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Molecular Interactions Between *Leptosphaeria maculans* and *Brassica* Species

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Abstract

Canola is an important oilseed crop, providing food, feed, and fuel around the world. However, blackleg disease, caused by the ascomycete *Leptosphaeria maculans*, causes significant yield losses annually. With the recent advances in genomic technologies, the understanding of the *Brassica napus–L. maculans* interaction has rapidly increased, with numerous *Avr* and *R* genes cloned, setting this system up as a model organism for studying plant–pathogen associations. Although the *B. napus–L. maculans* interaction follows Flor's gene-for-gene hypothesis for qualitative resistance, it also puts some unique spins on the interaction. This review discusses the current status of the host–pathogen interaction and highlights some of the future gaps that need addressing moving forward.

INTRODUCTION

QR: quantitative resistance

RMR: *R*-mediated resistance

HR: hypersensitive response

Canola (oilseed rape, *Brassica napus*) is the world's second most important oilseed crop, with more than 70 million metric tonnes being produced worldwide annually (134, 152). Canola is an amphidiploid (AACC) genome derived from a spontaneous hybridization event between *Brassica rapa* (AA genome, 2n = 20) and *Brassica oleracea* (CC genome, 2n = 18) and is now used for food, feed, and fuel, resulting in increased demands on production. Blackleg disease, caused by the fungal ascomycete *Leptosphaeria maculans*, is ranked in the top three diseases of canola across all growing regions of the world except China and causes on average annual losses of 10–15%, with epidemics resulting in up to 90% yield loss (33, 127, 152). Germinating sexual or asexual spores of the pathogen cause necrotic leaf lesions before switching to an asymptomatic phase, growing down the petiole and into the stem. As the plants mature, the pathogen then switches to a necrotrophic phase and colonizes the crown of the stem, restricting nutrient flow up the plant, causing yield loss, and, in severe situations, resulting in stem cankers that kill the plant (48). A closely related species, *Leptosphaeria biglobosa*, is also often associated with infection of canola; however, this species generally causes less-severe symptoms, is associated with upper stem infection, and is generally outcompeted by *L. maculans* when present (34).

The life traits of *L. maculans*, including its large population size (due to the colonization of crop debris) and ability to undergo both sexual and asexual reproduction, allow this pathogen to evolve and adapt to changes rapidly. Added to these biological features is the compartmentalized genome of *L. maculans*, which results in GC-rich, gene-abundant isochores compared to AT-rich, gene-poor regions that are riddled with transposable elements. Pathogenicity genes, known as effectors or avirulence genes, are located within these AT-rich regions that can undergo selection resulting in virulence. The less competitive species, *L. biglobosa*, is largely devoid of these AT-rich regions (44).

The impact of blackleg disease is minimized using cultural, chemical, and genetic strategies, with the last being the most effective and universal approach (27, 134). Genetic resistance associated with the control of blackleg disease is generally divided into qualitative and quantitative, also known as seedling resistance and adult plant resistance, respectively. In this review, we discuss the current understanding of the host resistance mechanisms and pathogen virulence, present hypotheses on the interactions between *Brassica* and *Leptosphaeria*, and highlight the gaps in the current knowledge.

GENETICS OF RESISTANCE TO BLACKLEG: PAST AND PRESENT

Discussion of qualitative and quantitative resistance (QR) in the *B. napus–L. maculans* pathosystem is somewhat confusing as multiple terms (monogenic versus polygenic, seedling versus adult plant, race-specific versus race-nonspecific, vertical versus horizontal) (107) have all been used and applied differently, and sometimes incorrectly, when describing agronomic, pathological, or molecular studies. One of the aims of this review is to try to clarify these concepts from a molecular point of view. Qualitative resistance is generally regarded as a single resistance (*R*) gene producing a rapid and robust defense response, usually in a gene-for-gene manner whereby the response is triggered by a pathogen carrying the matching avirulence (*Avr*) gene (12). The presence or absence of these *Avr* genes determines the race of the pathogen isolate, and thus *R*-mediated resistance (RMR) is usually considered to be race-specific. RMR is typically associated with the cell death known as hypersensitive response (HR). In the *B. napus–L. maculans* pathosystem, this is easily assessed using a cotyledon assay and many host *R* and pathogen *Avr* genes have been identified this way (see details below and **Table 1**). QR is based on a model in which multiple loci contribute, both positively and negatively, to the overall phenotypic value (53), and it is generally regarded to

Table 1 ***Leptosphaeria maculans Avr*** **and** ***Brassica R*** **interactions**

Avr phenotype	Avr locus	Avr protein	R protein	R locus	R phenotype	Reference(s)
A1	*AvrLm1-Lep3*	AvrLm1-Lep3	-	*Rlm1*	R1	43
AL3			LepR3	*Rlm2-LepR3*	LR3	72
A2	*AvrLm2*	AvrLm2	Rlm2		R2	40, 74
A3	*AvrLm3*	AvrLm3	-	*Rlm3-4-7-9*	R3	103
A4	*AvrLm4-7*	AvrLm4-7	Rlm4		R4	46, 99
A7		AvrLm7	Rlm7		R7	46, 99
A9	*AvrLm5-9*	AvrLm5-9	Rlm9		R9	41, 76
A5		AvrLm5	-	-	R5	104
A6	*AvrLm6*	AvrLm6	-	-	R6	36
A8	-	-	-	-	R8	8
A10	*AvrLm10A*	AvrLm10A	-	-	R10	101
	AvrLm10B	AvrLm10B				101
A11	*AvrLm11*	AvrLm11	-	-	R11	9
A12	-	-	-	*Rlm12*	R12	105
A13	-	-	-	*Rlm13*	R13	108
A14	*AvrLm14*	AvrLm14	-	-	R14	25
AS	*AvrLmS-Lep2*	AvrLmS-Lep2	-	-	RS	93
AL2			-	*LepR2*	LR2	148
AL1	*AvrLep1*	-	-	*LepR1*	LR1	148
AL4	-	-	-	*LepR4*	LR4	146
AL5	-	-	-	*LepR5*	LR5	76
AL6	-	-	-	*LepR6*	LR6	76
ASTEE98	*AvrLmSTEE98*	AvrLmSTEE98	-	*RlmSTEE98*	RSTEE98	61

Rows indicate corresponding avirulence and resistance phenotypes in *L. maculans* and *Brassica* spp., respectively. Columns list genetically defined loci and characterized proteins. Dashes indicate loci or proteins not yet identified.

provide partial yet race-nonspecific resistance, rather than the robust, race-specific resistance of RMR, and to be influenced by environmental factors (19, 112).

Part of the reason for the confusion regarding resistance in canola is that blackleg resistance research originated in the field, as part of breeding activities for a commercial crop, long before the modern concepts of molecular plant–pathogen interactions were widely applied. Blackleg resistance in *B. napus* germplasm was segregated into seedling and adult plant resistance based on the host growth stage at which the resistance was assessed, and this concept of two disparate resistance mechanisms has lingered in the literature to this day. Early efforts to identify seedling resistance through glasshouse trials proved unreliable when compared to field-based trials (20, 97). Disparities in inoculation techniques produced widely varying results (114), and attempts to transfer resistant material from one geographic location to another often met with failure, as an understanding of the race structure of *L. maculans*, or even the species structure of *Leptosphaeria*, was lacking.

With the application of single-spore-derived *L. maculans* isolates, a common wounded cotyledon inoculation procedure for the reliable detection of qualitative, *R*-induced resistance phenotypes (143) and Flor's gene-for-gene hypothesis (35), advancements in defining both *Brassica R* and *L. maculans Avr* interactions were made (6). Since then, efforts to screen commercial canola varieties and extensive *Brassica* germplasm collections have uncovered a large collection of blackleg *R* from the three progenitor genomes of most *Brassica* crops (A, B, C). Currently, there

QTLs: quantitative trait loci

are 22 defined, race-specific blackleg *R* (*Rlm1–14*, *RlmS*, *LepR1–6*, *RlmSTEE98*) identified from *B. rapa* (AA), *Brassica nigra* (BB), *B. oleracea* (CC), *B. napus* (AACC), and *Brassica juncea* (AABB) lines through either genetic mapping of the host resistance phenotype or *Avr* differentiation and/or genetic mapping in the pathogen (**Table 1**). Thorough genetic mapping and physical delimitation of host *R* loci within the *B. napus* genome, along with the use of *L. maculans* isolates transformed with complementary *Avr* constructs, have aided in clearing up confusion as to the identity of *R* within some cultivars, with many of the early genetically mapped resistance loci [*LmFr1* (29), *LEM1* (32), previously misidentified as *Rlm4* (112), *cLmR1* (90, 91) and *cRLM* (113)] later shown to be *Rlm3* (78). However, several *R* currently lack any published genomic location and are known only through their interaction with a characterized *Avr* (*Rlm5*, *Rlm6*, *Rlm10*, *Rlm11*, *Rlm14*); although *Rlm6*, *Rlm10*, and *Rlm11* are each featured as introgressions into *B. napus* material (9, 19, 27, 101) and thus presumably segregate as single genes. There are no established *Avr* or *R* loci yet described for *Rlm8*. There is also some apparent redundancy within the named *R*. *RlmS* (135) [later published as *BLMR2* by Long et al. (84)] was identified from *B. napus* cv. Surpass 400, carrying resistance derived from *B. rapa* ssp. *sylvestris* (23), and *LepR2* was identified separately from two different *B. rapa* ssp. *sylvestris* sources (147, 148). Both *RlmS* and *LepR2* have been mapped to the same region of chromosome A10 and have been recently shown to correspond to the same *L. maculans Avr* (*AvrLmS-Lep2*) (93) and are thus likely to be the same *R*.

The genetics governing QR remain poorly understood by comparison, despite much focus on blackleg QR over the years. The reasons for this are threefold: (*a*) The complexity of the underlying genetics, which are likely to be a range of genes active at different points in the infection process, each contributing to the overall resistance; (*b*) the large effect of environmental variation on the expression of the disease symptoms under the conditions used for testing (mostly in the field); and (*c*) difficulties with precise phenotyping of the resistance conveyed by individual, minor-effect *R* alleles. A recent review by Amas et al. (5) details the QR-associated regions that have been identified from a range of quantitative trait loci (QTLs) mapping and genome-wide association studies experiments carried out across different geographical regions and using different *B. napus* material, and some common resistance-associated genomic regions have been identified across those experiments.

The notion that RMR and QR represent two distinct resistance mechanisms, i.e., RMR is complete, race-specific, and seedling-expressed and QR is incomplete, race-nonspecific, and adult plant-expressed, needs to be retired, as more and more evidence points to these generalized characterizations being incorrect (see below). Blackleg resistance phenotypes are sometimes referred to as quantitative based purely on the expression of an incomplete or intermediate resistance phenotype observed in the stem or cotyledon, yet this is also incorrect. Quantitative is a description of the genotype, not the phenotype. RMR and QR are defined in terms of their genetics, i.e., whether a single locus or multiple loci are controlling the observed phenotypic variation. This requires segregating populations (either host or pathogen) and demonstrating either discrete (monogenic) or continuous (polygenic) variation of the host resistance phenotype. The objective of QTL mapping is to resolve non-Mendelian continuous variation into discrete Mendelian factors (100) (i.e., to resolve QR into a set of *R*), and this does not preclude the defined QTLs from being under gene-for-gene influence (i.e., race-specific; see *L. maculans* virulence below). Neither RMR nor QR requires the expression of resistance to be limited to a particular amplitude, tissue type, or growth stage. HR is not a requirement for RMR, as seen in several other pathosystems, nor is activation in response to pathogen avirulence proteins (Avr). In fact, the first *R* characterized through map-based cloning encoded an apoplast-secreted detoxifying enzyme from maize (62). Environmental effects on resistance are not limited to QR either, as has been demonstrated by the effect of temperature on *R* action in both *Brassica–Leptosphaeria* interactions and other pathosystems

(54, 139). The strength of the defense response induced by an *R* is not always complete and may result in intermediate phenotypes, and the amplitude of the response can also be highly dependent on the host genomic background in which the *R* is carried (45, 78). Although some blackleg *R* appear to be inactive in the stem when stems are directly inoculated (*Rlm1* and *Rlm4*) (31), some of the earliest blackleg *R* mapping was performed using stem inoculations (*Rlm3*, previously labeled as *LEM1*) (32). Likewise, some QR responses can be measured only in the stem (56), whereas several QTLs have been linked to cotyledon phenotypes (77, 110) and several recent studies have suggested that QR can be expressed throughout the life of the plant (55, 121). Blackleg QR can also be race-specific rather than have a general effect on all isolates (61, 89, 134). Furthermore, QR is not always stable and can be eroded over time. This erosion is evident through the decrease in the blackleg rating of Australian cultivars over time (**Supplemental Figure 1**), with the rate of decline varying across the individual cultivars, which may be indicative of variations in the genes underlying the QR in these lines. Variations in populations in their virulence toward QR lines may explain a portion of the environmental variance seen in many QR studies, particularly across diverse geographic locations (26, 106). This could be overcome through more precise screening methods, such as screening with pycnidiospores from individual clonal isolates (133). Alternatively, methods for the generation of artificial ascospore populations have recently been developed (17) and could be incorporated for screening to keep pathogen populations consistent over time.

From a molecular standpoint, there is little that separates RMR and QR other than the number of loci controlling the phenotype. All resistance responses, regardless of the presence or absence of known *R*, employ many genes that are expressed in concert in response to the recognition of pathogen-derived elicitors (22). Distinction in defense type can be made based on the type of elicitor that triggers the response: pathogen-associated molecular patterns (PAMPs), which lead to PAMP-triggered immunity (PTI), or pathogen effectors, which lead to effector-triggered immunity (ETI) (63). However, this is likely also an oversimplification, as many other modes of defense have been described to date for other systems (69) and both types of elicitors are under selection pressure by the host, with race specificity observable in both PTI and ETI systems. More recently, it has been suggested that a spatial immunity model is most appropriate, with plant defense being defined solely on the basis of either extra- or intracellular detection of the pathogen elicitor, as common signaling pathways govern these responses (136).

PTI: pattern-triggered immunity

ETI: effector-triggered immunity

MAMPs: microbe-associated molecular patterns

DAMPs: danger-associated molecular patterns

OGs: oligogalacturonides

RLP: receptor-like protein

LRR: leucine-rich repeat

Supplemental Material >

MOLECULAR DIALOGS BETWEEN *BRASSICA* AND *LEPTOSPHAERIA*

To date, five blackleg *R* have been cloned from *B. napus*, all of which encode membrane-bound cell surface–localized receptor proteins used to detect apoplastic elicitors. Plant defense receptors detect three types of elicitors present in the apoplast during pathogen invasion: (*a*) microbe-associated molecular patterns (MAMPs), which are highly conserved pathogen-derived molecules such as fungal chitin and bacterial flagellin; (*b*) danger-associated molecular patterns (DAMPs), which are host-derived molecules produced during invasion by pathogens, such as oligogalacturonides (OGs) released during cell wall degradation; and (*c*), particularly in the case of apoplastic fungal pathogens such as *L. maculans*, *Cladosporium fulvum*, and *Zymoseptoria tritici* (24, 92, 128), pathogen-secreted effector (Avr) proteins (18, 22). Two of the cloned *R* effective against *L. maculans* encode receptor-like protein (RLP) genes, *LepR3* and *Rlm2*, previously colocalized to the same genomic interval of chromosome A10 (73) and shown through their cloning to be alleles of the same RLP locus (72, 74). RLPs comprise a signal peptide that facilitates trafficking to the cell membrane, an extracellular ligand-binding domain containing multiple leucine-rich repeat (LRR) motifs likely involved in protein–protein interactions, a membrane-spanning transmembrane domain, and a short intracellular segment (18) (**Figure 1**). Despite being allelic, the LepR3

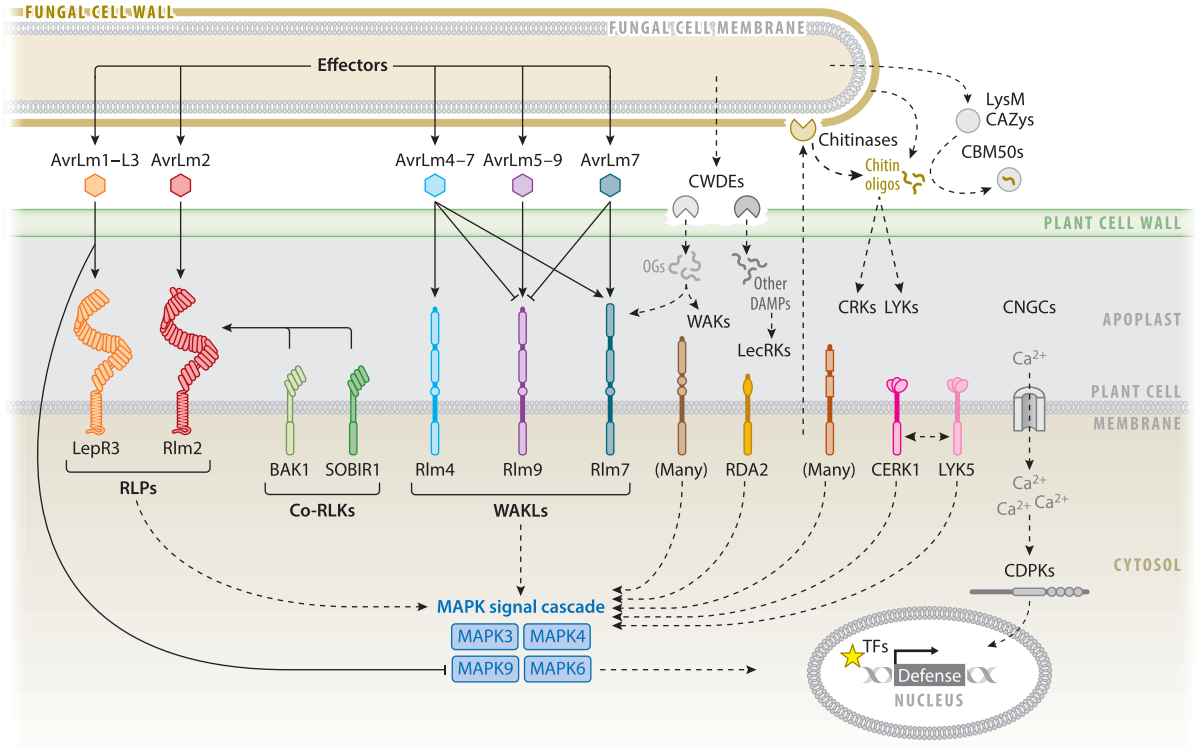

Figure 1

Proven and potential molecular interactions in the *Brassica–Leptosphaeria* pathosystem. Solid arrows indicate interactions between characterized proteins, and dashed arrows indicate potential interactions based on transcription-based or other studies. Abbreviations: CAZys, carbohydrate-active enzymes; CDPKs, calcium-dependent protein kinases; CNGCs, cyclic nucleotide-gated channels; CRKs, cysteine-rich receptor-like kinases; CWDEs, cell wall–degrading enzymes; DAMPs, danger-associated molecular patterns; LecRKs, lectin receptor kinases; LYKs, lysin motif receptor-like kinases; MAPK, mitogen-activated protein kinase; OGs, oligogalacturonides; RLKs, receptor-like kinases; RLPs, receptor-like proteins; TFs, transcription factors; WAKs, wall-associated kinases; WAKL, wall-associated kinase-like.

and Rlm2 RLPs differ greatly in their extracellular domains, with LepR3 sharing only four of its 22 LRR motifs with Rlm2 (74). Their matching Avr proteins, AvrLm1-Lep3 (43, 72) and AvrLm2 (40), originate from the same genetic cluster in the *L. maculans* genome (8) but share almost no homology.

The remaining three cloned *R* all encode wall-associated kinase-like (WAKL) proteins, *Rlm9* (75), *Rlm4*, and *Rlm7* (46), previously genetically clustered, along with *Rlm3*, on chromosome A07 (78). The cloning of these genes has revealed them to be allelic variants of one locus. WAKLs are a newly emerging class of Avr-responsive, ETI-inducing *R*, with the only other example being *Stb6* from wheat, which conveys race-specific resistance to another apoplastic fungal pathogen, *Z. tritici* (119), and little is known about their function. They share common domains (although not entirely) with the wall-associated kinase (WAK) proteins. This includes a GUB_WAK domain thought to bind either pectin components of the plant cell wall or OGs (DAMPs). However, although the binding of pectin or OGs has been demonstrated in WAKs (68), the same has not been shown for WAKLs, and homology between the domains of WAK and WAKL proteins is limited (75).

WAKL: wall-associated kinase-like

WAK: wall-associated kinase

It is not yet known how recognition of the cognate Avr proteins is established by the RLPs or WAKL proteins (see the section titled Avirulence Protein Interactions). We do know that the RLPs do not act alone but rather form a receptor complex with other proteins required for the perception of pathogen ligands and initiation of downstream signals (82, 140). Accumulating evidence, including our reports on the LepR3 and Rlm2 recognition complexes, show that RLP-type R proteins form a complex with SOBIR1 (SUPPRESSOR OF BIR1–1), an LRR receptor-like kinase, and BAK1 (BRI1-associated receptor kinase 1) following pathogen recognition (**Figure 1**). SOBIR and BAK1 have been reported to bind other effector-triggered RLPs such as the tomato Cf-2, Cf-4, Cf-9, and Ve1 as well as several other more distantly related RLPs from tomato (137). Similarly, perception of the MAMPs nlp20 peptide, found in the necrosis- and ethylene-inducing peptide-like (NLP) proteins of bacteria, fungi, and oomycetes, and VeM02, from the phytopathogenic fungus *Valsa mali*, by their respective RLPs, RLP23 and RE02, requires SOBIR1 (4, 94), and it is likely that coreceptors are involved in the recognition of MAMPs from *L. maculans* as well. The kinase domain activity of SOBIR1 is required for its function, suggesting a role for SOBIR1 in downstream signaling (137). Localization of Rlm2 and LepR3 with SOBIR1 in the absence of their respective effectors confirms previously reported ligand-independent constitutive association of RLP-SOBIR1 pairings. However, BAK1 associations with the RLP-SOBIR1 complexes are reportedly ligand dependent (3).

SOBIR1: SUPPRESSOR OF BIR1–1

BAK1: BRI1-associated receptor kinase 1

No genes associated with QR have yet been cloned for the *B. napus–L. maculans* interaction, and only a few candidate genes underlying the QR-associated regions have been identified (71, 77, 109). Genes associated with QR have been identified in other pathosystems, such as the wheat ATP-binding cassette transporter *Lr34* gene against rust and powdery mildew (70) and wheat *Fhb1* and *Fhb7* genes encoding a putative histidine-rich calcium-binding protein and a glutathione S-transferase (GST), respectively, that confer resistance to *Fusarium* head blight (81, 129, 141). Similarly, although there has been much work done looking at defense-associated gene expression with RMR during cotyledon infection (see the section titled Recognition and Response by *Brassica napus*), there are limited RNA-Seq data associated with defined QR-associated loci.

LEPTOSPHAERIA MACULANS VIRULENCE: BATTLE FOR THE CROWN

L. maculans infection of *Brassica* starts with the germination of fungal spores on the cotyledon and leaf, progresses through the initial biotrophic phase during which invasion of the leaf apoplastic spaces occurs (**Figure 2**), proceeds with symptomless growth of hyphae through the petiole and stem, and ends with the fungus reaching the crown of the stem, the juncture between stem and hypocotyl, where pathogen proliferation leads to stem canker (48). Therefore, suppression or evasion of the host defenses at multiple stages of the infection process is critical for *L. maculans* to complete its life cycle.

The *B. napus–L. maculans* interaction is proving to be a potential model for investigating gene-for-gene interactions at the molecular level, with many *Avr-R* partners now cloned. Currently, eleven avirulence (effector) genes have been characterized from *L. maculans*, more than any other apoplastic fungal plant pathogen (24, 115), including four *Avr* loci that encode the effectors to the five cloned *R* (**Table 1**). These effectors all meet the standard criteria for avirulence genes, i.e., small, secreted, typically cysteine-rich, highly upregulated in planta, and located within AT-rich regions of the *L. maculans* genome (for review, see 134). Although the effectors themselves meet the standard criteria for avirulence proteins, the interactions with the plant host are varied and push Flor's simple gene-for-gene hypothesis to the limits. Among the identified Avr effectors, there are those that interact in the standard gene-for-gene manner whereby a single Avr from the pathogen, e.g., AvrLm2, is recognized by a single R, e.g., Rlm2, in the host. In this scenario,

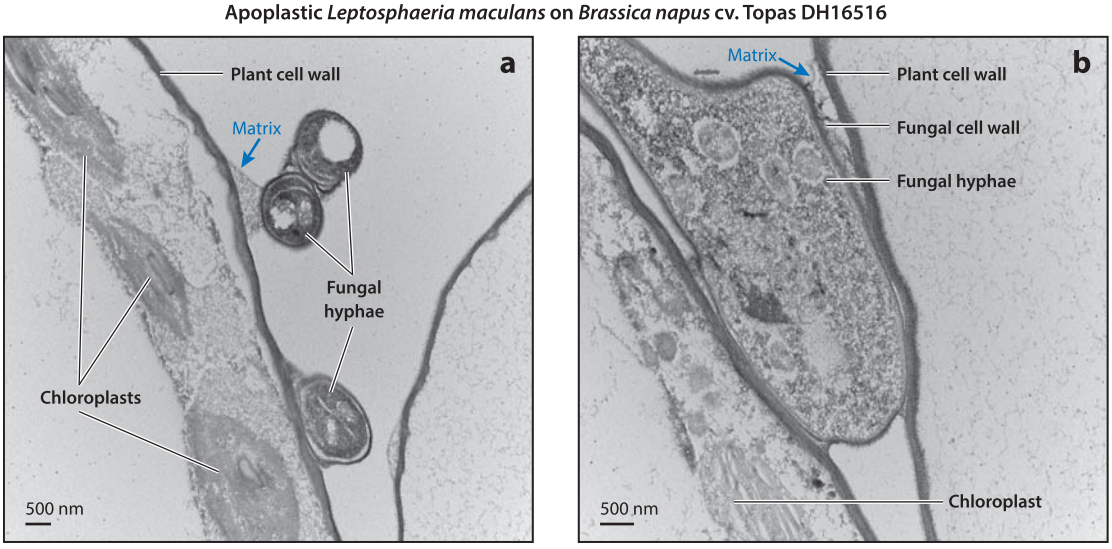

Figure 2

Electron microscopy of apoplastic *Leptosphaeria maculans*. (*a*,*b*) *L. maculans* accesses the host plant tissue via stomata and wound sites, with internal hyphal growth occurring between the host mesophyll cells. Blue arrows point to the formation of an unknown matrix between the hyphae and the host cell. Tissues were prepared from infected cotyledons of *Brassica napus* cv. Topas DH16516 (susceptible to *L. maculans*). Electron microscopy images produced by the University of British Columbia Bioimaging Facility (Vancouver, Canada).

a single-nucleotide polymorphism within the *AvrLm2* coding region results in a virulent protein that is no longer recognized by Rlm2 (40). However, there is also a range of other less typical gene-for-gene interactions. There are situations in which two Avr genes, *AvrLm10a* and *AvrLm10b*, are required to trigger RMR by a single resistance gene, *Rlm10* (101). However, in this scenario the corresponding *R* has yet to be cloned and no genetic mapping of the locus has been reported, so there is the possibility of two linked resistance genes being involved in the interaction. Alternatively, there is a situation whereby a single *Avr*, such as *AvrLm1-Lep3*, is recognized by two separate resistance genes, *Rlm1* and *LepR3*, and deletion of the *AvrLm1-Lep3* gene prevents recognition of the pathogen by either R protein (43, 72). In this scenario, the two *R* are located on different chromosomes (A07 and A10, respectively) within the A genome of *B. napus* in genomic regions that do not share any obvious homology (52), suggesting the independent convergent evolution of two separate *R* loci toward recognition of a single Avr. Although there are situations reported in other species in which two R recognize a single Avr, such as the *Avr-Pia* gene of *Magnaporthe oryzae* (96), in these scenarios, both genes must be present for recognition to occur. Currently, only *B. napus* is reported to have two independent R recognizing a single Avr.

In addition to the gene-for-gene interactions, there are epistatic interactions occurring between *Avr*. The *AvrLm4-7* avirulence locus produces both AvrLm4-7, recognized by both Rlm4 and Rlm7, and AvrLm7, recognized by Rlm7 only (46, 99). When either the avirulent AvrLm4-7 or AvrLm7 proteins are present, they are epistatic over AvrLm3 and AvrLm5-9, resulting in the *AvrLm3-Rlm3* and *AvrLm5-9–Rlm9* interactions being masked (41, 103). Therefore, when an isolate contains *AvrLm4-7*, it is phenotypically virulent toward *Rlm3* and *Rlm9*, irrespective of the *AvrLm3* and *AvrLm5-9* genotype. How this epistatic interaction occurs is currently unknown. As mentioned, five pairs of corresponding Avr and R have been characterized from the *B. napus–L. maculans* interaction: AvrLm2–Rlm2, AvrLm1-Lep3–LepR3, AvrLm5-9–Rlm9, AvrLm4-7–Rlm4, and AvrLm7–Rlm7 (**Table 1**); this sets the stage for investigating the functions and interactions of

these proteins in regard to plant defense (see discussion of Avr protein interactions in the section below titled Recognition and Response by *Brassica napus*: Apoplast as the Battleground).

In addition to the *Avr* that have been identified that correspond to known qualitative *R*, more recently a different class of effectors, termed late effectors, has been identified that are proposed to be involved in QR (61). Gervais et al. (39) examined the expression of effector genes during different stages of the *B. napus–L. maculans* infection process and characterized them as either early effectors, which are upregulated early in the infection of cotyledons and include all the currently characterized *Avr*, or late effectors, which represent a set of effector genes being expressed during the stem colonization phase of the interaction. Jiquel et al. (61) characterized a subset of these late effectors and identified an effector, *AvrSTEE98*, that can elicit a gene-for-gene-like interaction at the cotyledon stage (when expressed under the *AvrLm4-7* promoter) or the stem stage. The authors suggest that these late effectors are responsible for a gene-for-gene interaction corresponding to QR and that QR may be isolate specific rather than generally affecting all isolates. Consistent with this is the previous finding that when a set of isolates were screened against a set of *B. napus* cultivars with differing levels of QR, differential responses were detected rather than a general reduction in disease caused by all isolates (133). Again, this is consistent with the earlier suggestion that QR can be eroded over time (**Supplemental Figure 1**) as pathogens adapt to selection pressures and demonstrates how discrete, gene-for-gene traits can underlie a quantitative phenotype. Jiquel et al. (61) identified a single locus on chromosome A09 for the corresponding *RlmSTEE98* gene in the plant, using the Darmor × Yudal doubled haploid (DH) population, which did not correspond to the reported positions of other A09 blackleg QTLs. However, the resistance segregated into a resistant to susceptible ratio of 1:2 instead of the expected 1:1, although presumably 50% of the DH lines carry a homozygous-resistant allele. This suggests either difficulty in assessing resistance at the cotyledon stage or possibly the requirement for two linked genes for expression of the phenotype. Further studies using isolates able to overcome characterized QTL material, presumably due to the mutation of effectors governing the interaction, may prove fruitful in determining the *B. napus* genes controlling QR to blackleg.

Phytopathogenic fungi also secrete a diverse array of cell wall–degrading enzymes (CWDEs) during invasion of the host plant (85). Carbohydrate-active enzymes (CAZys) are secreted by the fungus during the infection process and are likely involved in nutrient acquisition for the growing hyphae via degradation of host cell wall components (**Figure 1**) (38, 39, 47, 86). A pathogen's lifestyle can be predicted by the suite of CAZy enzymes encoded by its genome (50), and in the case of *L. maculans*, different suites of CAZy-encoding genes are expressed at various infection stages throughout the pathogen's life cycle as it transitions between biotrophy and necrotrophy (38, 47, 86). *L. maculans* also secretes chitin-binding CAZys into the apoplast, including Carbohydrate-Binding Module 50 (CBM50) family proteins, which feature a LysM domain similar to those found on the plant lysin motif receptor-like kinase (LYK) receptors (2). These likely function to compete in the binding of chitin oligosaccharides in the apoplast; MAMP signals freed from the fungal cell wall during growth of the pathogen or during attack from host chitinases before they can be detected by the host LYKs suppress the host's chitin-triggered immune response (**Figure 1**) (39, 47, 86, 125).

DH: doubled haploid

CWDEs: cell wall–degrading enzymes

CAZys: carbohydrate-active enzymes

LYK: lysin motif receptor-like kinase

Supplemental Material >

RECOGNITION AND RESPONSE BY *BRASSICA NAPUS*: APOPLAST AS THE BATTLEGROUND

Perception of pathogens by the cell-surface receptors triggers downstream signaling pathways that mount an orchestrated defense response to counteract pathogen invasion. Forward and reverse genetics have advanced our understanding of downstream plant defense networks in model plants

and crop species. Efforts have been made to promote *Arabidopsis–Leptosphaeria* as a model system to discover *R* and defense responses against *L. maculans*, but other work points to the fact that *Arabidopsis*, as a nonhost, is not a suitable model and data obtained from such studies should be treated with caution (120). The majority of research has been conducted on the actual crop host, *Brassica napus*, and has been accelerated by the advances in genomics and genome-sequencing technologies. Genome sequencing of *Brassica* and *Leptosphaeria* species (21, 51, 118, 126), as well as deep RNA sequencing conducted on *B. napus* infected with *L. maculans*, have greatly advanced our knowledge of the *Brassica* defense response and *L. maculans* pathogenicity. Recent transcription-based dissections of the *Brassica–Leptosphaeria* interaction have highlighted several important aspects of the host defense response and are discussed below (10, 11, 38, 39, 45, 47, 57, 86, 125).

LecRKs: lectin receptor kinases

MAPK: mitogen-activated protein kinase

ABA: abscisic acid

CRK: cysteine-rich receptor-like kinase

DUF26: domain of unknown function 26

CDPKs: calcium-dependent protein kinases

MAMP and DAMP receptors are upregulated early in the infection process and are also regulated as part of the RMR along with other components of cell-surface receptor complexes. Of note are the WAK receptors (65), which detect apoplastic OGs produced during degradation of pectin in the plant cell wall by pathogen-secreted CWDEs (**Figure 1**) (7). WAK genes have been shown to underlie QTLs for resistance to several fungal pathogens (37, 58, 153). Lectin receptor kinases (LecRKs) are another form of cell-surface receptor and feature an extracellular carbohydrate-binding lectin domain and intracellular kinase domain (**Figure 1**), which are widespread in vascular plants and play a key role in plant immunity (142). Review of the RNA-Seq data generated by Haddadi et al. (45) revealed that among the *B. napus* LecRKs induced in response to *L. maculans* at 3 days post-inoculation (dpi) was an ortholog of *Arabidopsis RDA2* (resistant to DFPM-inhibition of ABA signaling; AT1G11330) that plays an important role in activating immune signaling through the mitogen-activated protein kinase (MAPK) pathway (see below) while inhibiting the abiotic stress–related abscisic acid (ABA) signal pathway to favor immune response (98). Other carbohydrate-binding receptors include chitin-sensing receptors such as cysteine-rich receptor-like kinases (CRKs) (16) and LYKs (124) (**Figure 1**); both show greater upregulation in *R*-mediated incompatible interactions when compared to compatible (no *R*) interactions (11, 45). Chitin-sensing receptors bind chitin oligosaccharides produced either during remodeling of the fungal cell wall during growth of the fungus or through direct attack against the fungal cell wall by plant chitinases secreted into the apoplast (124) (**Figure 1**). *B. napus* orthologs of CRK2, 4, 10, and 11 and the LYKs CERK1 and LYK5 are upregulated within 3 dpi. The extracellular domain of CRKs possesses two copies of DUF26 (domain of unknown function 26) domains containing a cysteine motif (C-8X-C-2X-C) in its core. The DUF26 domain is specific to land plants and is likely involved in carbohydrate binding based on structural similarity to the fungal carbohydrate-binding lectin protein (132). Phenotyping of the *Arabidopsis* CRK mutants has identified several CRKs, including CRK2 and CRK10, that function in chitin-triggered immunity and have been shown to regulate stomatal closure as part of the defense response to fungal invasion (16). The upregulation of chitin-sensing receptors is consistent with earlier reports of *L. maculans* cell wall extracts enhancing host resistance (67), with the association of a major field resistance QTL with a CRK gene cluster (77) as well as the involvement of CRKs in many other fungal pathosystems (111, 124, 144).

Perceived apoplastic signals released during *L. maculans* invasion trigger a cascade of downstream signals. MAPKs, transducers of external signals through phosphorylation of downstream target proteins, are key regulators of several cellular processes, including response to biotic stress (28). Activation of MAPK cascades, along with Ca^{2+} signaling and activation of calcium-dependent protein kinases (CDPKs) (see below), is a common output response for all cell-surface receptor signaling (**Figure 1**) (136). MAPKs have been shown to be the target of bacterial and fungal effectors (13) such as *L. maculans* AvrLm1 (88). Among the MAPK pathway genes, MKK9 and downstream MPK3 were upregulated at 3–9 dpi (45, 47). Using laser microdissection combined

with RNA sequencing, Becker et al. (10) were able to detect upregulation of MPK4 and MPK6 within the first day of the infection process. MKK9 and MPK3, 4, and 6 function has been linked to the regulation of the salicylic acid (SA), jasmonic acid (JA), ethylene, and ABA plant hormone pathways (59).

SA: salicylic acid

JA: jasmonic acid

CNGCs: cyclic nucleotide-gated channels

The roles of plant hormones in *B. napus* cotyledons infected with *L. maculans* are defined by the timing of their expression. Induction of SA-related genes, which are important for defense against biotrophic pathogens, correlated with the initial (2–4 dpi) biotrophic stage of *L. maculans* infection, whereas the JA pathway, associated with defense against necrotrophic pathogens, was induced at a later (6–8 dpi) stage. The importance of SA in race-specific resistance has been substantiated by several studies through observation of the induction of SA marker genes, ICS1 and PR1, and increase in SA levels (10, 45, 47, 80, 95, 120). SA and other plant hormone pathways can be targets of pathogen effectors in many pathogens, such as *Ustilago maydis*, *Sclerotinia sclerotiorum*, and *Verticillium dahliae* (49). It is very likely that similar strategies are employed by *L. maculans* to counteract the *B. napus* hormonal defense. Expression of SA marker genes and SA production were suppressed in the *B. napus* line Columbus in response to the *L. maculans* effector *AvrLm4-7* (95).

The role of calcium signaling in both MAMP/DAMP and Avr responses has also been made evident by recent reports (10, 45). Induction of calcium sensors, transporters, and calcium-dependent signal transducer genes was reported as early as 1 dpi in infected tissue. Furthermore, pretreatment of cotyledons of the *B. napus* Topas-*Rlm2* resistant line with the calcium chelator EGTA abolished resistance against *L. maculans* (10). One of the earliest cellular responses is activation of cyclic nucleotide-gated channels (CNGCs). CNGCs, many of which have been implicated in pathogen defense, form heteromeric channel complexes located mostly in the plasma membrane (60), leading to an increase in the cytosolic calcium level and alkalization of the apoplast. Consistent with this, searches of DEG at 3 dpi (45) revealed the induction of genes homologous to *A. thaliana* CNGC3 (BnaC04g01250D, BnaA05g01380D), CNGC12 (BnaC03g31050D), and CNGC19 (BnaA03g34680D, BnaCnng45430D). CNGC12 and CNGC19 induce Ca^{2+} influx (151) and CNGC3 participates in efflux of Na^{+} and K^{+} (42). The association of CNGC19 and CNGC12 directs influx of calcium in response to MAMPs and effectors (151). In a recent paper, Yu et al. (149) reported the involvement of CNGC19 and CNGC20 in the regulation of BAK1-mediated cell death in *Arabidopsis* through BAK1 phosphorylation of CNGC20. The role of CNGC12 in plant defense was discovered through cloning of the *Arabidopsis constitutive expresser of PR genes 22* (*cpr22*), whereby a mutation in the *cpr22* gene resulted in the fusion of CNGC11 and CNGC12 and constitutive expression of multiple defense genes (145). CDPKs, which play roles in oxidative burst, hormone signaling, and defense gene expression (123), are also upregulated during the *L. maculans* infection process (10, 45). Several CDPKs have been shown to act downstream of RLP-mediated defense in response to elicitation by fungal Avr effectors (116, 117) and regulate a separate defense pathway to the MAPK signal cascade in a concerted manner (87).

The integrated inter- and intracellular defense network culminates in the induction of transcription factors (TFs), leading to transcriptional reprogramming (130) and production of primary and secondary metabolites (102). The most prominent examples of TFs with a possible role in defense against *L. maculans* are WRKY 18, 33, 51, and 70, identified by a time-course RNA-sequencing analysis conducted by Haddadi et al. (45, 47). Upregulation of WRKY33 is associated with the induction of the JA pathway and suppression of SA (14), whereas WRKY 18, 51, and 70 are positive regulators of the SA pathway (122). Other *B. napus* TFs upregulated in response to *L. maculans* infection included members of the MYB (MYB37, 48, 51, 52, 73, and 77), and NAC (NAC 36, 42, and 47) families. Genes involved in the early steps of lignin biosynthesis, sugar and amino acid transporters, proteases, and protease inhibitors were also upregulated (45, 47).

Y2H: yeast two-hybrid

Co-IP: co-immunoprecipitation

To gain insight into the observed effect of host genotype on the strength of *R*-mediated HR to *L. maculans* (45, 77), researchers took advantage of *B. napus* introgression lines carrying the *R LepR1* and *LepR2* in either Topas DH16516 (78) or Westar (147) *B. napus* backgrounds, using RNA-Seq to analyze an infection time-course study. This study revealed a significantly higher intensity and more rapid defense gene expression in Topas compared to Westar, both with and without the *R* present. Early in the *L. maculans* infection process, genes observed to have higher expression in Topas than Westar included genes regulating SA and JA pathways and plant cell wall strengthening as well as genes encoding secreted plant chitinases, which presumably target the pathogen's cell wall (**Figure 1**). These host background–dependent differences explain the weaker overall phenotypic response observed in Westar-derived lines (45), which likely contributes to the low reported incidence of *AvrLep1* and *AvrLep2* in *L. maculans* populations in studies using the Westar-derived *LepR1* and *LepR2* lines (1065 and 1135, respectively) for their pathotyping.

Although RNA-Seq analyses allow for temporal and special dissection of both the host and pathogen transcriptome during the infection process, it should be noted that upregulation of genes does not necessarily mean they are indispensable for pathogenicity/resistance. CRISPR–Cas9 disruption of 11 individual highly upregulated *L. maculans* genes did not prevent infection of *B. napus* cotyledons by the affected mutants, and only one mutant produced significantly smaller lesions (131). This suggests a concerted attack on the host involving multiple pathogen-derived factors, as well as functional redundancy among genes evolved to disrupt components of the host cell, each contributing to the overall infection process, allowing for evolutionary adaptability during the ongoing battle between plant and pathogen. There is also the potential for read-mapping bias inherent in RNA-Seq analyses using a reference genome that differs from the actual fungal isolate or host plant line used in the experiment. This can result in the underestimation of transcript abundance for alleles that differ from the reference (150).

AVIRULENCE PROTEIN INTERACTIONS

To date, only the host target and virulence function for the *L. maculans* effector protein AvrLm1 have been identified (88). Yeast two-hybrid (Y2H) screening identified that AvrLm1 interacts with the *B. napus* MAPK 9 (MPK9), which was validated by coimmunoprecipitation (Co-IP) and bimolecular fluorescence complementation assays. Binding of AvrLm1 results in stability and phosphorylation of MPK9. Transient expression of MPK9 in *Nicotiana benthamiana* induces cell death, a phenotype that is enhanced in the presence of AvrLm1. Therefore, AvrLm1 contributes to *L. maculans* virulence by inducing MPK9-mediated cell death at the necrotrophic stage of *L. maculans* infection, which also coincides with the peak of AvrLm1 expression (4–6 dpi) in planta. Transgenic *B. napus* overexpressing MPK9 are more susceptible to *L. maculans*. AvrLm1 is the only non-cysteine-rich (contains only one cysteine) *L. maculans* effector identified to date, pointing to its translocation into the cell and targeting a host intracellular protein. Our attempt to visualize the host cytoplasmic localization of AvrLm1 by generating an *L. maculans* transgenic isolate that expressed C-terminally tagged AvrLm1-mCherry was not successful and only resulted in the detection of AvrLm1 being accumulated at the focal points at the interface of contact between the hyphae and *B. napus* mesophyll cells (88). Challenges with the localization of filamentous plant-pathogen effector proteins during infection are likely due to factors such as sensitivity of detection methods, low or transient expression level of effectors, posttranslational modifications, and stability of the effector proteins, to name a few. Pathogen-independent (i.e., in planta transient expression of effector genes) approaches have been applied to determine cellular location of effector proteins such as *L. maculans* AvrLm4-7 (15). Blondeau et al. (15) reported that AvrLm4-7 is localized to the cytoplasm and based on this suggested that Rlm4 and Rlm7 are likely cytoplasmic

R. However, as we have recently reported (46), Rlm4 and Rlm7 are extracellular WAKL receptors, and although this is not direct evidence to dispute the cytoplasmic location for AvrLm4-7, it strongly suggests the apoplast as the site of AvrLm4-7 encounter with Rlm4 and Rlm7. Results of pathogen-independent localization of effectors should be treated with caution because application of this method to localize effectors of the filamentous plant pathogens has produced conflicting results (142).

The masking effect of AvrLm4-7 on AvrLm3 and AvrLm5-9 has been functionally validated (41, 103). However, at the molecular level, no direct interaction between AvrLm5-9 and Rlm9, AvrLm4-7 and Rlm9, or AvrLm4-7 and AvrLm3 or AvrLm5-9 has been detected, suggesting that an intermediate molecule in the host is involved (41, 79, 103). Similarly, our attempts to detect direct interaction between effector and R proteins using Y2H and Co-IP of in planta transiently expressed proteins or identify other host targets of several *L. maculans* effector proteins, including AvrLm2, AvrLm3, AvrLm4-7, and AvrLm5-9, were inconclusive (L. Ma & M.H. Borhan, unpublished data). In some cases, RLP effector recognition has been shown to be mediated in the apoplast by host-secreted proteins in what has been termed the Guard Model (30, 66, 138), such as in the case of Rcr3 (the guardee), a papain-like cysteine protease that is targeted by the *C. fulvum* effector Avr2, which is in turn recognized by the RLP Cf-2 (the guard). Recognition of AvrLm1-Lep3 and AvrLm2 by LepR3 and Rlm2, respectively, may also require an intermediary protein, whereby the RLPs are binding to a host apoplastic protein after its deactivation by the *L. maculans* effector rather than through direct interaction.

There is no sequence similarity between AvrLm4-7, AvrLm5-9, and AvrLm3; however, it has recently been shown that these three Avr proteins are structurally similar to each other and to Ecp11-1, an effector from *Fulvia fulva* (79). Lazar et al. (79) showed that this structurally similar effector from another species could complement AvrLm3 in the recognition of Rlm3 and that this recognition can be suppressed by AvrLm4-7. Interestingly, in this situation, the AvrLm5–Rlm5 interaction is not masked by AvrLm4-7 despite the *AvrLm5-9* gene locus being responsible for both the AvrLm5 and AvrLm5-9 proteins. This suggests that AvrLm4-7 is able to disrupt some component of the WAKL signaling complex that is specific to Rlm3 and Rlm9. *Rlm4* and *Rlm7* could have then evolved to recognize this disrupted component while *Rlm5* evolved to somehow evade the disruption. Alternatively, *Rlm5*, identified in *B. juncea* (8, 27), may encode a different class of R and represent another case of convergent *R* evolution toward recognition of the same Avr, as seen with *LepR3* and *Rlm1*.

THE ROAD AHEAD

Technological advances in the field of genomics, particularly in the past two decades, and the burst of information from these advances have revolutionized biology research. Prior to the new era in genetics and genomics research, plant pathology, like many other areas of plant biology research, relied on model plant species *Arabidopsis thaliana* and *Nicotiana benthamiana*. Advances in genome sequencing, data science, and gene/genome manipulation techniques have shifted plant pathology research from model plants to crop species. The new genomic era has greatly expanded our knowledge of the molecular interaction between *Leptosphaeria* and *Brassica*. However, there are still many challenges ahead.

There is still limited information regarding the components of receptor complexes and how the perception of *Leptosphaeria* elicitors occurs. Advances in proteomics and the application of new tools such as proximity labeling (83) could provide insight into the formation of receptor complexes, the perception of pathogen effectors and MAMPS, the potential intermediaries that act between elicitor and receptor to facilitate recognition, how receptor specificity is generated, the

identification of the host targets of *L. maculans* avirulence proteins, and downstream signal transduction. Such approaches could shed light on current questions, such as how AvrLm4-7 interferes with the perception of AvrLm3 and AvrLm5-9 via their respective R proteins Rlm3 and Rlm9.

Our understanding of the virulence function of *L. maculans* effectors has been hindered by their sequence diversity and lack of known functional domains. However, structural similarity of effector proteins could imply functional homology and reveal their biological activities, as has been reported for a handful of the *L. maculans* effectors. Rapid expansion of databases and advances in the field of computational biology, data mining, and AI hold great promise. An example is the recent release of AlphaFold 2, a software that predicts 3D protein structure with near experimental accuracy even in the absence of known similar protein structures (64).

Although there have been considerable advances in understanding the biology of *Leptosphaeria* virulence, there are still many unknowns, the most intriguing of all is the question of delivery of effector proteins into the host cell and determining an intra- or extracellular site of action for each of them. Furthermore, our knowledge of spatial and temporal expression of *Leptosphaeria* effectors is increasing, although other aspects of *Leptosphaeria* effector biology, such as posttranslational modification and possible effector oligomerization are entirely unknown. Comparative genomics with closely related fungal species should be explored more extensively and could prove to be very instrumental in finding answers to some of the current unknowns of the *Brassica–Leptosphaeria* pathosystem. The development of CRISPR technology (1) has the potential to aid in these investigations through the development of isolates with specific effectors knocked out, allowing for comparison of different virulent isolates that differ in only single genes. Accumulating genome and transcriptome sequence data for *Brassica* and *Leptosphaeria* species can be investigated to map the *Brassica–Leptosphaeria* interactome networks and identify key host targets (hubs) of the pathogen to be used in engineering novel and broad-spectrum resistance. Years of data regarding QR-associated loci and defense-related gene expression have been amassed, and we are hopeful that this will soon lead to the identification of genes underlying QR, which would greatly aid modern breeding efforts globally.

As with all fields of science, the future progress in the *Brassica–Leptosphaeria* field also requires effective coordination of research and sharing of data and resources among the blackleg research community. Although continued rapid technological advances coupled with ever-expanding biological data and computational power herald an exciting future in *Leptosphaeria–Brassica* research, classical research approaches such as cell biology, histopathology, field observation, and data collection remain a critical component of holistic research.

As global demand continues to increase for this important oilseed crop, disease pressure from *L. maculans* and other pathogens will also increase. Understanding how the interaction occurs, how it can be manipulated to reduce disease pressure, and how to develop the resources for scientists to answer these questions will remain a top priority in the coming years.

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