Leptosphaeria maculans Effector Protein AvrLm1 Modulates Plant Immunity by Enhancing MAP Kinase 9 Phosphorylation

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HIGHLIGHTS
Leptosphaeria maculans effector AvrLm1 interacts with the Brassica napus MPK9 (BnMPK9)
AvrLm1 increases the accumulation and enhances the phosphorylation of BnMPK9
AvrLm1 enhances BnMPK9-dependent cell death in Nicotiana benthamiana
Stable expression of BnMPK9 in B. napus facilitates L. maculans infection

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Leptosphaeria maculans Effector Protein AvrLm1 Modulates Plant Immunity by Enhancing MAP Kinase 9 Phosphorylation

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SUMMARY
Leptosphaeria maculans, the causal agent of blackleg disease in canola (Brassica napus), secretes an array of effectors into the host to overcome host defense. Here we present evidence that the L. maculans effector protein AvrLm1 functions as a virulence factor by interacting with the B. napus mitogen-activated protein (MAP) kinase 9 (BnMPK9), resulting in increased accumulation and enhanced phosphorylation of the host protein. Transient expression of BnMPK9 in Nicotiana benthamiana induces cell death, and this phenotype is enhanced in the presence of AvrLm1, suggesting that induction of cell death due to enhanced accumulation and phosphorylation of BnMPK9 by AvrLm1 supports the initiation of necrotrophic phase of L. maculans infection. Stable expression of BnMPK9 in B. napus perturbs hormone signaling, notably salicylic acid response genes, to facilitate L. maculans infection. Our findings provide evidence that a MAP kinase is directly targeted by a fungal effector to modulate plant immunity.

INTRODUCTION
Fungal pathogens of crop plants cause significant yield losses and affect global food security, particularly in the face of increased demand due to a growing world population (Fisher et al., 2012). Diverse lifestyle, genome plasticity and evolution, prolific reproduction, and longevity of spores under harsh environment impede the efficient control of fungal diseases. Like bacterial and oomycete plant pathogens, fungi also secrete small proteins, known as effectors, which counter plant defense and modulate plant physiology to promote pathogen growth and reproduction (Presti et al., 2015; Stergiopoulos and de Wit, 2009). Accumulating evidences show that the secreted effectors are key players in suppressing pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI) induced upon fungal recognition by plant (Presti et al., 2015). As an example, the lysine motif (LysM) effector ECP6 secreted by the tomato pathogen Cladosporium fulvum suppresses chitin triggered immunity. ECP6 binds chitin via the intramolecular chitin-binding groove formed by the LysM domains, resulting in the sequestering of chitin released from the cell walls of invading hyphae (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). Plants, on the other hand, have evolved disease resistance (R) genes encoding receptor proteins that detect and respond to pathogen effector proteins, leading to the activation of effector-triggered immunity (ETI) (Jones and Dangl, 2006). Effectors that trigger ETI are referred to as avirulence (Avr) genes. Discovery of the host targets of effectors is essential for our understanding of effector activity and molecular mechanisms of plant defense (Win et al., 2012). There are many examples of plant proteins that are targeted by bacterial effectors secreted by the type III secretion system (Block and Alfano, 2011; Deslandes and Rivas, 2012). However, despite the economic importance of diseases caused by the fungal plant pathogens, for most fungal effectors, the host targets and underlying molecular mechanisms remain unknown.

Mitogen-activated protein (MAP) kinase cascades are highly conserved signaling modules in eukaryotes and have a central role in plant immunity against pathogen attack (Meng and Zhang, 2013; Pitzschke et al., 2009). A MAP kinase cascade is commonly composed of three types of kinases including a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MKK), and a MAP kinase (MPK). In Arabidopsis, pathogen-responsive MPK signaling has been reported to be involved in both PTI and ETI, in which the best characterized MPKs are MPK3, MPK4, and MPK6 (Asai et al., 2002; Rasmussen et al., 2012). PAMPs, such as flg22 (a conserved 22-amino acid flagellin peptide) and elf18 (elongation factor-Tu peptide), activate the kinase signaling cascades involving MPK3, MPK4, and MPK6 (Felix et al., 1999; Gao et al., 2008;
Zipfel et al., 2006). In contrast, plant pathogens have evolved mechanisms to target the MPK signaling pathways to enhance plant susceptibility. For example, *Pseudomonas syringae* HopA11 targets MPK3 and MPK6 and inactivates their kinase function to suppress plant defense responses (Zhang et al., 2007). *P. syringae* HopF2 targets MKKS and can inactivate MKKS via ADP-ribosylation of the C terminus of MKKS in vitro (Wang et al., 2010). AvrB is a *P. syringae* effector that interacts with MPK4 to perturb hormone signaling and promote infection (Cui et al., 2019). *Phytophthora infestans* RXLR effector PexRD2 as a virulence factor interacts with the kinase domain of the host MAPKKK to suppress MAPKKK-dependent phosphorylation of MPKs to modulate plant immunity (King et al., 2014). These findings highlight the importance of MPK pathways in plant immunity and as targets of bacterial and oomycete pathogen effectors. However, to date no MAPKs have been identified as targets of effectors from fungal plant pathogens.

Plant MPKs can be classified into four groups (A, B, C, D) based on the conserved amino acid sequences of the Txy motif present in the activation loop of MPKs (Ichimura et al., 2002). Among the best studied MPKs, MPK3 and MPK6 are in group A and MPK4 belongs to group B. However, MPK9 belongs to the D group with the TxY motif present in the activation loop of MPKs (Ichimura et al., 2002). Among the best studied MPKs, MAPKKs have been identified as targets of effectors from fungal plant pathogens.

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*Leptosphaeria maculans*, the fungal agent of blackleg disease (phoma stem canker), causes major yield loss on *Brassica napus* (canola/rapeseed) crops worldwide (West et al., 2001). During infection it remains extracellular and exhibits a range of modes of infection from biotrophy to necrotrophy on Brassica hosts. Genome-wide transcriptomic analyses in the *B. napus–L. maculans* pathosystem revealed that the majority of known and predicted effectors had no expression during in vitro culture but were highly up-regulated during infection, supporting their roles as virulence factors (Haddadi et al., 2016). Resistance against *L. maculans* at the cotyledon stage is race specific. So far, 19 race-specific resistance (R) genes have been reported from *Brassica* species (Larkan et al., 2016), but only two R genes, *LepR3* and *Rlm2*, have been cloned, both encoding membrane-localized receptor-like proteins (RLPs) (Larkan et al., 2013, 2015). LepR3 perceives *L. maculans* AvrLm1 and triggers Brassica defense, leading to hypersensitive response (HR) at the site of infection (Larkan et al., 2013). Transient expression of both LepR3 and AvrLm1 induces HR in *Nicotiana benthamiana* leaves, and SOBIR1 and BAK1 receptor-like kinases (RLK), two components of LepR3 complex, are required for the perception of AvrLm1 (Ma and Borhan, 2015). To date, seven Avr genes (AvrLm1, 2, 3, 4–7, 5–9, 6, and 11) have been cloned from *L. maculans*, all encoding cysteine-rich proteins, except for AvrLm1, which contains only one cysteine residue (Gout et al., 2006). Cysteine enrichment is a feature found in most of the effectors of apoplastic fungi such as *L. maculans* and is believed to protect the effector protein, by formation of disulfide bonds, from plant proteases released into the apoplastic space during pathogen invasion (van den Burg et al., 2003). Lack of cysteine enrichment in the AvrLm1 protein could indicate that AvrLm1 is translocated inside the plant host cells. Given that LepR3 is a cell surface receptor, recognition of AvrLm1 by LepR3 likely occurs in the apoplast (Gout et al., 2006; Larkan et al., 2013; Ma and Borhan, 2015). However, it is still possible that AvrLm1 is translocated inside the plant cells to modulate host immunity. Owing to technical challenges, host cellular location for a vast majority of effector proteins of fungal and oomycete plant pathogens has not been determined and localization of pathogen effectors in the host plant is often inferred from their structure and their plant target proteins.

Although AvrLm1 was cloned a decade ago, the host target and molecular mechanism underlying AvrLm1 virulence have remained unknown. In this study, we report the identification of MPK9 as a novel target of *L. maculans* effector AvrLm1. Stable expression of *BnMPK9* in *B. napus* enhances the growth of *L. maculans*
by suppressing the salicylic acid (SA) pathway, suggesting that BnMPK9 negatively regulates plant defense. Our findings indicate that AvrLm1 as a virulence factor functions by stabilizing BnMPK9 and inducing its phosphorylation, leading to enhanced cell death and plant susceptibility to the hemibiotrophic pathogen *L. maculans*.

**RESULTS**

**AvrLm1 as a Virulence Factor Modulates Plant Hormone Signaling**

Effectors contribute to fungal virulence by protection of fungal structures and modulation of host metabolism. Comparison of near-isogenic *L. maculans* isolates with and without the effector AvrLm1 suggested that isolates carrying AvrLm1 were more aggressive on susceptible *B. napus* cultivars (Huang et al., 2009). To further examine the virulence function of AvrLm1, we generated transgenic Arabidopsis Columbia-0 (Col-0) lines carrying AvrLm1 in which expression of the full-length AvrLm1 gene was driven by the constitutive CaMV 35S promoter. A homozygous single-insertion line was generated (Col-AvrLm1), and expression of AvrLm1 was confirmed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) (See Figure S1). Expression of SA, jasmonic acid (JA), ethylene (ET), and ABA signaling response marker genes (*PR1*, *LOX2*, *ABF2*, and *PR4*) in Col-AvrLm1 and Col-0 plants were compared. Expressions of *PR1* and *LOX2* were significantly decreased in Col-AvrLm1, whereas expressions of *ABF2* and *PR4* were the same as in Col-0, indicating that AvrLm1 suppresses SA and JA signaling but does not affect the ABA and ET pathways (Figure 1). Col-AvrLm1 plants were challenged with the bacterial pathogen *Pst* DC3000. Col-0 was also inoculated with *Pst*-DC3000 as a control. As shown in Figure 2, Col-AvrLm1 showed enhanced disease symptoms (severe chlorosis) and higher bacterial growth compared with the Col-0 control. These findings indicate that AvrLm1 functions as a virulence factor by suppressing plant defense.

**AvrLm1 Is Secreted into the Apoplast of Brassica upon L. maculans Infection**

*L. maculans* resides in the plant extracellular space during *Brassica* infection. To monitor AvrLm1 during *L. maculans* infection, AvrLm1 driven by its native promoter was tagged with the fluorescent protein...
mCherry and transferred to L. maculans isolate 3R11 (3R11:AvrLm1-mCherry). An additional transgenic isolate carrying the fluorescent gene YFP driven by a fungal constitutive promoter was generated to serve as a control. To ensure that addition of mCherry tag to AvrLm1 did not disrupt its Avr function (being recognized by LepR3) or the overall fitness of 3R11 isolate, the 3R11:AvrLm1-mCherry was inoculated on the susceptible B. napus cultivar Topas DH16516 and the Topas:LepR3 transgenic line NLA8-2 (Larkan et al., 2013). The 3R11:AvrLm1-mCherry was virulent on Topas DH16516 (see Figure S2). As anticipated, the transgenic strain 3R11:AvrLm1-mCherry was avirulent on the LepR3-transformed NLA8-2 line, confirming that addition of mCherry tag did not interfere with the function of AvrLm1 (Figure S2). Subsequently, 3R11:AvrLm1-mCherry was inoculated on the host Topas DH16516 plants for the localization study. Infected cotyledons of Topas DH16516 were examined by confocal microscopy, and red fluorescent signals of the tagged AvrLm1 were detected along the cell wall of the hyphae. Intense focal fluorescent spots were observed where intercellular hyphae were in contact with the host mesophyll cells. In contrast, yellow fluorescent signals in the control 3R11-YFP were more uniformly observed throughout the fungal hyphae (Figure 3). We were not able to detect AvrLm1-mCherry inside the cell or apoplastic space of the host infected tissue. However, accumulation of mCherry signals along the hyphal cell wall and particularly focal accumulation where hyphae were in the vicinity of host cells could be an indication of localized AvrLm1 secretion into the apoplast or accumulation within the cytoplasm of the host plant cells.

To further investigate if AvrLm1-mCherry protein accumulates in the apoplast, we extracted the apoplastic fluids from L. maculans-infected cotyledons at 4 days post-inoculation (dpi). Apoplastic fluid was probed with anti-mCherry antibody, and AvrLm1-mCherry fusion protein was detected from the apoplastic fluids isolated from the infected plants (Figure S3). In conclusion, the localization of AvrLm1-mCherry on the surface of the fungal hyphae and detection of secreted AvrLm1-mCherry proteins in the apoplastic fluids indicate that AvrLm1-mCherry is very likely secreted into the apoplast.

AvrLm1 Physically Associates with BnMPK9

To investigate the molecular mechanism underlying the AvrLm1 virulence function, yeast two-hybrid screening was performed using AvrLm1 without signal peptide (ΔspAvrLm1) as a bait against a prey cDNA library prepared from young Arabidopsis seedlings. Four AvrLm1-interacting candidate proteins from Arabidopsis were identified including EBF1 (EIN3-binding F box protein 1), MPK17, MPK9, and T21P5.2 (hypothetical protein) (see Table S1). To validate the interaction for the candidate proteins, ΔspAvrLm1 was used as the bait against each of the protein product of Arabidopsis AtEBF1, AtMPK17, AtMPK9, and AtT21P5.2 with full-length open reading frames as the respective preys. Among the four candidates only full-length AtMPK9 (At3G18040) was validated as interacting with AvrLm1 (Figure S4). BLAST search against the genome of B. napus Darmor-bzh (Chalhoub et al., 2014) identified two Brassica homologs of AtMPK9, BnaA05g22170D and BnaCnng17720D, residing in the A and C genomes of B. napus, respectively. Protein sequence alignment (Figure S5) identified that both shared the same level of homology with AtMPK9. Therefore only one gene, BnaCnng17720D (hereafter referred to as BnMPK9), was chosen for further analysis. Interaction between AvrLm1 and BnMPK9 was confirmed by yeast two-hybrid assay.
(Figure 4A). We next confirmed this interaction with bimolecular fluorescence complementation (BiFC) in planta. To generate the BiFC constructs, pENTR/Zeo:BnMPK9 and pENTR/Zeo:DspAvrLm1 were recombined into the binary vectors pDEST-GW-VYCE and pDEST-GW-VYNE. BiFC assay was performed by transiently co-expressing DspAvrLm1-VYNE or BnMPK9-VYNE with BnMPK9-VYCE in N. benthamiana leaves. As shown in Figure 4B, a strong yellow fluorescent signal was observed in cells co-expressing AvrLm1-VYCE and BnMPK9-VYNE, but not with the negative control (BnMPK9-VYNE and BnMPK9-VYCE). To further validate this association in vivo, the co-immunoprecipitation assay was performed upon transient expression of HA-BnMPK9 and AvrLm1-GFP constructs in N. benthamiana. Hemagglutinin (HA)-tagged BnMPK9 co-immunoprecipitated with green fluorescence protein (GFP)-tagged AvrLm1, and vice versa (Figures 4C and S6). Taken together, these observations support that BnMPK9 is the host target for AvrLm1.

AvrLm1 Specifically Interacts with BnMPK9 and Its Small N-Terminal Region Is Dispensable for the Interaction

It has been reported that Arabidopsis MPK9 and MPK12 positively regulate ABA, SA, and MeJA signaling in Arabidopsis (Jammes et al., 2009; Khokon et al., 2017) and MPK9 and MPK12 are functionally redundant. Two other Arabidopsis MPKs, AtMPK3 and AtMPK6, are well known to be involved in plant immunity (Asai et al., 2002). To assess whether AvrLm1 specifically interacts with BnMPK9, we tested the interaction of AvrLm1 with either AtMPK12 or BnMPK3 in yeast two-hybrid (Y2H) assay. Neither AtMPK12 nor BnMPK3 interacted with AvrLm1, confirming the specificity of interaction between AvrLm1 and BnMPK9 (Figure S7).

To define which region of AvrLm1 is required for the interaction with BnMPK9, three N-terminally and one C-terminally truncated AvrLm1 proteins were generated based on the predicted AvrLm1 secondary structure (Ma and Borhan, 2015). Four variants were constructed: AvrLm1-Δ40, AvrLm1-Δ73, AvrLm1-Δ108, and CTΔ14. Only AvrLm1-Δ40 interacted with BnMPK9 (Figure 5A), indicating that a small (40-amino acid [aa]) N-terminal region of AvrLm1 is not required for the interaction but the remaining region of AvrLm1 is indispensable for its interaction with MPK9. To further define the region of BnMPK9 that is required to interact with AvrLm1, we generated three truncations of BnMPK9 that were used for Y2H with AvrLm1. One of them carrying a small N-terminal deletion region (95 aa) enabled the growth of yeast on selective plates, indicating that deletion of up to 95 N-terminal amino acid from the BnMPK9 protein does not disrupt its interaction with AvrLm1 (Figure 5B).
AvrLm1 Enhances BnMPK9-Dependent Cell Death

Transient expression in tobacco leaves has been extensively used to rapidly establish gene function. For instance, transient expression of tomato LeMKK4 and MAPKKKa, N. benthamiana MKK1, and B. napus MAPKKK4, MAPKKK18, and MAPKKK19 in tobacco leaves causes HR-like cell death (del Pozo et al., 2004; Li et al., 2015; Pedley and Martin, 2004; Sun et al., 2014; Takahashi et al., 2007). BnMPK9 was transiently expressed in N. benthamiana leaves via agroinfiltration. HR-like cell death developed extensively by 5 days after BnMPK9 infiltration, whereas no HR was observed with the control BnMPK3 (Figure 6A).

To examine whether BnMPK9-dependent cell death is regulated by its phosphorylation activity, a mutated version of BnMPK9 was generated, in which the predicted TDY phosphorylation sites were mutated (BnMPK9-mTDY). As shown in Figure 6A, BnMPK9-mTDY failed to induce cell death when infiltrated in tobacco leaves, indicating that phosphorylation activity is required for the BnMPK9-induced cell death.

AvrLm1 Stabilizes Accumulation and Enhances BnMPK9 Phosphorylation

Based on the presented evidences that AvrLm1 interacts with BnMPK9 and enhances BnMPK9-induced cell death, we investigated the stability and level of accumulation of BnMPK9 in the presence of AvrLm1.
protein. HA and StrepII tags were added to BnMPK9 and DspAvrLm1, respectively. BnMPK9-HA was co-expressed in N. benthamiana with DspAvrLm1-StrepII or GFP (negative control) using agroinfiltration. As shown in Figure S8, BnMPK9-HA accumulated to a higher level in the presence of DspAvrLm1-StrepII compared with the GFP control. The ubiquitin/26S proteasome pathway is the major proteolysis machinery in eukaryotic cells, removing most abnormal peptides and short-lived cellular regulators (Sadanandom et al., 2012). We examined and compared the stability and accumulation of BnMPK9 in the presence or absence of MG132, a proteasome inhibitor. N. benthamiana leaves co-expressing BnMPK9-HA and DspAvrLm1-StrepII or GFP were pre-treated with proteasomal inhibitor MG132. MG132 application resulted in increased accumulation of BnMPK9 in the leaves of control treatment (co-expressing BnMPK9-HA and GFP), to a similar level of accumulation of BnMPK9 that was observed in the leaves co-expressing BnMPK9-HA and DspAvrLm1-StrepII (Figure 7A). However, to ensure that overexpression of AvrLm1 does not interfere with the MPK9 expression level, we measured the AtMPK9 expression in wild-type Col and Col-AvrLm1 transgenic lines. AtMPK9 expression profile in Col plants overexpressing AvrLm1 was similar to the Col wild-type (Figure S9), proving that the effect of AvrLm1 on MPK9 is only at the protein level. Taken together, these observations suggest that AvrLm1 stabilizes BnMPK9 by preventing its degradation via the ubiquitin/26S proteasome pathway.

It has been demonstrated that the TDY phosphorylation site of BnMPK9 is essential for its cell death function. Thus, we hypothesized that AvrLm1 might affect the phosphorylation of BnMPK9. HA-tagged BnMPK9 was co-expressed with DspAvrLm1-StrepII in N. benthamiana leaves. BnMPK9 and GFP co-expression served as a negative control. HA-magnetic beads were used to immunoprecipitate transiently expressed BnMPK9 from the N. benthamiana leaves in the presence of MG132. The level of phosphorylation of BnMPK9 was determined by immunoblot using anti-phosphothreonine monoclonal antibodies. As shown in Figure 7B, the AvrLm1
transgene specifically enhanced the phosphorylation of BnMPK9. BnMPK9 purified from N. benthamiana leaves infiltrated with Agrobacterium carrying BnMPK9 or truncated BnMPK9 encoding the kinase domain (BnMPK9-KD) or mutated TDY motif (BnMPK9-mTDY). BnMPK3 was included as a control. Representative pictures were taken 5 days post-infiltration. Overexpression of BnMPK9 Enhances Production of Hydrogen Peroxide in Brassica napus

Since AvrLm1 stabilizes BnMPK9 accumulation and enhances its phosphorylation, we examined the effect of BnMPK9 in response to L. maculans infection. We initially performed RNA interference (RNAi) silencing to knockdown the expression of BnMPK9 in Topas DH16516. Despite several attempts, we failed to generate the transgenic B. napus plant carrying the BnMPK9 RNAi construct. Failure to generate MPK9 knockouts in Arabidopsis has also been reported (Jammes et al., 2009). However, we were successful in generating BnMPK9 overexpression B. napus lines. Three homozygous single-insert B. napus Topas

Figure 6. AvrLm1 Enhances the Development of BnMPK9-Dependent Cell Death
(A) BnMPK9 triggers cell death, and the plant-specific TDY phosphorylation motif is required for the induction of cell death. N. benthamiana leaves were infiltrated with Agrobacterium carrying BnMPK9 or truncated BnMPK9 encoding the kinase domain (BnMPK9-KD) or mutated TDY motif (BnMPK9-mTDY). BnMPK3 was included as a control. Representative pictures were taken 5 days post-infiltration. (B) Co-expression of AvrLm1 and BnMPK9 enhanced the development of BnMPK9-induced cell death. N. benthamiana leaves co-infiltrated with Agrobacterium strains carrying AvrLm1 and BnMPK9 or GFP. The representative pictures were taken 3 and 4 days post-infiltration.
DH16516 lines in which the BnMPK9 expression was driven by the 35S promoter were generated. Overexpression of BnMPK9 (15–20 fold) in these lines was confirmed by qRT-PCR (Figure S10). As described above, transient expression of BnMPK9 induces cell death in N. benthamiana plants (Figure 6); however, transgenic B. napus lines overexpressing BnMPK9 grew normally and did not display any visual cell death symptoms. High accumulation of hydrogen peroxide has been used as an indicator of cell death. To detect the H2O2 production, BnMPK9-overexpressing lines were stained by 3,3-Diaminobenzidine (DAB). Interestingly, we observed pronounced staining for hydrogen peroxide in the cotyledons of BnMPK9-overexpressing lines. By contrast, the cotyledon of control line Topas DH16516 showed only weak staining (Figure 8). To further confirm this observation, we measured the production of hydrogen peroxide in cotyledons infected with L. maculans isolate v23.1.3. The intensively stained area, due to H2O2 production, was much larger in cotyledons overexpressing BnMPK9 than in control plant (Figure 8). Taken together, it could be concluded that overexpression of BnMPK9 enhances the production of hydrogen peroxide, while no cotyledon of transgenic lines shows any macroscopically visible cell deaths.

Overexpression of BnMPK9 Enhances L. maculans Growth in Brassica napus
Expression of SA marker genes PR1 and WRKY70, and JA-related genes LOX3 and AOS, was measured by qRT-PCR in Topas DH16516 overexpressing BnMPK9 and the control Topas DH16516 lines. PR1 and WRKY70 expression levels were reduced to 70% and 30% compared with the control, respectively. In contrast, the expression levels of LOX3 and AOS were slightly elevated in the BnMPK9 overexpression lines (Figure 9). Two BnMPK9 transgenic lines were inoculated with the L. maculans isolate v23.1.3. Topas DH16516 that is susceptible to L. maculans was included as a positive control. At 8 dpi enhanced disease development was evident as formation of larger lesion on the cotyledons of Topas:BnMPK9 compared with the control (Figures 10A and 10B). Next, disease development was monitored over time. Asexual spore (pycnidiospore) formation, seen as black dots formed within the lesion, was more prevalent in the BnMPK9 overexpression lines at 10, 12, and 14 dpi (Figure 10A). Furthermore, the amount of fungal DNA in the cotyledons of BnMPK9-overexpressing and wild-type Topas lines was quantified by quantitative PCR (qPCR) analysis of L. maculans’ LmITS1 DNA at 8 dpi. Higher levels of L. maculans DNA in the...
Topas: BnMPK9 provided additional support that overexpression of BnMPK9 enhances the susceptibility of B. napus to L. maculans (Figure 10C).

**DISCUSSION**

B. napus resistance at the seedling stage to L. maculans is conferred by race-specific resistance genes. Despite significant advances in defining the genetics of the B. napus-L. maculans interaction, including isolation of two R genes and several Avr genes (Raman et al., 2013), up to now information about the molecular interaction and function of R/Avr proteins for the B. napus-L. maculans pathosystem has been lacking. The study presented here is the first report that describes the virulence function of an L. maculans effector by identifying its target in B. napus. We identified BnMPK9 as the host target of AvrLm1 and determined their interaction both in vitro and in planta. The BnMPK9-AvrLm1 interaction enhances cell death caused by BnMPK9, and mutation within kinase domain of BnMPK9 abolishes cell death. We further showed that AvrLm1 stabilizes the accumulation and facilitates the phosphorylation of BnMPK9, leading to the development of enhanced cell death in tobacco. Furthermore, overexpression of BnMPK9 perturbs hormone signaling pathways and enhances the growth of L. maculans in B. napus. Together, these results uncover a novel role for BnMPK9 in plant defense and its modulation by a fungal effector, causing enhanced plant susceptibility.

L. maculans is an intercellular pathogen, and not surprisingly, the LepR3 protein, the B. napus receptor for AvrLm1, is a membrane-bound RLP. To gain insight into the localization of AvrLm1, we tracked the AvrLm1-mCherry fusion protein expressed in L. maculans during infection of B. napus cotyledon tissues. Although we could not detect mCherry fluorescent signals inside the host plant cells, we frequently observed accumulation of mCherry signals at the focal points of contact between the fungal hyphae and plant host mesophyll cells. In addition, proof of function of AvrLm1 signal peptide in yeast (Figure S11) and enrichment of AvrLm1 protein in the apoplastic fluid of B. napus tissue infected with L. maculans (Figure S3) provided further evidence that L. maculans secretes AvrLm1 into the host apoplast. AvrLm1 encodes a protein with one cysteine residue, which is atypical for the commonly reported apoplastic fungal effector proteins (Fudal et al., 2007), indicating that AvrLm1 may enter into plant cell to avoid the harsh environment of the apoplast. Therefore, lack of discernible red fluorescent signals inside the host cell may be due to the rapid diffusion of AvrLm1-mCherry protein, reducing fluorescent signals to a level below our detection limits. Similar technical limitations have been reported for the translocation studies of fluorescently labeled effectors from other filamentous pathogens such as the RXLR effectors from oomycetes or effectors from Colletotrichum higginsianum (Jing et al., 2016; Kleemann et al., 2012). Alternative localization techniques reported recently, such as the split fluorescent GFP system, may overcome these technical limitations (Henry et al., 2017; Park et al., 2017).

The physical interaction between AvrLm1 and BnMPK9 results in increased accumulation and enhanced phosphorylation of BnMPK9 (Figure 7). So far, there has been no report of a fungal effector directly targeting the MAPK pathway. The only example from filamentous pathogens is the P. infestans RXLR effector PexRD2, which has been reported to interact with host MAPKKKs and suppress MAPKKKs-dependent cell death to perturb plant immune signaling (King et al., 2014). However, accumulating evidences from
studies on a variety of bacterial pathogens support that manipulation of components of MAPK kinase signaling cascade is an important virulence strategy of bacterial pathogens (Bigeard et al., 2015; Feng and Zhou, 2012). Two known MAPK modules, MKK4/MKK5-MPK3/MPK6 and MEKK1-MKK1/MKK2-MPK4 involved in plant defense signaling, are targeted by different bacterial effectors either to inactivate or enhance their kinase activities. For example, HopAI1 inactivates AtMPK3, AtMPK4, and AtMPK6 by irreversibly removing the phosphate group from the threonine residue of the MAPK activation loop (Zhang et al., 2007). P. syringae effector HopF2 targets AtMKK5, and the P. syringae effector AvrB interacts with AtMPK4 and induces the phosphorylation and activation of MPK4 (Cui et al., 2010; Wang et al., 2010). Our results provide the first example that a fungal pathogen also targets MAPK modules to manipulate plant defense response. The RLK proteins, SOBIR1 and BAK1, are components of the LepR3 receptor complex and are required for the recognition of AvrLm1 (Ma and Borhan, 2015), indicating the importance of MAPK signaling cascade downstream of the LepR3 receptor complex.

Previous studies showed that AtMPK9, together with AtMPK12, functions in ABA- and methyl jasmonate-induced stomatal closure in Arabidopsis (Lee et al., 2016; Jammes et al., 2009; Khokon et al., 2015) and that they act redundantly. However, our data show that AvrLm1 does not interact with MPK12, proving the specificity of AvrLm1-MPK9 interaction (Figure S7) and indicating that in the Brassicaceae, MPK12 is dispensable for the virulence function of AvrLm1. Furthermore, transient overexpression of BnMPK9 in N. benthamiana resulted in cell death. However, we did not detect any visible cell death phenotype when BnMPK12 was overexpressed in tobacco (Figure 6). These observations indicated that MPK9’s function in inducing cell death and mediating L. maculans virulence in B. napus is independent of MPK12. The importance of MPKs in regulating plant immune response is well documented (Pitzschke et al., 2009; Popescu et al., 2009). Overexpression of the N. benthamiana homologs of AtMPK3 and AtMPK6 and Arabidopsis

**Figure 9. Relative Expression of Salicylic Acid (SA) and Jasmonic Acid (JA) Marker Genes in BnMPK9-Overexpressing B. napus Lines**

Cotyledons of 7-day-old seedlings of BnMPK9-overexpressing B. napus plants were collected and used for RNA extraction. Expressions of B. napus signaling marker genes including PR1, WRKY70, LOX3, and AOS were determined by quantitative reverse-transcriptase polymerase chain reaction and normalized to B. napus actin. Values represent means ± standard error (SE) from three independent experiments. *, **, and *** represent the significant differences (p < 0.05, p < 0.01, and p < 0.001, respectively, one-way analysis of variance [ANOVA] followed by the Turkey post-test).
MKK4, MKK5, MKK7, and MKK9 cause cell death in tobacco (Popescu et al., 2009; Ren et al., 2006; Yang et al., 2001). Also, overexpression of *B. napus* MAPKKK4 causes up-regulation of genes related to reactive oxygen species accumulation, cell death, and defense response (Li et al., 2015). Plant cell death is an effective mechanism of containing the growth of biotrophic pathogens, whereas induction of cell death is a virulence mechanism employed by hemibiotrophic and necrotrophic fungi to more readily absorb nutrients from the host plant (Ding et al., 2011; Greenberg and Yao, 2004). Interestingly, previous study of the *B. napus* transcriptome in response to *L. maculans* infection revealed that the peak expression of AvrLm1 and NLP (necrosis- and ethylene-inducing protein) coincide with the transition from biotrophy to necrotrophy of *L. maculans* during *B. napus* cotyledon infection (Haddadi et al., 2016). Taken together, heightened expression of AvrLm1 by *L. maculans* leading to enhanced induction of cell death through the interaction of AvrLm1 with BnMPK9 could be perceived as a virulence mechanism supporting the transition from biotrophy to necrotrophy to facilitate the acquisition of host nutrients by *L. maculans*.

Our results show that in the BnMPK9 transgenic *B. napus* plants, transcription of PR-1 and WYKR70 is significantly reduced but expression levels of LOX3 and AOS are elevated, indicating suppression of the SA-dependent pathway and activation of the JA-dependent pathway. However, expression of LOX2 gene in AvrLm1-overexpressing Arabidopsis is down-regulated. This apparent discrepancy in LOX gene expression could be attributed to the suggested presence of a cytoplasm- and a chloroplast-localized pathway for JA biosynthesis. LOX2 is reported to be targeted to the chloroplast (He et al., 2002). Although LOX3 is possibly targeted to the chloroplast, its expression pattern is not necessarily similar to that of LOX2. For instance, it was reported that LOX3 was up-regulated during the leaf senescence, but expression of LOX2 was significantly down-regulated (He et al., 2002). Importance of SA and JA pathways during the *L. maculans*- *B. napus* interaction has also been previously reported (Becker et al., 2017; Haddadi et al., 2016). In *B. napus* cotyledon tissues infected with *L. maculans*, SA-related genes were induced at the initial

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**Figure 10. Expression of BnMPK9 Results in Increased Susceptibility to L. maculans**

Cotyledons of 7-day-old Topas DH16516 seedlings and two BnMPK9-expressing lines (BnMPK9-6B9 and BnMPK9-7A6) were inoculated on wound sites using a spore suspension of *L. maculans* isolate v23.1.3. (A) Representative cotyledons at 8, 10, 12, and 14 dpi were photographed. (B) The size of the lesions was measured at 8 dpi and statistically analyzed. Values represent means ± standard error (SE) (n > 30). Asterisks indicate statistically significant differences (***, p < 0.001, one-way analysis of variance [ANOVA]). Three independent biological experiments were conducted. (C) qRT-PCR analysis showing the fungal mass in *L. maculans*-infected cotyledons of *B. napus* at 8 dpi. Constitutively expressed LmITS1 gene was used as a marker showing DNA level and pathogen mass. LmITS1 gene expression levels were normalized to expression of *B. napus* actin. Values are means ± SE of triplicate reactions of three independent biological samples. Significant differences are represented by ** (**, p < 0.01, one-way ANOVA).
phases of infection (2-4 dpi), but as the infection progressed and at the initiation of necrotrophic stage, genes related to the JA pathway were up-regulated (Haddadi et al., 2016). Thus, we hypothesize that AvrLm1 as a virulence factor facilitates the phosphorylation of BnMPK9, leading to the suppression of SA-dependent pathway and supporting the colonization of L. maculans at the initial infection stage. AvrLm4-7, another L. maculans effector, was recently reported to act as a virulence factor by suppressing the host SA signaling pathway (Novákova et al., 2016). AvrLm1 and AvrLm4-7 also contribute to the aggressiveness of L. maculans during leaf infection (Huang et al., 2006, 2009).

To the best of our knowledge, this is the first study to show that an effector from a fungal plant pathogen targets the MPK pathway to suppress plant host defense. Our results show that AvrLm1 supports the L. maculans infection by stabilizing the accumulation and inducing the phosphorylation of BnMPK9. Owing to technical limitations for genetic manipulation and also the complexity of the B. napus genome, most of the experiments that were carried out in the present study had to be done using the model plants A. thaliana and N. benthamiana. These model plants have been extensively used in plant biology research and proved to be invaluable in the translation of such research to crop species. However, further experiments in B. napus will provide additional support for the role of AvrLm1 and its target BnMPK9 in defense against L. maculans in its the natural host plant.

Until recently the molecular interaction of B. napus with the economically important blackleg pathogen L. maculans remained largely unknown. The research presented here expands our knowledge of the Brassica-Leptosphaeria pathosystem and highlights the importance of the MPK pathway, adding to our recent advances in understanding the perception of L. maculans and induction of downstream signaling in B. napus.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Transparent Methods, 11 figures, and 2 tables and can be found with this article online at https://doi.org/10.1016/j.isci.2018.04.015.

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**AUTHOR CONTRIBUTIONS**

L.M. and M.H.B. designed the experiments and prepared the manuscript. L.M. conducted the majority of the experiments. M. D., H.W., N.J.L., P.H., E.B., and G.G. contributed to the experiments and read the manuscript. N.J.L. edited the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no conflict of interest.

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Supplemental Information

*Leptosphaeria maculans* Effector Protein

AvrLm1 Modulates Plant Immunity
by Enhancing MAP Kinase 9 Phosphorylation

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Supplemental Information

Figure S1. Related to figures 1 and 2. qPCR showing \textit{AvrLm1} expression in \textit{AvrLm1}-overexpressing Arabidopsis line. \textit{AvrLm1} gene expression level was normalized to Arabidopsis gene \textit{Ubiquitin}. Values represent means ± standard error (SE) from three independent experiments.
Figure S2. Related to figure 3. *L. maculans* 3R11-AvrLm1-mCherry causes disease on susceptible Topas DH16516, but not on Topas:LepR3 plants. Cotyledons of 7-day-old seedlings of susceptible *B. napus* Topas DH16516 and Topas:LepR3 (NLA8) lines were inoculated with a transgenic *L. maculans* 3R11 isolate carrying AvrLm1-mCherry. A). Images were taken from infected cotyledons of seedlings 10 days after inoculation. B). Average disease index representing data collected from 12 cotyledons of susceptible Topas DH16516 and Topas:LepR3 transgenic plants (NLA8).
Figure S3. Related to figure 3. AvrLm1-mCherry fusion protein is detected from apoplastic fluids isolated from cotyledons infected with *L. maculans* 3R11 carrying *AvrLm1-mCherry*. Cotyledons of 7-day-old seedlings of susceptible *B. napus* Topas DH16516 were inoculated with a transgenic *L. maculans* 3R11 isolate carrying *AvrLm1-mCherry* or 3R11. Apoplastic fluids isolated from cotyledons 4 days after inoculation were concentrated and probed with anti-mCherry antibody and expected protein band with molecular weight 48 KDa was detected.
Figure S4. Related to figure 4. Testing the interaction of AvrLm1 with full length of candidate genes obtained from yeast two hybrid screening. Four candidate genes as prey (P) including AtEBF1, AtMPK9, AtMPK17 and AtT21P5.2 were co-transformed with AvrLm1 as bait (B) or empty vector to yeast, respectively. All transformants were able to grow on SD-WL medium. Yeast were able to grow on selective medium (SD-HAWL+X-α-Gal) with development of blue coloration indicating a protein-protein interaction. Representative pictures were photographed 3 days after incubation at 30 °C.
Figure S5. Related to figure 4. Protein sequences alignment between AtMPK9 and the two homologous ones from *B. napus.*
Figure S6. Related to figure 4. Avrlm1 associates with BnMPK9. Co-immunoprecipitation of AvrLm1 and BnMPK9 from total plant protein extracts. GFP-tagged *AvrLm1* and HA-tagged BnMPK9 were co-expressed in *N. benthamiana*. Proteins were extracted after 48 h and subjected to immunoprecipitation by GFP-Trap beads. Immunoprecipitated proteins (IPs) were analysed by immunoblotting by probing with either anti-HA (α-HA) or anti-GFP (α-GFP).
Figure S7. Related to figure 4. AvrLm1 specifically interacts with BnMPK9.
Arabidopsis MPK12 (AtMPK12) or B. napus MPK3 (BnMPK3) or BnMPK9 was co-transformed with AvrLm1 to yeast. There were no interactions between AvrLm1 and AtMPK12 or BnMPK3.

Figure S8. Related to figure 7. AvrLm1 is able to stabilize the accumulation of BnMPK9 in planta. Western blot probed with anti-HA and anti-StrepII antibodies showing the stabilization of BnMPK9 in the presence of AvrLm1 in transient expression. HA-tagged BnMPK9 was co-expressed with StrepII-tagged AvrLm1 or GFP in N. benthamiana leaves using agroinfiltration. Proteins were extracted after 48 h post infiltration and subjected to western blotting.
Figure S9. Related to figure 7. qPCR showing \textit{AtMPK9} expression in \textit{AvrLm1} overexpressing Arabidopsis lines. \textit{AtMPK9} gene expression levels are normalized to Arabidopsis Ubiquitin gene. Values represent means ± standard error (SE) from three independent experiments.

Figure S10. Related to figures 8, 9 and 10. qPCR showing \textit{BnMPK9} expression in \textit{BnMPK9} overexpressing \textit{B. napus} lines. \textit{BnMPK9} gene expression levels are normalized to \textit{B. napus} actin gene. Values represent means ± standard error (SE) from three independent experiments.
Figure S11. Related to figures 3 and S3. Yeast signal sequence trap assay of the predicted signal peptide of AvrLm1 protein. The predicted signal peptide sequences plus the following two amino acids (1–24) of AvrLm1 and the negative control pSUC2 vector were used for the assay. CMD-W (minus Trp) plates were used to select yeast strain YTK12 carrying the pSUC2 vector. Sucrose and YPRAA media were used to indicate invertase secretion.
Table S1. Related to figure 4. Arabidopsis candidates from yeast two-hybrid screening.

| Gene names                                      | Accession number | Independent clones |
|-------------------------------------------------|------------------|--------------------|
| Arabidopsis thaliana EBF1 (EIN3-BINDING F BOX PROTEIN 1) | AT2G25490.1      | 28                 |
| Arabidopsis thaliana - MPK17                     | AT2G01450.1      | 32                 |
| Arabidopsis thaliana - MPK9                      | AT3G18040.1      | 30                 |
| Arabidopsis thaliana - T21P5.2 (hypothetical protein) | AT3G03560.1      | 18                 |
Table S2. Primers used in this study

| Primer names | Sequences |
|--------------|-----------|
| BnMPK9-FB    | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| BnMPK9-RB    | ACCACCTTGTGACAAAAAGCTGGTGCTACGATGCCACAC |
| YFP-FB       | ACAAGTTTGTGACAAAAAAGCTGGTGCTACGATGCCACAC |
| YFP-RB       | ACCACCTTGTGACAAAAAAGCTGGTGCTACGATGCCACAC |
| BnMPK9-NdeI  | GCGGCATATGAGATGGGTGCTACTCACAGCACCAAC |
| BnMPK9-KD-FB | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| BnMPK9-KD-RB | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| BnMPK9-EcoRI | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnMPK9-NdeI  | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AvrLm1-FB    | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| AvrLm1-RB-S  | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| AtEBF1-NdeI  | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtEBF1-EcoRI | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtMPK17-NdeI | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtMPK17-Xhol | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtMPK17-QF    | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtMPK17-QR    | GCCGGGAATTTCTCAGATGCCGAGCAG |
| ΔspAvrLm1-FB | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| ΔspAvrLm1-RB-S | ACAAGTTTGTACAAAAAAGCTGGTGCTACGATGCCACAC |
| AtUbiquitin-QF | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtUbiquitin-QR | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnActin-QF    | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnActin-QR    | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnPR1-QF      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnPR1-QR      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnLOX3-QF     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| BnLOX3-QR     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtUbiquitin-QF | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtUbiquitin-QR | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtUBI2-QF     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtUBI2-QR     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtPR1-QF      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtPR1-QR      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtLOX2-QF     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtLOX2-QR     | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtPR4-QF      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtPR4-QR      | GCCGGGAATTTCTCAGATGCCGAGCAG |
| AtABF2-QF     | GCCGGGAATTTCTCAGATGCCGAGCAG |
Transparent methods

Plant growth and infection conditions

The susceptible doubled-haploid (DH) *B. napus* cv. Topas DH16516, NLA8-2 (Topas: *LepR3*) and single-spore cultures of the *L. maculans* isolates v23.1.3 and 3R11 were used in this study. Plant growth and *L. maculans* infection conditions were described previously (Larkan et al., 2014). Briefly, seedlings were grown in a growth chamber at 20°C, 16 h days, and light intensity c. 450 µmol m$^{-2}$ s$^{-1}$ at bench level, and 18°C, 8 h nights. Pycnidiospore inoculum, (10 µL of $2 \times 10^7$ spores/mL suspensions) was placed on the wounded site of a cotyledon of 7-day-old seedlings (4 infection sites per seedling). After inoculation, the seedlings were kept in the same growth condition as described above.

Generation of transgenic *L. maculans* strains

AvrLm1-mCherry insert DNA with Gateway attB linkers was synthesized (GenScript, USA) and recombined into the Gateway entry clone pDONR/Zeo and then into the destination binary vector GW-pLM4 (Ma and Borhan, 2015) as instructed by the manufacturer (Invitrogen) resulting in the plasmid pLM4::AvrLm1-mCherry. The YFP gene was amplified with the primer set YFP-FB/YFP-RB using pEarlyGate101 as a template. YFP fragment was first recombined into the entry clone pDONR/Zeo and then into the binary vector pJU472 (Utermark and Borhan, unpublished) to obtain pJU472::YFP construct. pLM4::AvrLm1-mCherry and pJU472::YFP were transformed into Agrobacterium AGL1 and used for subsequent *Agrobacterium tumefaciens*-mediated transformation of pycnidiospores from the *L. maculans* isolate ‘3R11’ (avrLm1), as described by (Utermark and Karlovsky, 2008).

Binary vector constructions

For stable plant transformation, the *Brassica napus* MPK9 (*BnMPK9*) ORF was amplified using primers BnMPK9-FB and BnMPK9-RB (Table S2) from the *L. maculans-B. napus* cDNA library. The PCR product was introduced into entry clone pDONR/Zeo (Invitrogen) and then into the binary vector pEarlyGate100 (Earley et al., 2006). At the same time, the AvrLm1 coding sequences with or without signal peptide plus AttB gateway linker and C-terminal StrepII tag was synthesized (GenScript). The resulting plasmid pUC57:AvrLm1 and pUC:ΔspAvrLm1 were recombined, via entry vector pDONR/Zeo, followed by recombination into destination vector pEarlyGate100. pEarlyGate100:BnMPK9, pEarlyGate100:AvrLm1-StrepII and pEarlyGate100:ΔspAvrLm1-StrepII were used for agrotransformation.

For transient expression, full length *AvrLm1* was PCR-amplified from *L. maculans-B. napus* cDNA library using primers AvrLm1-FB and AvrLm1-RB. The truncated *AvrLm1* lacking the signal peptide sequence was amplified using primers ΔspAvrLm1-FB and AvrLm1-RB. The PCR products were introduced into entry clone pDONR/Zeo (Invitrogen) to obtain
pENTR/Zeo:AvrLm1 and pENTR/Zeo:ΔspAvrLm1. The kinase domain of BnMK9 was amplified with primer set BnMPK9-KD-F8/BnMPK9-KD-RB. pENTR/Zeo:AvrLm1 and pENTR/Zeo:ΔspAvrLm1 were recombined into the binary vector pEarlyGate100. The BnMPK9 insert encoding the protein with mutated TDY motif was synthesized (GenScript). pUC57:BnMPK9mTDY was recombined via entry vector pDONR/Zeo into pEarlyGate100. The resulting plasmids pEarlyGate100:AvrLm1, pEarlyGate100:BnMPK9-KD, pEarlyGate100:ΔspAvrLm1 and pEarlyGate100: BnMPK9mTDY were used for Agrobacterium-mediated transformation and transient assay.

To generate the BiFC constructs, pENTR/Zeo:BnMPK9 and pENTR/Zeo:ΔspAvrLm1 were recombined into the binary vectors pDEST-GW-VYCE and pDEST-GW-VYNE, respectively (Gehl et al., 2009). The resulting plasmids pDEST:BnMPK9-VYCE, pDEST:BnMPK9-VYNE and pDEST: ΔspAvrLm1-VYNE were used for Agrobacterium transformation.

For co-immunopreipication, the pENTR/Zeo:ΔspAvrLm1 was recombined into the binary vector pGBW451 containing the C-terminal GFP (Nakagawa et al., 2007), which resulted in the pGBW451:ΔspAvrLm1. pENTR/Zeo:BnMPK9 was recombined into binary vector pEarlyGate201 containing the N-terminal HA tag fused to MPK9. All pDONR/Zeo clones were confirmed by sequencing. All resulting binary plasmids were transformed to A. tumefaciens strain GV3101 (pMP90). Agrobacterium-mediated transient expression in N. benthamiana was performed according to methods described previously (Ma et al., 2012).

For the yeast two-hybrid, AvrLm1 lacking signal peptide sequencing was cloned into pGBKKT7 bait vector with primer set ΔspAvrLm1-Ndel/ΔspAvrLm1-EcoRI and the BnMPK9 was cloned into pGADT7 prey vector with primer set BnMPK9-Ndel/BnMPK9-EcoRI (Clontech). The Brassica napus MPK3 (BnMPK3), AtMPK12, AtMPK17, AtEBF1, AtMPK9 and AtT21P5.2 ORFs were amplified using primers listed in Table S2 from L. maculans-B. napus cDNA library or Arabidopsis cDNA. Series of truncated ΔspAvrLm1 and BnMPK9 were amplified using the primers listed in Table S2. The PCR products were cloned into pGADT7 (Clontech, Mountain View, USA).

For yeast signal sequence trap experiment, the signal peptide fragment of AvrLm1 gene was obtained via PCR. The amplification was performed with primer pair SP-AvrLm1-F/SP-AvrLm1-R using pENTR/Zeo:AvrLm1 as a template. The obtained product, carrying EcoRI and XhoI restriction sites, was cloned into the vector pSUC2 digested with the same restriction enzymes.

**Plant transformation**

Arabidopsis thaliana ecotype Columbia (Col) was transformed by the floral dip method (Clough and Bent, 1998). First-generation transformations were selected with herbicide BASTA. Several independent homozygous single insertion lines were selected based on segregation analyses and homozygous T4 lines were used for further experiments. Transformation of B. napus Topas DH16516 with pEarlyGate100:BnMPK9 construct was conducted as described by De Block et al. (De Block et al., 1989).

**Yeast two hybrid library screening and assays**
Initial screening of *A. thaliana* cDNA library was performed by Hybergenics (France) using ULTImate Y2H. *AvrLm1* lacking signal peptide sequence was cloned into pB27 vector as the bait.

To confirm the potential target genes identified after the first round of screening we used the matchmaker GLA4 two-hybrid system and yeast strain Y2HGold (Clontech, Mountain View, USA). The yeast strain Y2HGold was co-transformed with bait and prey plasmid combinations using lithium-acetate and polyethylene glycol 3350 as described previously (Daniel Gietz and Woods, 2002). 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. One colony per combination was picked from SD-WL plates to inoculate 1 mL SD-WL culture. After 36 h growth, cells were collected by centrifugation and resuspended in 25 µL 0.9% NaCl from OD$_{600}$=1 to OD$_{600}$=0.00001 and spotted on SD-WL and SD-AH威尔 plates supplementing with 40 µg/mL X-α-Gal (Clontech, Mountain View, USA) and 200 ng/mL Aureobasidin A (Clontech, Mountain View, USA). After 3 days incubation, the plates were checked for growth and photographed.

**RNA isolation and quantitative PCR**

Cotyledons discs (2mm in diameter) from 10-day-old Topas DH16516 and Topas:*BnMPK9* seedlings were collected, frozen in liquid nitrogen and stored at −80 °C. Leaves of 4 to 5-weeks-old Arabidopsis plants were collected and immediately frozen in liquid nitrogen, and stored at −80 °C prior to extraction of total RNA. Total RNA from the samples was extracted with TRIZol LS reagent (Invitrogen) and subsequently purified with RNeasy Mini kit (Qiagen). DNA was removed by on-column treatment with RNase-free DNase (Qiagen). qRT-PCR was performed using a CFX96 qPCR machine (Bio-Rad) and SsoAdvanced™ Universal SYBR® Green Supermix (BIO-RAD). RT-PCR was performed for all tested genes with three biological samples. The primers used for qRT-PCR are described in Table S2. Ct values were analysed according to the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The *B. napus* actin and Arabidopsis ubiquitin served as reference genes. The statistical significance of differences was calculated using GraphPad Prism 6 (GraphPad Software, Inc., USA) with one-way ANOVA followed by the Turkey post-test or with the two-tailed Student's *t*-test to obtain the P-value. Data are shown as mean ± SEM of three biological replicates from one representative experiment. Significant differences between treatments and controls are represented by three (P < 0.001), two (p<0.01) or one asterisk (p<0.05).

**Bimolecular fluorescence complementation**

For BiFC assays, infiltration was done on the leaves of 4- to 5-week-old *N. benthamiana* plants with Agrobacteria containing the corresponding constructs at an absorbance density of 0.5. Leaf discs 48 h after infiltration were imaged. Confocal microscopical analysis was performed with the LSM610 (Zeiss, Germany). Excitation of the mCherry was detected at 543 nm with HeNe laser. The 590-610 nm filter captured emission.

**Apoplastic fluids isolation**

Cotyledons of 7-day-old Topas DH16516 were inoculated with *L. maculans* isolate 3R11(naturally lacks *AvrLm1*) transformed with *AvrLm1-mCherry* or wild type 3R11 4-day-after inoculation, 96 cotyledons were collected for each inoculum and the apoplastic fluids (AF) were
isolated according to the previously described method (Joosten, 2012). Briefly, collected cotyledons were placed in a beaker filled with deionised water and a weight was placed on the top of cotyledons. Next, a vacuum was applied with a final pressure of about 60 mbar (hPa). The infiltrated cotyledons were centrifuged at 1000 g for 10 min at 4°C. After centrifugation, the obtained AF was concentrated with Amicon® Ultra (Millipore) unit to a final volume of 200-300 µL. Concentrated AF was used for immunoblotting with monoclonal anti-mCherry antibody (Sigma) at a dilution 1:2000.

**Protein extraction**

Protein from agroinfiltrated leaves was extracted as described previously (Ma et al., 2013; Liebrand et al., 2013). Briefly, the total protein fractions were extracted from *N. benthamiana* leaves 48 h after infiltrating with mixture of *A. tumefaciens* GV3101 containing either pEarlyGate201::BnMPK9 and GFP or pEarlyGate100::ΔspAvrLm1-StrepII and pEarlyGate201::BnMPK9 in the presence or absence of 26S proteasome inhibitor MG132 (100 mM) and DMSO, which was infiltrated into the leaves at 24 h after agroinfiltration. The frozen leaves were ground in liquid nitrogen and suspended in 1 ml protein extraction buffer per gram of tissue (25 mM Tris, pH 8, 1 mM EDTA, 5 mM DTT, 150 mM NaCl, 0.1% IGEPAL CA-630 [NP-40], 1x Roche complete protease inhibitor cocktail and 2% PVPP). Extracts were centrifuged at 12,000 g at 4°C for 10 min, and the supernatant was passed over four layers of Miracloth to obtain a total protein lysate. After running the SDS-PAGE gels, the samples were blotted on PVDF membranes using semi-dry blotting. Skimmed milk powder (5%) was used as blocking agent (PBS, 0.1% Tween 20).

**Co-immunoprecipitation**

Co-IP was performed as previously described (Ma et al., 2013). Briefly, the total protein fractions were extracted from *N. benthamiana* leaves 48 h after infiltrating with mixture of *A. tumefaciens* GV3101 containing either pEarlyGate201::BnMPK9 and GFP or pGWB451::ΔspAvrLm1 and pEarlyGate201::BnMPK9. Extracts were centrifuged at 12,000 g for 15 min, and 1 ml supernatant was added to 50 µL of HA-tag beads (Roche) or GFP-Trap (ChromoTek), which was incubated for 1.5 h at 4°C in a rotator. After washing the beads four times with extraction buffer, immunoprecipitated proteins were separated by 8% SDS-PAGE gels and blotted onto PVDF membrane (Bio-Rad) overnight using wet blotting. Skimmed milk powder (5%) was used as blocking agent. A 1:1000 dilution of anti-GFP antibody (Sigma) or 1:8000 diluted anti-HA-HRP (Pierce) was used. The secondary antibody goat-anti-mouse (Pierce) was used as a 1:15000 dilution. The luminescent signal was visualized by Immobilon Western Chemiluminescent HRP Substrate using Bio-Rad ChemiDoc imager (Bio-Rad).

To isolate BnMPK9 for MPK phosphorylation assays, 2 mL protein lysate was incubated for 2 hr with 3 mg anti-MPK9 antibodies at 4°C followed by incubation for 1 hr with 50 µL Goat-anti mouse magnetic beads (NEB). After washing the beads four times with the extraction buffer, proteins were eluted by boiling in 60 mL Laemmli loading buffer. Immunoprecipitated proteins were separated by 8% SDS-PAGE gels and blotted onto PVDF membrane (Bio-Rad) using wet blotting for overnight. BSA (5%) was used as blocking agent. A 1:8000 dilution of anti-HA-HRP (Pierce) and 1:1000 dilution of monoclonal anti-Phosphothreonine antibody (Sigma) were used. The secondary antibody goat-anti-mouse (Pierce) was used as a 1:15000 dilution. The
luminescent signal was visualized by Immobilon Western Chemiluminescent HRP Substrate using Bio-Rad ChemiDoc imager (Bio-Rad).

**Pseudomonas syringae infection assays in Arabidopsis**
The *Pseudomonas syringae* infection assays in Arabidopsis was performed as described previously (Yao et al., 2013). The virulent strain of *Pst* DC3000 was used in this study. Bacterial cells were grown overnight in King’s B medium broth at 28°C. Leaves of the 5-week-old plants were syringe inoculated with a bacterial suspension of *Pst* DC3000 [1×10⁸ colony-forming units (cfu) mL⁻¹] in 10 mM MgCl₂ containing 0.02% Silwet L-77. The inoculated plants were kept at 100% humidity for 24 h and then raised in a growth chamber under the same condition as described above. Bacterial growth in the infected leaves of each plant was determined according to the previously described method (Van Pooeke et al., 2007). Three days after inoculation, two leaf discs (total surface 0.57 cm²) were punched from a single leaf using a new plant for each time point. Leaf discs were pulverized in 400 µL 5 mM MgSO₄, and a dilution series of the suspension was made. Of each dilution, 10 µL was streaked on King’s B plates containing appropriate antibiotics. After 2 d, colonies were counted from the dilution that resulted in 15 to 150 colonies per streak. From this data, the log₁₀ of the number of cfu per cm² leaf surface was calculated. The experiment was repeated three times, with eight replicates per genotype per treatment in each experiment.

**Quantification of *L. maculans* in cotyledons of *B. napus***
Quantification of *L. maculans* in inoculated infected cotyledons of *B. napus* was conducted according to the method described previously (Sasek et al., 2012). DNA was extracted from cotyledons using hexadecyltrimethylammonium bromide (CTAB) based method. DNA (100 ng) was used as a template for a qPCR performed with *LmITS1* and *BnActin* primers and the following program: 95°C for 10 min; followed by 45 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s; followed by a melting curve analysis. The relative quantity of *LmITS1* was normalized to *B. napus Actin*. The statistical significance of differences was calculated using GraphPad Prism 6 (GraphPad Software, Inc., USA) with one-way ANOVA to obtain the P-value. Data are shown as mean ± SEM of three biological replicates from one representative experiment. Significant differences between treatments and controls are represented by three (P < 0.001), two (P<0.01) or one asterisk (P<0.05).

**DAB staining to detect hydrogen peroxide**
3,3-Diaminobenzidine (DAB) staining was conducted following the protocols described previoulsy (Sasek et al., 2012). Briefly, DAB solution was prepared by dissolving 30 mg of DAB (Sigma-Aldrich) in 150 µl of dimethylformamide and mixing it with 30 ml of water. Six detached cotyledons collected from individual plants without inoculation of *L. maculans* or four-day post inoculation were immersed in the staining solution and infiltrated under vacuum. Subsequently, cotyledons were kept at room temperature in a closed petri dish in darkness until reddish-brown staining was visualized. After that, cotyledons were destained in 96% ethanol to remove Chlorophyll. Next, cotyledons were rehydrated in water and scanned in a reflective mode.

**Yeast signal sequence trap system**
Yeast transformation was performed according to the protocol listed in the YeastmakerTM Yeast Transformation System 2 (Clontech, USA). The invertase negative yeast strain YTK12 was transformed with 20 ng of the pSUC2:SP(AvrLm1) or empty vector pSUC2. After transformation,
yeast was plated on CMD-W (minus Trp) plates (0.67% yeast N base without amino acids, 0.075% W dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar). Transformed colonies were transferred to fresh CMD-W plates and incubated at 30°C for 3 days. For the invertase secretion assay, transformed colonies were replica plated on CMD-W plates and YPRAA plates (1% yeast extract, 2% peptone, 2% raffinose, and 2 µg/mL antimycin A) containing raffinose and lacking glucose. After 3 days incubation at 30 °C, the plates were checked for growth and photographed.

Data and Software Availability
AvrLm1 yeast-two hybrid screening candidates using Arabidopsis cDNA library with Arabidopsis accession number: AT2G25490.1, AT2G01450.1, AT3G18040.1 and AT3G03560.1

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