Fig. 2 Syntenic alignment of candidate gene region from *Arabidopsis thaliana* and *Brassica rapa*. The *B. rapa LepR3* candidate receptor-like protein 'Bra008930', which had no syntenic match in *A. thaliana*, was revealed after using *A. thaliana* as a 'bridging genome' to find matching *B. rapa* BACs. Homologues are indicated by a dashed line.

Identification of candidate gene

Annotation of the *B. rapa* BAC 'KBr080E24' containing the chromosome A10 region matching the *LepR3* interval revealed the presence of a 1890 bp predicted receptor-like protein (RLP)-coding gene that was nonsyntenic to the *A. thaliana* interval, with its best match (*e*-value of $5e^{-66}$) being AtRLP32 (At3g05650). Upon the release of the *B. rapa* genome (Wang *et al.*, 2011) the gene was annotated as 'Bra008930' (Fig. 2). No other candidate genes were detected within the target interval. Amplification and sequencing of the candidate locus from *B. napus* revealed a predicted gene of 2853 bp (one exon) from the susceptible 'Topas DH16516' and 2556 bp (one exon) from the resistant cv 'Surpass 400'. The *B. napus* homologues appeared to be larger than the predicted *B. rapa* gene (1890 bp). While the 'Topas DH16516' and 'Surpass 400' genes showed conserved homology over most of their lengths they also contained large indel variations: an indel in the 'Topas DH16516' 5′ region, two indels in the 'Surpass 400' LRR region and a large indel 3′ of the 'Surpass 400' CDS (Fig. 3), along with numerous amino acid variations within the CDS (Fig. S2).

Analysis of *LepR3* transcript

Amplification of a near-full length *LepR3* transcript from 'Surpass 400' cotyledon total cDNA produced two fragments: one with the expected size of 2431 bp (*LepR3* candidate) as well as a second fragment of only 2262 bp. The second, smaller fragment was identical to the first except for the removal of 169 bp from within the CDS (Δ386..536). Rapid amplification of cDNA ends (RACE) analysis of the *LepR3* transcript allowed for the determination of a 37 bp 5′ UTR, though the 3′ UTR was not successfully amplified. A transcript of the susceptible (*lepR3*) open reading frame (ORF) was also amplified from 'Topas DH16516' cDNA using unique primers, demonstrating that the *LepR3* and *lepR3* alleles are both expressed in their respective cultivars.

The predicted full-length transcript encodes one exon producing a protein 851 amino acids in length. InterProScan analysis of the predicted LEPR3 protein revealed a typical receptor-like protein motif structure (Fritz-Laylin *et al.*, 2005): a signal peptide located at the *N*-terminal (residues 1–26) followed by an extensive eLRR region (34–731), a GXXXG-type transmembrane motif and terminating with a 24 amino acid cytoplasmic *C*-terminal region. The smaller, partially deleted fragment contained

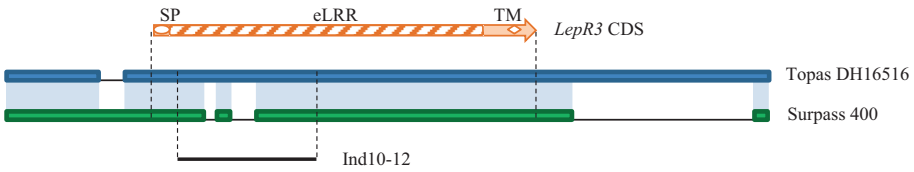

Fig. 3 Alignment of 'Surpass 400' *LepR3* and 'Topas DH16516' *lepR3* alleles. Cartoon representation of the alignment of resistant 'Surpass 400' and susceptible 'Topas DH16516' *LepR3*/*lepR3* alleles detailing the coding region (CDS) of each allele and insertion/deletions both 5′ and 3′ of the CDS and within the eLRR-coding region of the gene. The position of the marker Ind10-12 is also noted. SP, signal peptide; LRR, leucine-rich repeat; TM, transmembrane domain. Full alignment presented in the Supporting Information, Fig. S2.

a premature stop codon after only 123 amino acids, meaning the resulting peptide would only contain a small portion of the eLRR domain.

Analysis of *LepR3* transformants

A *LepR3* genomic clone, driven by its native promoter, was transferred to the susceptible *B. napus* cv 'Topas DH16516'. A total of 13 transformants (T_0) were screened with the 'Ind10-12' marker positioned within the *LepR3* CDS (Table S1) to confirm the presence of an insertion event, revealing five positive and eight negative transformants. Of the five positive plants, only one ('NLA8') displayed a balanced heterozygous-type polymorphism for 'Ind10-12' while the other four plants displayed polymorphisms heavily favoring the 'Surpass 400' allele, suggesting multiple insertion events (data not shown). After allowing self-pollination, eight T_1 seedlings produced from each of four mature T_0 plants (two negative lines 'NLA6' and 'NLA7' and two positive lines 'NLA8' and 'NLA9') were tested with '3R5', the same *L. maculans* isolate used to map the *LepR3* locus (data not shown). Both negative lines proved to be 100% susceptible, equal to the 'Topas DH1516' susceptible control, 'NLA8' displayed six resistant and two susceptible T_1 individuals (Fig. 4) and 'NLA9' showed 100% resistance. To test the segregation ratio for resistance in the 'NLA8' T_1 progeny, an additional 40 T1 individuals were screened with '3R5', revealing a segregation for resistance (38R to 10S) conforming to a 3 : 1 ratio (χ^2 (3 : 1) = 0.44, $P = 0.501$), as expected from the segregation of a single dominant gene, suggesting a single insertion event and further confirming cloning of the *LepR3* gene (Table 1). Several resistant 'NLA8' T_1 individuals were self-pollinated and the resulting T_2 seed was screened with '3R5' to select *LepR3*-homozygous lines (data not shown).

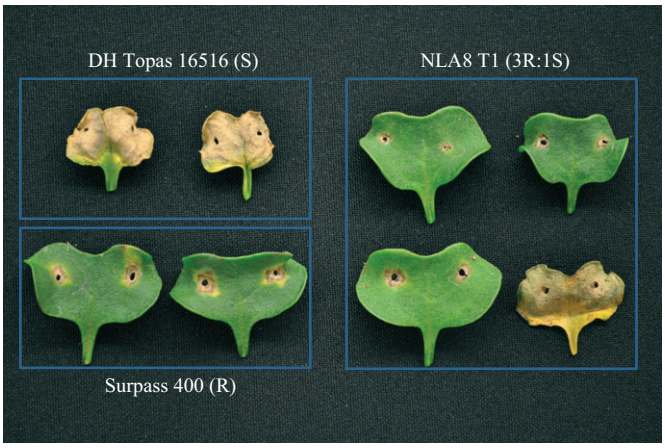

Fig. 4 Reaction of *LepR3*-transgenic T_1 plants to *Leptosphaeria maculans* isolate '3R5'. The 'NLA8' T_1 generation showed resistance to '3R5' in a 3R : 1S ratio, confirming the candidate gene as *LepR3*.

Both the near full-length *LepR3* transcript and the second, partially deleted *LepR3* transcript (first detected from 'Surpass 400' cDNA) were also detected in the resistant, *LepR3*-transformed 'NLA8' T_1 plants. However, no *LepR3* transcript, partial or otherwise, was detected in the 'Topas DH16516' susceptible control, showing it to likely be a product of the *LepR3* gene and not a related transcript from the *B. napus* genome (data not shown).

The *L. maculans* effector *AvrLm1* confers avirulence to *LepR3*

Resistance of *LepR3*-containing *B. napus* cv 'Surpass 400' to *L. maculans* isolates carrying *AvrLm1* had previously been attributed to the presence of *Rlm1* in this cultivar. When several *L. maculans* isolates were inoculated on the *LepR3*-transgenic 'NLA8' T_1 lines, all six of the isolates that contained *AvrLm1* were avirulent on the *LepR3*-transgenic material while the three isolates lacking *AvrLm1* were virulent. This suggested that *AvrLm1* conferred avirulence to *LepR3*. The interaction of the *L. maculans* isolates with the control *B. napus* lines and 'NLA8' T_1 plants is summarized in Table 1. The functionality of *AvrLm1* in these isolates was confirmed by their avirulence on 'Columbus' (*Rlm1, Rlm3*) and virulence on 'Quantum' (*Rlm3*) and the *AvrLm1* genotypes were also confirmed via PCR (data not shown). One isolate, '99-53', was shown to be virulent towards *Rlm1*, yet still induced a resistance response on 'Surpass 400', and was therefore designated as '*avrLm1*, *AvrLmS*', as described by Van de Wouw *et al.* (2009). This isolate was also virulent on the *LepR3* transgenic material. We were unable to determine the '*AvrLmS*' status of the *AvrLm1* isolates 'WA74', '87-41' or '2354' at the time of scoring as the phenotype was masked by the isolates' reaction to *LepR3*.

In total, 88 T_1 individuals were screened with '*AvrLm1*' isolates, producing a total segregation of 68 resistant to 20 susceptible plants (χ^2 (3 : 1) = 0.24, $P = 0.622$), consistent with a single insertion event for the *LepR3* construct in the 'NLA8' T_0 individual. Our results for the isolates 'S005' and 'P042' confirmed the *AvrLm1* designations reported previously (Van de Wouw *et al.*, 2009).

To further validate the recognition of *AvrLm1* by *LepR3* we transferred an *AvrLm1* genomic clone driven by its native promoter to the virulent *L. maculans* isolate '3R11' (*avrLm1*, *avrLmS*). The untransformed isolate and the transgenic '3R11 : *AvrLm1*G' were both virulent on the 'Topas DH16516' and 'Quantum' controls, yet differed in their reaction to *Rlm1* and *LepR3* lines (Table 2). '3R11' was virulent on the *Rlm1* lines 'Columbus 6-1' and 'Quinta DH24288', the *LepR3* line 'Surpass 400' and all three T_2 *LepR3*-transgenic homozygous lines ('NLA8-2, 'NLA8-3' and 'NLA8-9') as expected. However, the transgenic '3R11 : *AvrLm1*G' was avirulent on all *Rlm1* or *LepR3* material, including the *LepR3*-transgenic T_2 lines, demonstrating the complementation of both *R*-genes by *AvrLm1* (Fig. 5).

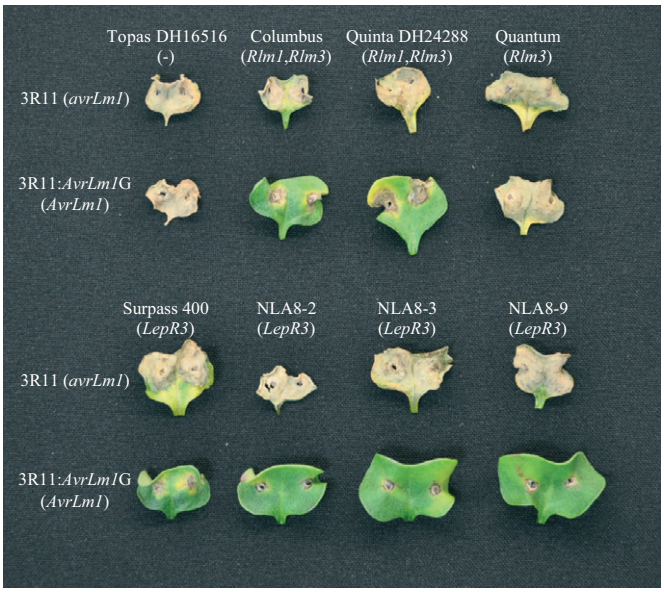

Fig. 5 Phenotypic interaction of *AvrLm1*-transgenic *Leptosphaeria maculans* and *LepR3*-transgenic *Brassica napus* lines. The addition of *AvrLm1* to the *L. maculans* isolate '3R11' shifted it to avirulence towards all *Rlm1* and *LepR3 B. napus* lines tested, confirming the interaction of *AvrLm1* with both *R*-genes.

Discussion

The region of the *B. napus* chromosome A10 in which the *LepR3* locus is situated had previously been shown to share a high degree of low-resolution collinearity with chromosome 5 of *A. thaliana* (Parkin *et al.*, 2002, 2005). The results of our study highlight the conserved nature of this collinear region at high resolution, with perfect marker order conservation. No change in marker order was detected over this region of *A. thaliana* (not shown), *B. rapa* or *B. napus* (Fig. 1). This close synteny allowed the development of targeted markers that reduced the size of the *LepR3* interval to 0.4 cM (Fig. 1).

In a previous study of the *LmR1* gene on linkage group A07 of *B. napus* (Mayerhofer *et al.*, 2005) collinearity with *A. thaliana* chromosome 1 (At1) was exploited to identify a number of candidate genes associated with disease resistance, though this study was complicated by an inversion that differentiated *B. napus* and *A. thaliana* in this region. By contrast, the search for candidate genes for *LepR3* was facilitated by the close collinearity over the interval between markers sN8502 and sR0685 on A10 of *B. napus* and the corresponding region between At5g13810 and At5g11700 on At5. Although none of the *A. thaliana* genes were likely homologues of *LepR3*, the collinear nature of the relationship between *A. thaliana* and *Brassica* spp. over this region allowed the retrieval of *B. rapa* BAC sequences related to the target interval, one of which harbored the *LepR3* homologue. While this approach to finding a candidate gene was employed successfully in this study, the recent release of the *B. rapa* genome (Wang *et al.*, 2011) makes this approach mostly obsolete for finding *Brassica* A genome genes. However, using *A. thaliana* as a bridging genome may still be useful for placing *Brassica* B and C genome BACs within target intervals, at least until sequencing of these genomes is completed.

The *LepR3* gene discovered in this study is a member of the receptor-like protein family of genes first linked to disease resistance in plants with the cloning of the *Cf-9* gene for resistance to *Cladosporium fulvum* in tomato (Jones *et al.*, 1994). Both *Cf-9* and the well-characterized tomato RLP *Ve1* (Kawchuk *et al.*, 2001) have been previously clustered in the same 'superclade' as At3g05650 (Fritz-Laylin *et al.*, 2005); the best match to *LepR3* in *A. thaliana* (this study). A large family of 56 RLP genes is found in the *A. thaliana* genome (Fritz-Laylin *et al.*, 2005). Each of the two *Brassica* genomes in *B. napus* (AACC) have evolved through the whole-genome triplication of an ancestral Brassicaceae genome closely related to *A. thaliana* (Lagercrantz, 1998; Nelson

Table 2 Phenotypic reaction of *AvrLm1*-transgenic *Leptosphaeria maculans* on *Brassica napus* lines

	Control lines[b]					T_2 lines[b]		
Isolates[a]	Topas DH16516 (–)	Columbus (*Rlm1*,*Rlm3*)	Quantum (*Rlm3*)	Quinta DH24288 (*Rlm1*,*Rlm3*)	Surpass 400 (*LepR3*,*RlmS*)	NLA8-2 (*LepR3*)	NLA8-3 (*LepR3*)	NLA8-9 (*LepR3*)
3R11 (*avrLm1*)	S (9.0)	S (8.5)	S (9.0)	S (8.8)	S (8.0)	S (9.0)	S (9.0)	S (8.8)
3R11 : *AvrLm1*G (*AvrLm1*)	S (8.8)	R (3.5)	S (9.0)	R (3.8)	R (3.3)	R (2.1)	R (2.5)	R (2.8)

Both wild-type *L. maculans* isolate '3R11' and transgenic '3R11' carrying the GW-pPK2-*AvrLm1*G construct were used to demonstrate the interaction of *AvrLm1* with *B. napus* lines containing the *R*-genes *Rlm1* and *LepR3*, including three homozygous *LepR3*-transgenic lines (NLA8-2, -3 & -9). S = susceptible reaction, R = resistant reaction, mean disease score (0–9 scale, 0 most resistant, eight plants per test) in brackets after phenotype. *L. maculans* Avr gene alleles (a) and *B. napus* genes (b) are given in brackets after the name. Resistant reactions, shaded green; susceptible reactions, pink.

& Lydiate, 2006; Ziolkowski *et al.*, 2006) thus the RLP gene family in *B. napus* could number close to 300 members.

The nested PCR strategy employed in this study to clone *LepR3* enabled us to efficiently amplify a unique fragment without interference from any other RLP locus in the genome and without knowing the precise sequence of the target, or the added time and expense of building and screening a large-insert genomic library. This method may also prove useful for the cloning of genes from other large gene families. However, this approach has the limitation of only being applicable for use in examining candidate genes detected in the *B. rapa* genome. A full *B. napus* reference genome sequence would likely be a better resource for this approach; however, a full pathological examination of the reference *B. rapa* var. 'Chiifu' for the presence of resistance genes for all of the major *B. napus* pathogens would be very useful.

Investigation of the *LepR3* locus in the resistant 'Surpass 400' and the susceptible 'Topas DH16516' revealed three regions harboring insertion/deletion (indel) differences between the two loci (Figs 3, S2). However, these indels do not disrupt the expression of the two alleles as we were able to detect transcripts from both loci. It is not yet known what role, if any, the truncated form of the *LepR3* transcript plays in the resistance response, though we speculate that it may be the product of post-transcriptional control of transcript abundance. While alternative splicing has been described in NBS-LRR *R*-genes (Gassmann *et al.*, 1999; Marathe *et al.*, 2002; Schornack *et al.*, 2004; Ferrier-Cana *et al.*, 2005) we are unaware of any reports of alternative splicing or 'exon editing' in RLPs.

Here we provide evidence that the *L. maculans* effector *AvrLm1* confers avirulence to both *LepR3* and *Rlm1*. It has already been demonstrated that the addition of *AvrLm1* to an *avrLm1, AvrLmS L. maculans* isolate produces a stronger avirulence reaction on 'Surpass 400' than the untransformed isolate (Van de Wouw *et al.*, 2009). The recognition of a single Avr gene by two plant *R*-genes is not unprecedented in the *L. maculans-B. napus* pathosystem, since the *L. maculans* Avr gene *AvrLm4-7* was shown to trigger a defense response in both *Rlm4* and *Rlm7*-carrying *B. napus* lines (Parlange *et al.*, 2009), although in this case both of the *R*-genes are located in the same genetic cluster, and may be allelic variants (Delourme *et al.*, 2004). Only three *L. maculans* Avr genes have been cloned to date, yet two of them have been shown to trigger resistance responses from two separate *Brassica* resistance loci. This is suggestive of the broad use of these Avr genes by the pathogen and possible chromosomal rearrangement and/or horizontal transfer of *R*-genes among the *Brassica* spp.

Are *Rlm1* and *LepR3* the same gene? *Rlm1* occupies a different chromosomal location (A07 – Delourme *et al.*, 2004) than *LepR3* (A10 – this study) thus, genetically, they should be considered separate resistance loci. Van de Wouw *et al.* (2009) speculated that the *LepR3* resistance locus may have been produced via the translocation of *Rlm1* from A07 to A10 during the original Crouch *et al.* (1994) resynthesis used to create 'Surpass 400', or that 'Surpass 400' contained both *Rlm1* and *LepR3*, in addition to *RlmS*. During our mapping of the *LepR3* locus there was no evidence to support either of these hypotheses; all markers behaved as predicted by their syntenic location on chromosome A10 of *B. rapa*, including the *LepR3* locus itself. Had the *LepR3* gene been nonsyntenic (i.e. located on A07) in *B. rapa*, we would not have been able to identify the candidate RLP gene on A10 of the *B. rapa* genome sequence. Alternatively, if both *Rlm1* (A07) and *LepR3* (A10) were present in 'Surpass 400' we would have observed a 3R : 1S segregation ratio for the BC population (two independently-assorting loci) when characterizing the plants with '3R5′ (*AvrLm1, avrLmS*). Instead, we saw the expected 1R : 1S ratio, with all of the observed resistance being explained by the A10 *LepR3* locus and no distortion of associated markers to suggest it was the product of a recent translocation. It is still possible that *Rlm1* and *LepR3* are homologous sequences located at two independent loci within the *B. napus* genome, as nonsyntenic transposition of genes has been observed in *Brassica* genomes (Cheung *et al.*, 2009). However, the chromosomal regions of A07 and A10 harboring the two genes do not share any homology (Parkin *et al.*, 2005) and while recombination between homoeologous A and C genome chromosomes is frequently observed in resynthesized material (Szadkowski *et al.*, 2010), there are no reports of nonhomoeologous translocation events occurring within the A genome of *B. napus*. Indeed, the *Rlm1* locus was previously mapped to the same A07 location in both *B. rapa* and in a resynthesized *B. napus* (Leflon *et al.*, 2007). The relationship between the *LepR3* and *Rlm1* resistance loci will be elucidated through the future fine mapping and study of the genomic region surrounding the *Rlm1* gene. Meanwhile, we may be able to determine if the protein products of the redundant *R*-genes recognize the same or different epitopes of AVRLM1 through the study of naturally occurring or induced mutants. In this way we may be able to identify a version of *AvrLm1* that confers avirulence to *LepR3* and not *Rlm1*, similar to *AvrLm7*, a mutated allele of *AvrLm4-7* which triggers *Rlm7* but not *Rlm4* (Parlange *et al.*, 2009). However, at present, we have no evidence for such a gene.

While direct *R*-gene–*Avr* interactions have been demonstrated in a few plant–pathogen interactions (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006), other evidence has suggested that *R*-genes are not always triggered by direct interaction with pathogen effectors. The 'guard hypothesis' (reviewed in Jones & Dangl, 2006) suggests many *R*-genes are triggered indirectly by recognizing the disruption of other host cellular targets by pathogen effectors. In this scenario the *R*-protein is likely to be part of a multiprotein complex involved in monitoring the basal defense machinery of the cell and in triggering defense signaling by modifying the stability of the *R*-protein (reviewed in Belkhadir *et al.*, 2004). Given that *LepR3* codes for a protein with extracellular LRRs, it would seem likely that it would detect a fungal elicitor present outside of the cell, produced by the invading *L. maculans* during infection, suggesting a direct interaction. However, in studies with the well-characterized *Cf* RLP gene family of tomato, which is structurally similar to *LepR3*, no direct interaction between the RLPs and their corresponding AVR proteins has been detected. This suggests that the *Cf* proteins act as guards of the avirulence targets and interact with the AVR proteins in an indirect manner (Kruijt *et al.*, 2005). For example, recognition of

C. fulvum Avr2 effector by the Cf2 protein is mediated by the tomato cysteine protease Rcr3. Avr2 is a cysteine protease inhibitor that binds and inhibits Rcr3 (Rooney *et al.*, 2005). However, unlike many fungal effectors, which are cysteine-rich, AVRLM1 only contains one cysteine residue. Cysteine-rich effectors are believed to withstand plant proteases, which are secreted into the host apoplastic space. Based on this, Gout *et al.* (2006) suggested that AVRLM1 may be localized to the host cytoplasm. Cloning of *LepR3* and future determination of its protein interactions will help in elucidating the function and potential host target of AVRLM1.

Our results, demonstrating that *AvrLm1* confers avirulence to *LepR3*, also offer a possible explanation as to the rapid loss of effective *LepR3* resistance in *B. napus* material, including 'Surpass 400′, in some parts of Australia soon after it was deployed (Sprague *et al.*, 2006). Given that *Rlm1* varieties were already in use in Australia before the release of 'Surpass 400′ (Rouxel *et al.*, 2003) and that the 'breakdown' of resistance was coincident with a large decrease in *AvrLm1* allele frequency (Van de Wouw *et al.*, 2010) it is possible that some populations of *L. maculans* had been driven towards a high proportion of virulent *avrLm1* pathotypes because of previous exposure to *Rlm1*. This could have effectively enriched entire populations of the pathogen for virulence on *LepR3*, drastically reducing the gene's effective lifespan in these areas.

Van de Wouw *et al.* (2009) showed the *L. maculans* avirulence gene '*AvrLmS*' triggers a second *AvrLm1*-independent *R*-gene in 'Surpass 400' referred to as '*RlmS*'. Our results, demonstrating that the *avrLm1* isolate '99-53' induces a resistance response from 'Surpass 400' but not *Rlm1* lines or *LepR3*-transgenic 'NLA8' plants, corroborates the presence of both *AvrLmS* in *L. maculans* and *RlmS* in 'Surpass 400'. This is also supported by the findings of a recent survey of western Canadian *L. maculans* isolates where 97.9% of the '*avrLm1*' isolates tested still produced a resistance response on 'Surpass 400' (Kutcher *et al.*, 2010). It also shows that the *AvrLmS* does not complement *LepR3*. In light of this evidence we believe the correct designation for the resistance genotype of 'Surpass 400' should be '*LepR3*, *RlmS*'. Mapping of the *RlmS* resistance locus and study of its interaction with *AvrLmS* isolates is currently in progress. In another recent report, 'Surpass 400' resistance was once again investigated and two resistance loci (named '*Blm1*' and '*Blm2*') were mapped to *B. napus* chromosome A10 (Long *et al.*, 2011) using the *L. maculans* isolate '87-41', which we have shown also carries *AvrLm1* (Table 1). The *Blm1* locus reported in that study corresponds to the same *B. rapa* BAC (KBrB080E24) described here for cloning the *LepR3* gene. As the *Blm1* resistance locus was mapped from the same cultivar, corresponds to the same genomic location and produces a resistance response to an *AvrLm1* isolate we believe that it is *LepR3*.

Our results highlight the need to consider redundancy in *R*-gene specificities when using differential isolate sets to determine the *R*-gene content of *Brassica* lines. *Rlm1* and *LepR3* cannot currently be distinguished by differential phenotypic reactions; one needs to either determine the chromosomal linkage of the resistance phenotype or amplify and sequence the *LepR3/lepR3* locus in order to discriminate between the presence of the two genes. We should not rely on a literal interpretation of the 'gene-for-gene' hypothesis, where any given avirulence protein only ever interacts with one specific *R*-protein.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Infection phenotype scoring guide.

Fig. S2 Alignment and translation of 'Surpass 400' *LepR3* and 'Topas DH16516' *lepR3* loci.

Table S1 Primer sequences

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