Transcriptome Analysis of *Rlm2*-Mediated Host Immunity in the *Brassica napus*–*Leptosphaeria maculans* Pathosystem

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Our study investigated disease resistance in the *Brassica napus*–*Leptosphaeria maculans* pathosystem using a combination of laser microdissection, dual RNA sequencing, and physiological validations of large-scale gene sets. The use of laser microdissection improved pathogen detection and identified putative *L. maculans* effectors and lytic enzymes operative during host colonization. Within 24 h of inoculation, we detected large shifts in gene activity in resistant cotyledons associated with jasmonic acid and calcium signaling pathways that accelerated the plant defense response. Sequencing data were validated through the direct quantification of endogenous jasmonic acid levels. Additionally, resistance against *L. maculans* was abolished when the calcium chelator EGTA was applied to the inoculation site, providing physiological evidence of the role of calcium in *B. napus* immunity against *L. maculans*. We integrated gene expression data with all available information on cis-regulatory elements and transcription factor binding affinities to better understand the gene regulatory networks underpinning plant resistance to hemibiotrophic pathogens. These in silico analyses point to early cellular reprogramming during host immunity that are coordinated by CAMTA, BZIP, and bHLH transcription factors. Together, we provide compelling genetic and physiological evidence into the programming of plant resistance against fungal pathogens.

Keywords: defense signaling pathways, elicitors, ethylene, fungus–plant interactions, genomics, hormones, jasmonic acid, MAMPs,

metabolomics, PAMPs, plant antifungal responses, plant responses to pathogens, proteomics, resistance genes, salicylic acid

Blackleg disease, caused by the hemibiotrophic fungus *Leptosphaeria maculans*, is a major cause of yield loss in the family Brassicaceae (Fitt et al. 2008). Following spore germination on the cotyledon or leaf surface, pathogen hyphae bypass the epidermis through stomata or surface wounding (Hammond et al. 1985) and move into the apoplast, where the fungus grows biotrophically. Activation of host immune receptors in response to *L. maculans* triggers cell signaling systems that initiate defense (Ma and Borhan 2015). These receptors include race-specific resistance (*R*) genes effective at juvenile stages of the plant lifecycle (Raman et al. 2013). When plant *R*-gene products and corresponding pathogen avirulence (*Avr*) gene products interact, an incompatible interaction occurs, leading to pathogen arrest. Sixteen *R* genes have been named in *Brassica* germplasm (*LepR1* to *R4*, *RlmS*, and *Rlm1* to *11*) and their corresponding effectors have been identified (Raman et al. 2013). Many of the *L. maculans* *Avr* genes have been cloned; however, only two of the *R* genes, *Rlm2* (Larkan et al. 2015) and *LepR3* (Larkan et al. 2013), have been cloned and identified as membrane surface receptor-like proteins (RLP).

L. maculans is assumed to remain undetected in the apoplast for several days while adapting to host antimicrobial compounds (Stotz et al. 2014). Although the genetics of the *L. maculans*–*B. napus* interaction is fairly well explained, the interplay between *L. maculans* and its host during establishment is largely unresolved. Several recent reports indicate the complexity of *L. maculans* effector-mediated suppression of host defense. For example, *AvrLm4-7* affects the host immune responses associated with salicylic acid (SA) and ethylene (ET) signaling and reactive oxygen species (ROS) production (Nováková et al. 2015); however, the observed effects of *AvrLm4-7* may be indirect because it prevents host detection of *AvrLm3* and *AvrLm5-9* (Ghanbarnia et al. 2018; Plissonneau et al. 2016). The production of cytokinins by *L. maculans* (Trdá et al. 2017) or the activation of additional, yet-to-be-identified fungal effectors may also affect immune signaling and colonization of the host plant. Despite the growing body of evidence dissecting this pathosystem, little is known about early host immune signaling during pathogen establishment and certainly nothing about fungal gene activity early in the infection process directly at the infection site.

The receptor-like R protein *Rlm2* interacts with membrane receptor kinase *SOBIR1* (Larkan et al. 2015) and likely partners

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with BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) to form a fungal receptor complex (Ma and Borhan 2015). Pathogen detection through this receptor complex triggers host phosphorylation cascades that integrate with signals from pathogen-associated molecular pattern (PAMP) immune receptors, including chitin-elicited receptor kinase 1 (CERK1) (Ma et al. 2016; Peng et al. 2018; Yamada et al. 2017). These cascades function synergistically with signals from calcium decoders such as calcium-dependent protein kinases (CPKs/CDPKs) (Boudsocq et al. 2010) and cues from intracellular redox states. Further, these signals may be regulated and amplified through intracellular nucleotide-binding-leucine-rich-repeat (NLR) receptors (Li et al. 2015; Wu et al. 2019). In *Arabidopsis*, this combination of signals leads to widespread transcriptional reprogramming (Yuan et al. 2017) that involves CAMTA, WRKY, BZIP, ERF, and BES transcription factors (TFs) (Li et al. 2016; Seybold et al. 2014). Additionally, a SOBIR1 homolog in cotton was recently shown to directly activate bHLH TFs (Zhou et al. 2018), emphasizing the need to characterize transcriptional reprogramming during RLP-mediated immunity. In *Brassica napus* and other members of the Brassicaceae family, global transcriptional regulation of defense remains largely unexplored. Rather, research has focused on individual TFs or TF families (He et al. 2016; Zhou et al. 2017).

Widespread transcriptional changes during defense lead to specific hormone responses that modulate cellular signaling and propagate defense signals across the cotyledon. Previously, SA signaling has been generally associated with defense against biotrophic fungal pathogens, whereas ET and jasmonic acid (JA) have been associated with defense against necrotrophs (Glazebrook 2005). However, this paradigm is heavily scrutinized because an increasing number of studies show essential roles for JA signaling in biotrophic and hemibiotrophic interactions, especially at early infection stages, and exceptions suggest more complex interactions of defense hormones (Derksen et al. 2013). In the grapevine–*Plasmopara viticola* (powdery mildew) pathosystem, JA accumulates specifically in incompatible lines in the first 24 h of infection (Guerreiro et al. 2016). Both JA and abscisic acid (ABA) play important roles in early defense signaling in the wheat–*Fusarium graminearum* pathosystem (Qi et al. 2016), and both SA and JA are associated with potato defense against the hemibiotroph *Verticillium dahliae* (Zhu et al. 2017). Previous RNA-seq studies have shown increased JA-associated gene activity in RLP-SOBIR1 mediated immunity (Becker et al. 2017b; Zuluaga et al. 2016); however, JA concentrations have never been directly measured and the signaling mechanisms upstream of JA activation are unclear.

Signaling molecules responsible for immunity such as receptors, kinases, TFs, and regulators of hormone production are cell specific and mainly targeted at the host–pathogen interface (Chandran et al. 2010; Lenzi et al. 2016). Because available transcriptome data for the *B. napus*–*L. maculans* pathosystem is derived from the response of whole- or hand-dissected cotyledons (Becker et al. 2017b; Haddadi et al. 2016; Lowe et al. 2014; Sonah et al. 2016), these signals are often too weak to be detected. The combination of laser microdissection (LMD) and transcriptome profiling can monitor gene activity within the first host cells responding to pathogen. This technique has improved our understanding of the soybean response to *Heterodera glycines*, identifying specific activation of heat-shock and *LOX* genes during host incompatibility (Klink et al. 2005). Roux et al. (2014) used a similar strategy to identify tissue-specific gene sets associated with nodule formation in *Medicago truncatula*, capturing known and novel symbiotic genes validated by in situ mRNA hybridizations. Although LMD has been used to investigate host–pathogen interactions previously (Becker et al.

2017b; Chandran et al. 2010; Hacquard et al. 2010; Klink et al. 2005; Roux et al. 2014; Tang et al. 2006), these studies generally performed downstream analysis with quantitative PCR (qPCR) or arrays. To our knowledge, this is the first study to combine the resolution of LMD with the depth of next-generation RNA-seq in the study of a plant host–microbe interactions.

This study captures gene activity in *B. napus* and *L. maculans* directly at the host–pathogen interface in a susceptible (Topas) and resistant transgenic line (T-*Rlm2*) (Larkan et al. 2015). Our predictive TF networks suggest that bHLH, CAMTA, and bZIP TFs are active by 1 day postinoculation (dpi) and are likely involved in cellular reprogramming during host–pathogen incompatibility. TF activity was associated with rapid JA and calcium signaling responses unique to the resistant line. Quantification of endogenous JA levels showed increased production of this important phytohormone in resistant hosts. Application of calcium chelators at the inoculation site prevented host defense activation and completely abolished resistance, providing physiological evidence that calcium influx is required for *Rlm2*-mediated immunity. Additionally, this research suggests that prolific pathogen-induced repression of host defenses at the inoculation site coincides with activation of known *L. maculans* effectors. Taken together, our research provides a high-resolution analysis of gene activity guiding plant immune responses during the earliest stages of host–pathogen interactions and is complemented by physiological investigation.

RESULTS

Overview of plant responses to *L. maculans* (AvrLm2).

First, we examined the phenotype of susceptible Topas DH16516 and the transgenic line NLA51-1 containing *Rlm2* (Topas:*Rlm2*) to characterize disease progression over time. At 1 and 3 dpi, no difference in lesion color or size was visible between genotypes (Fig. 1A). By 10 dpi, lesion spread was significant in susceptible plants, encompassing the entire leaf by 14 days. In transgenic plants containing *Rlm2*, cotyledons remained healthy and green at 14 dpi, with a necrotic ring observed around the inoculation site.

Combined LMD and RNA sequencing improves pathogen detection at the earliest stages of infection.

To capture gene activity at the host–pathogen interface, we used LMD-RNA-seq, targeting the first 100 µm of healthy plant tissue adjacent to the inoculation site, equal to approximately two plant cell layers (Fig. 1B). Following histological processing, cellular structure was well preserved in cross sections (Fig. 1B). To capture early signaling events during *L. maculans*' biotrophic phase, we focused our analysis on 1 and 3 dpi, before visible lesion spread.

Following read quality control and alignments to the *B. napus* genome, hierarchical clustering was performed to identify relationships between genotypes and treatments (Fig. 1C). Samples clustered based on treatment and time point, apart from inoculated susceptible plants at 3 dpi that clustered with mock controls, indicating a lack of a measurable defense response at this early stage in the susceptible line. Next, we plotted detection levels of *L. maculans* transcripts within each treatment (Fig. 1D). In all mock controls, few reads aligned to the *L. maculans* genome (<0.2%). At 1 dpi, pathogen alignment rates were similar in inoculated susceptible (5.7%) and resistant lines (5.2%). At 3 dpi, alignment rates were 14.6 and 3.6% in susceptible and resistant lines, respectively (Fig. 1D), suggesting that resistant plants may be effectively stopping the pathogen by 3 dpi. Additionally, we compared our alignment rates to those of RNA-seq reads derived from hand-dissected inoculation sites at 3 dpi (Fig. 1D). Data show a marked increase

in pathogen detection through the application of LMD, with an average 37-fold higher alignment rate in LMD-collected samples.

L. maculans gene expression profiles are different in resistant and susceptible *B. napus* lines.

We next explored differences in the pathogen's gene activity during infection of susceptible and resistant lines. Principal component analysis suggests similar mRNA profiles at 1 dpi regardless of host genotype but divergent responses at 3 dpi, as indicated by independent clusters of samples (Fig. 2A). To elucidate the genes contributing to these responses, we investigated differential gene expression between *L. maculans* colonizing resistant and susceptible plants at both time points (Fig. 2B) and between time points (Fig. 2C). At 1 dpi, only 13 *L. maculans* genes, including hypothetical proteins and two putative pectate lyases (*LEMA_T033320* and *LEMA_T001630*) (Supplementary File S1) were differentially expressed between the two interactions (Fig. 2B). At 3 dpi, this increased to 927 *L. maculans* differentially expressed genes (DEGs) (approximately 8% of the *L. maculans* genome) that were influenced by host genotype. Differential gene expression analyses between time points identified 633 and 928 *L. maculans* DEGs that responded during infection of susceptible and resistant lines, respectively, and an additional 554 DEGs that were shared between interactions (Fig. 2C). Together, data revealed global shifts in *L. maculans* gene expression during infection that are influenced by host genotype.

Expression of known *L. maculans* Avr genes during infection showed that *AvrLm2* and *AvrLm5-9* are the first Avr genes expressed by the pathogen, with detectable expression at 1 dpi and approximately 100-fold higher expression at 3 dpi (Fig. 2D). Expression of *AvrLm6* was first detected at 3 dpi, and *AvrLm1*, 4-7, and 11 were undetected (fragment per kilobase per million reads [FPKM] = 0) in this dataset and are likely absent in strain 00-100.

To determine putative *L. maculans* effectors involved in pathogen establishment, we identified secretory signals within the N-terminal domain of translated DEGs (Supplementary File S2). The top five most upregulated genes (3 versus 1 dpi) within the predicted *L. maculans* secretome contained *AvrLm2* and *AvrLm6*, along with three hypothetical proteins of unknown function (*LEMA_T082980.1*, *LEMA_T054900.1*, and *LEMA_T086540.1*), highlighting the ability of our analysis to identify a combination of putative and characterized pathogen effectors.

Further analysis of the secretome identified *L. maculans* degradative enzymes and Avr genes with higher gene expression during colonization of susceptible plants at 3 dpi (Fig. 2D). This included *AvrLm2*, 5-9, and 6, putative carbohydrate binding molecules (CBMs) with homology to starch hydrolyzing amylases (*LEMA_T082710.1* and *LEMA_T018480.1*), glucoside hydrolases (*GH6*, 12, 16, 17, 35, and 39), peptidases (*LEMA_T110360.1*, *LEMA_T040600.1*, *LEMA_T082270.1*, and *LEMA_T079720.1*), and a ribonuclease (*LEMA_T108620.1*).

The production of cytokinins by *L. maculans* has been shown in vitro, and cytokinin biosynthetic enzyme AK1 activity increases disease severity (Trdá et al. 2017). Both ADENOSINE KINASE 1 (*AK1*) and ISOPENTENYLTRANSFERASE 1 (*IPT1*), involved in cytokinin production, were expressed at 1 dpi, with decreasing expression by 3 dpi in both lines. In resistant plants, there was no detectable expression of *AK1* at 3 dpi.

Early differences in gene expression between resistant and susceptible plants.

With notable differences in pathogen gene activity observed between compatible and incompatible interactions, we were next interested in examining host responses in plant cells directly adjacent to the inoculation site. At 1 dpi, 2,572 (47%) upregulated DEGs (uDEGs) were shared between both genotypes,

Fig. 1. Phenotypic response of resistant and susceptible hosts and preliminary data analysis. **A**, Disease progression in resistant Topas Rlm2 (top) and susceptible Topas DH16516. Lesion spread is observed in susceptible hosts by 10 days postinoculation (dpi). Scale = 1 cm. **B**, Cross section of histologically processed cotyledon showing excision of plant tissue by laser microdissection. Collected fragments were used to generate sequencing libraries. **C**, Dissimilarity matrix, showing relationships between treatments and genotypes; R = resistant, S = susceptible, Lm = *Leptosphaeria maculans*, and mk = mock. **D**, Average mapping rates of all treatments to the *L. maculans* genome. Data show highest mapping rates in susceptible hosts at 3 dpi, and lowest in hand-dissection cotyledons (cot) and mock controls.

with an additional 541 (9.9%) uDEGs specific to susceptible plants and 778 (14.2%) specific to resistant plants. At 3 dpi, the number of shared uDEGs decreased to 2,484 (31.0%), with 1,193 (14.9%) and 2,105 (26.3%) uDEGs specific to susceptible and resistant plants, respectively. Similar trends in the number and distribution of downregulated DEGs were observed in response to *L. maculans* inoculation (Fig. 3B). Together, data indicate a more conserved response between lines at 1 dpi and an overall stronger response to *L. maculans* in resistant plants at the mRNA level. All gene expression data are presented in Supplementary File S3.

JA and calcium-associated transcription factors influence the cellular response to *L. maculans* in resistant plants.

Gene regulatory networks from our coexpressed gene sets were then studied using SeqEnrich. A putative gene regulatory network was identified in resistant plants early in the defense response (Fig. 3C). Data predict TF homologs canonically associated with JA (MYC3 and JAM1), ABA (AKS2 and ABF3), and calcium (CAMTA5 and CAMTA3/SR1) signaling that regulate essential defense processes at the inoculation site in resistant plants. This includes overarching regulation of calcium ion binding, response to ABA, JA-mediated signaling, brassinosteroid-mediated signaling, response to chitin, and g-protein coupled receptor activity (Fig. 3C).

To validate gene activity, we measured mRNA levels of *JAM1* and *MYC3* in all treatments using LMD-qPCR and found that expression of *JAM1* and *MYC3* was 8.8- and 1.8-fold higher, respectively, in resistant plants compared with their susceptible counterparts (Supplementary Fig. S1). Both genes

were upregulated in susceptible and resistant lines when compared with mock treatments.

Genes associated with growth and development have contrasting expression profiles at the inoculation site of susceptible and resistant plants at 1 dpi.

We were also interested in genes at the host–pathogen interface that contribute to susceptibility. We discovered a putative regulatory network specific to susceptible plants at 1 dpi associated with development, energy production, and plastid maintenance (Fig. 3D). Specifically, four DNA sequence motifs (MYB-like, ZF, CAMTA, and GATA) and one CG-1 CAMTA TF were predicted to coordinate bioprocesses associated with the chloroplast, mitochondrial membrane, respiratory chain complex I, polysaccharide metabolism, ribosome biogenesis, and glucose 6-p transport (Fig. 3D). In contrast, genes associated with photosynthesis and primary metabolism were identified in a putative regulatory network downregulated in resistant plants (Supplementary Fig. S2). Downregulated DEGs were associated with response to cytokinin, photosynthesis, amino acid biosynthesis, metabolism, and unsaturated fatty acid biosynthesis, indicating that these processes may be inhibited in resistant plants.

A heightened defense response in resistant hosts coincides with attenuated defense in susceptible cotyledons.

We analyzed a complex predictive network active specifically in resistant plants at 3 dpi composed of WRKY, BZIP, bHLH, CAMTA, and heat-shock TFs (Fig. 3E). These TFs were predicted to regulate plant immune responses, including protein

Fig. 2. *Leptosphaeria maculans* gene expression during its infection of susceptible and resistant hosts. **A**, Principal component analysis showing relationships between pathogen transcriptomes in infected treatments. Transcriptomes cluster together at 1 day postinoculation (dpi) and diverge by 3 dpi. **B**, Significantly ($P < 0.05$) differentially expressed genes between *L. maculans* infection on resistant hosts versus susceptible hosts. **C**, Significantly ($P < 0.05$) differentially expressed genes between treatments at 3 and 1 dpi. **D**, Selected gene set of virulence factors and known effectors, and their expression in reads per kilobase per million mapped (RPKM).

ubiquitination; hypersensitivity; autophagosome; epigenetic regulation; mitogen-activated protein kinase (MAPK) activity; response to ET, SA, AND ABA; and calcium binding. We also identified a putative regulatory network active in all inoculated plants except susceptible plants at 3 dpi, suggesting that their expression may become attenuated in susceptible cotyledons. This network contains WRKY, heat-shock, ABA INSENSITIVE GROWTH 1, and CAMTA TFs that putatively regulate genes associated with kinase activity, JA-mediated signaling, PAMP-receptor signaling, and a diverse set of other plant defense responses.

Calcium signaling is required for resistance and basal defense against *L. maculans*.

Because our network analyses associated resistance with CAMTAs and calcium-related biological functions, we

investigated mRNA levels of calcium transporters, signal transducers, and binding proteins (Fig. 4A). Homologs of calcium sensors *CAM1*, *CAM5*, *CAM7*, and *CAM-LIKE 40* were 2.15-fold more expressed in resistant plants at 1 dpi. Similarly, calcium transporters (*CYCLIC NUCLEOTIDE GATED CALCIUM CHANNEL 3*, *12*, and *19*; and *CALCIUM ATPASE 2* and *11*) and calcium dependent signal transducers (*CALMODULIN-DOMAIN PROTEIN KINASE 5* and *9*; *CALCIUM-DEPENDENT PROTEIN KINASE 6* and *28*; and *CALCINEURIN B-LIKE GENE 1*) were an average of 1.41-fold higher in resistant plants at 1 dpi, increasing to 4.15-fold by 3 dpi.

Previous studies have used the calcium chelator EGTA to explore the role of calcium in bioprocesses such as defense (Hu et al. 2007; Lee and Back 2016; Ma et al. 2017; Qiao et al. 2015). Here, we applied EGTA before inoculation with

Fig. 3. Differential gene expression analyses and predictive transcription factor networks. **A**, Significantly ($P < 0.05$) upregulated genes in each treatment as compared with their mock-inoculated controls. Four-way VENN diagram shows number of unique or shared genes between treatments. **B**, Significantly ($P < 0.05$) downregulated genes in each treatment as compared with their mock-inoculated controls. Four-way VENN diagram shows number of unique or shared genes between treatments. **C**, Predicted network showing transcription factors (squares), DNA binding motifs (diamonds), and gene ontology terms (circles) enriched in resistant-specific differentially expressed genes at 1 day postinoculation (dpi) (hexagon). Gray lines show predicted connections between transcription factors, motifs, gene ontology terms, or the resistant-specific gene set. **D**, Predicted network showing genes specifically upregulated in susceptible hosts at 1 dpi. **E**, Predicted network showing genes specifically upregulated in resistant hosts at 3 dpi.

L. maculans to explore how the interaction would occur at a calcium-depleted interface and to provide physiological support that calcium is required for *Rlm2*-mediated immunity. EGTA alone caused minor chlorosis around the inoculation site at 3 dpi (Fig. 4C). EGTA pretreatment abolished resistance and caused rapid pathogen proliferation in both lines (Fig. 4C). By 3 dpi, fungal load was 19.5-fold higher in EGTA-pretreated resistant cotyledons versus *L. maculans* alone, and similar to pretreated susceptible cotyledons (Supplementary Fig. S3). Lesions continued to spread aggressively in EGTA-pretreated plants, with complete cotyledon coverage and visible pycnidia by 11 dpi.

We used *PR1* mRNA level as an indicator of SA-mediated defense activation in all treatments at 1 and 3 dpi (Fig. 4D). Inoculation without EGTA application induced *PR1* gene activity at both time points, with higher expression in resistant

lines (1.48- and 8.45-fold higher at 1 and 3 dpi, respectively). EGTA application without *L. maculans* inoculation transiently activated host defense, with a 149-fold increase in *PR1* mRNA level in both treatments at 1 dpi that diminished by 3 dpi that may be related to observed chlorosis. EGTA pretreatment preceding inoculation with *L. maculans* strongly increased *PR1* accumulation at both time points with no difference between genotypes, indicating that SA signaling was not compromised by apoplastic calcium depletion (Fig. 4D).

Hormone signaling during *Rlm2*-mediated immunity to *L. maculans*.

Because hormone signaling is essential for host defense programming, we examined the expression of biosynthetic genes and markers directly at the inoculation site for ET, JA, SA,

Fig. 4. Role of calcium signaling in resistance and inhibition of pathogen establishment. **A**, Log₂ fragment per kilobase per million reads fold change of calcium transporters and signal transducers. Darker shading denotes upregulation and lighter shading downregulation versus mock; dpi = days postinoculation. **B**, Relative mRNA abundance of select genes from A as measured by quantitative PCR (qPCR). **C**, Treatment of cotyledons with calcium chelator EGTA prior to infection at 3 dpi. EGTA + Lm = coinoculation with calcium chelator and pathogen *Leptosphaeria maculans*, Lm = treatment with chelator alone, EGTA = inoculation with water. Scale = 1 mm. **D**, relative mRNA abundance of *PR1* in following EGTA application. Significance of qPCR results was determined with a one-way analysis of variance ($P < 0.05$) between the different treatments, where significance is indicated by different letters. Significant deviation from mock controls is denoted by an asterisk (*), as determined by a Student's *t* test ($P < 0.05$).

pipecolic acid (PiP), auxin, and ABA (Fig. 5). *ACC OXIDASE 2* and *ACC SYNTHASE 6*, essential for ET production, were an average of 1.97- and 2.01-fold higher in resistant cotyledons at 1 and 3 dpi, respectively. Primary JA biosynthesis genes *ALLENE OXIDE SYNTHASE* and *ALLENE OXIDE CYCLASE 3* had similar expression levels between lines at 1 dpi; however, at 3 dpi, they were downregulated specifically in susceptible cotyledons (Fig. 5A). Additionally, a marker of JA/ET signaling, *PLANT DEFENSIN 1.2* (*PDF1.2*), was 3.2-fold downregulated in susceptible plants, in contrast to a 9.6-fold increase in resistant plants. *COI1* was the only JA marker tested with higher overall expression in susceptible plants, with 59% higher expression. Other markers of JA activity (*ETR1*, *JAZ1*, *JAR1*, and *MPK5*) were an average of 83% higher in resistant hosts. Together, data indicate a robust JA response at the host–pathogen interface at 3 dpi specifically in resistant plants.

Because of the higher expression of JA-related biosynthesis genes and markers in resistant plants, we measured the physiological JA concentration in all treatments (Fig. 5B). At both time points, JA levels were higher in resistant cotyledons. Interestingly, JA decreased in both susceptible and resistant cotyledons at 1 dpi as compared with mock controls (Fig. 5B). At 3 dpi, JA concentrations returned to near control levels in susceptible cotyledons and increased 1.8 times in resistant cotyledons.

Homologs of *ISOCHORISMATE SYNTHASE 1*, critical to SA production, were an average of 1.6- and 3.1-fold higher in resistant cotyledons at 1 and 3 dpi, respectively, as compared with their susceptible counterpart (Fig. 5D). In addition, regulators of ISC1 gene activity (*TGA1*, *SARD1*, and *CBP60g*) were an average of 2.64-fold higher in resistant plants at 3 dpi; however, the receptors *NPR1*, -3, and -4 and transcription factors *TGA5* and -6 showed no overall difference in expression between the two lines. EDS5, another downstream regulator of SA activity, was fourfold higher in resistant plants at 3 dpi. PiP biosynthesis branches from the SA pathway following *SARD1* and *CBP60g* signaling, and has emerged as an important signaling hormone. Markers for PiP biosynthesis and activity were an average of 2.45-fold higher in resistant plants at 3 dpi, suggesting that this pathway may play a role in resistance (Fig. 5C).

Auxin markers showed similar expression in both hosts at 1 dpi. At 3 dpi, expression of biosynthetic gene *NITILASE 2* along with markers *AFB1*, *TIR1*, *HAT2*, and *ARF1* was an average of 53% higher in susceptible hosts (Fig. 5E). ABA markers showed no clear pattern between the two hosts at 1 and 3 dpi, with mixed expression (Fig. 5F). For example, putative homologs of markers *ABSCISIC ACID RESPONSIVE ELEMENTS-BINDING FACTOR 1* (*AREB1*) and *AREB2* were upregulated only in susceptible plants, whereas a homolog of key biosynthetic enzyme *NCED3* was specifically downregulated.

Pathogen detection and immune signaling is compromised in susceptible plants and maintained in *Rlm2*-mediated immunity.

From our network analyses, we identified cohorts of genes expressed specifically in resistant *Rlm2* plants at 3 dpi (Fig. 3E) or attenuated in susceptible plants at the same time point directly at the host–pathogen interface. These genes coded for core signaling machinery involved in fungal detection and defense elicitation through the receptors *SOBIR1* and *CERK1*. We plotted the mRNA levels of these receptors along with their coreceptors and downstream signal transduction machinery (Fig. 6A and B). With the exception of *MAPK KINASE 9* (*MKK9*) and *BOTRYTIS-INDUCED KINASE 1*, the entire pathway was more highly expressed in resistant plants at 3 dpi (Fig. 6B). This includes homologs of *SOBIR1* and *CERK1*, along with functional partners *BAK1* and *LYK5* and signal

transducers *MAPK/ERK KINASE KINASE 1* (*MEKK1*); *MKK1*, -2, and -4; and *MPK3*, -4, and -6 that were an average of 4.0-fold higher at the host–pathogen interface during *Rlm2*-mediated immunity.

We then used targeted transcript profiling to measure mRNA abundance of *SOBIR1*, *MAPK/ERK KINASE KINASE 1* (*MEKK1*), *MPK4*, and *MPK6* directly at the inoculation site (Fig. 6C). At 1 dpi, all targets were upregulated during infection and showed similar expression profiles between genotypes (Fig. 6C). At 3 dpi, data show no difference between susceptible interactions and mock controls; however, all genes were significantly upregulated in resistant plants, with mRNA levels 2.4- to 8.2-fold higher than those observed in mock treatments (Fig. 6C).

Fig. 5. Expression of hormone biosynthesis genes and signaling markers, and jasmonic acid (JA) concentration in cotyledons, in response to *Lep-tosphaeria maculans*. **A**, Relative log₂ fragment per kilobase per million reads (FPKM) fold change of biosynthetic genes and signaling markers for ethylene (ET) and JA in resistant and susceptible hosts; dpi = days post-inoculation. **B**, Concentration of JA in hand-dissected infection sites as compared with mock controls. Error bars show standard error. **C**, Relative log₂ FPKM fold change of biosynthetic genes and signaling markers for pipecolic acid; **D**, salicylic acid (SA); **E**, auxin (IAA), and **F**, abscisic acid (ABA).

Because intracellular NLRs also play a role in amplifying the host immune response, we plotted the expression of all differentially expressed NLRs along with known NLR lipase-like proteins involved in signal transduction or yet-to-be-identified functions (Supplementary Fig. S4). We observed patterns of gene activity with similar expression of NLRs and markers for NLR-related signaling between hosts at 1 dpi, and a 93% overall expression increase in resistant hosts at 3 dpi.

DISCUSSION

We profiled *B. napus* and *L. maculans* gene activity directly at the host–pathogen interface to better understand *Rlm2*-mediated immunity. A major challenge in dual transcriptome analysis of this pathosystem is overcoming low pathogen abundance at early infection stages. At 3 dpi, *L. maculans* mapping rates of <0.5% are commonly recorded, with researchers performing analyses with <1× coverage at these early disease stages (Haddadi et al. 2016; Sonah et al. 2016). Here, LMD markedly increased sensitivity with achieved *L. maculans* mapping rates up to 5.7% at 1 dpi and 14.5% at 3 dpi, thus providing the necessary resolution to confidently monitor *L. maculans* gene activity during establishment in *B. napus* cotyledons.

Approximately 11.9% of the *L. maculans* genome responded during the first 3 days of infection, with major differences observed based on host genotype. Putative starch hydrolyzing CBMs, glucoside hydrolases, peptidases, and ribonucleases were strongly upregulated during infection of susceptible plants, suggesting that *L. maculans* may be capable of mobilizing and utilizing host resources during establishment in susceptible cotyledons. The activation of bioprocesses related to plastid maintenance and photosynthesis in susceptible cotyledons at 24 h postinoculation may explain the elevated expression of *L. maculans* digestive enzymes in this line. In immune *Rlm2* cotyledons, these photosynthetic processes are downregulated along with primary production and cytokinin responses, perhaps to restrict nutrient production that would support *L. maculans* progression into host tissues.

We observed rapid and widespread activation of calcium, JA, and ABA signaling during incompatible interactions directly at the host–pathogen interface that is associated with the activity of bHLH, CAMTA, and BZIP TFs, including *CAMTA3*, *CAMTA5*, and *BZIP1*. In *Arabidopsis*, *CAMTA3* and *CAMTA5* transcriptionally activate genes associated with ABA signaling, including the master regulator *DREB1* (Kidokoro et al. 2017). In *Arabidopsis*, *BZIP1* is transcriptionally activated by calcium signaling cascades and initiates ABA-mediated changes to glucose and amino acid metabolism (Hartmann et al. 2015). Further, we predict that *MYC3* and *JAM1*, along with uncharacterized bHLHs, regulate genes canonically associated with wounding, heat-shock, salinity, and plasma membrane signaling in resistant (*Rlm2*) plants. In *Arabidopsis*, *MYC3* is a

Fig. 6. Expression levels of cell-surface receptors and signal transduction machinery responsible for initiating host defense. **A**, Partial model of signaling events following activation of cell-surface receptors SOBIR and CERK1. Activation of signal transduction cascades results in regulation of gene transcription. TFs = transcription factors. **B**, Gene expression changes (log₂-fold change versus mock) of signaling pathway components as measured by RNA-seq. Genes associated with surface receptors and mitogen-activated protein kinase activity are upregulated in resistant hosts but not susceptible at 3 days postinoculation (dpi). **C**, Gene expression of selected genes measured by quantitative PCR (qPCR). Significance of qPCR results for each target individually was determined with a one-way analysis of variance ($P < 0.05$) between treatments, where significance is indicated by different letters. Significant deviation from mock controls is denoted by an asterisk (*), as determined by a Student's *t* test ($P < 0.05$).

positive regulator of JA (Cheng et al. 2011) and JAM1 is a modulator of JA signaling (Sasaki-Sekimoto et al. 2013) that coordinates JA defense responses. The important role of bHLH TFs in RLP-SOBIR1 immunity is also supported by the recent discovery that bHLH TFs can be directly activated by SOBIR1 in cotton (Zhou et al. 2018). Together, this provides a putative regulatory framework activated during *Rlm2*-mediated immunity that reprograms cells to respond to the hemibiotrophic pathogen. Because little information is available regarding transcriptional control of *B. napus* immunity, the uncharacterized BZIP and bHLH TFs identified through our analyses serve as targets for future functional studies.

Marked distinctions in the expression of JA biosynthesis genes and markers suggest that JA is repressed in susceptible cotyledons and is consistent with reports showing repression of JA markers during compatible *L. maculans*–*B. napus* interactions (Becker et al. 2017b; Lowe et al. 2014), despite endogenous JA levels never being assayed. Here, we confirm through quantification of endogenous JA levels that this important signaling hormone is present at higher concentrations in resistant *Rlm2* cotyledons as compared with susceptible plants. JA signaling plays a central role in defense against necrotrophs by limiting cell death (Rossi et al. 2011) and favoring accumulation of antifungal compounds (Antico et al. 2012). Because *L. maculans* has been described as a primarily necrotrophic fungus (Staal and Dixelius 2008), an effective response to the pathogen would likely involve extensive JA signaling. Accordingly, our data suggest that *Rlm2*-mediated immune signaling at the inoculation site triggers production of JA that can modify gene expression before the necrotrophic transition of *L. maculans*. The ability of JA to antagonize cell death provides an explanation for the delayed onset of cell death commonly observed in *B. napus* cotyledons during the incompatible response to *L. maculans* (Stotz et al. 2014).

Rlm2-mediated immunity may fortify defense and protect signaling pathways from pathogen interference through the production of calcium signals such as those initiated through the *CPKs/CDPKs* upregulated in our dataset. For example, *CPK5* stimulates hormone production in response to fungi (Gravino et al. 2015) and phosphorylates RBOHD to produce ROS (Dubiella et al. 2013). Additionally, core calcium sensors upregulated in resistant plants activate strong defensive cues; *CAM1* increases nitric oxide (Choi et al. 2009; Zhou et al. 2016) and ROS (Choi et al. 2009) production, which is amplified in the presence of CBL1 (Drerup et al. 2013). Thus, calcium signaling likely in concert with *Rlm2*/SOBIR1-mediated signaling is likely to direct and amplify the immune response.

When the calcium chelator EGTA was applied to the inoculation site, *Rlm2*-mediated immunity was abolished and infection progressed rapidly in both lines, suggesting that calcium signaling is required to initiate resistance. The ability of EGTA to affect disease progression has been demonstrated previously. Pretreatment of wheat leaves with EGTA prevented NO production and delayed the peak hypersensitive response to *Puccinia triticina* by 24 h (Qiao et al. 2015), and also increased *Tobacco mosaic virus* load in *Arabidopsis* leaves by obstructing systemic acquired resistance and preventing *PR1* accumulation (Zhao et al. 2018). In our study, accumulation of *PR1* suggests that SA signaling was not compromised by EGTA treatment and occurs upstream or independent of calcium influx. Additionally, because calcium deficiency in cotyledons initiates abiotic stress responses (Simon 1978), SA production was likely stimulated by calcium chelation in combination with *L. maculans* inoculation. SA production may have induced cell death and contributed to observed susceptibility. Although EGTA was applied locally at the inoculation site and its effect is

transient, *L. maculans* colonized the entire cotyledon despite the presence of *Rlm2*. This suggests that early signaling events that prevent fungal establishment are likely critical for determining host fate.

Although network analyses identified a robust defense response in *Rlm2* cotyledons at 3 dpi associated with pathogen detection and MAPK signaling, this pathway is largely responsible for hormone balance and may explain the observed lack of JA responses in susceptible plants at 3 dpi. For example, *mpk4* plants constitutively activate SA-dependent defenses and have severely diminished JA signaling (Brodersen et al. 2006), and interference with MPK4 and MPK6 signaling decreases *PDF1.2* and causes hypersusceptibility to *Botrytis cinerea* (Schweighofer et al. 2007). Similarly, *mekk1*, *mkk1/mkk2*, and *mpk4 Arabidopsis* have severe dwarfism caused by constitutive SA signaling and compromised JA signaling (Pitzschke et al. 2009). Reflective of its importance, pathogen effectors are known to target features of this signaling cascade, including SOBIR1 (Wu et al. 2018), BAK1 (Zhou et al. 2014), CERK1 (Yamaguchi et al. 2013), and MAPKs (King et al. 2014; Teper et al. 2018; Zhang et al. 2017), which is consistent with the recent identification of MAPK9 as a target of AvrLm1 (Ma et al. 2018). The activity of *Rlm2* and other *R* genes likely protects the integrity of this MAPK signaling pathway, allowing the host to maintain JA signaling and respond appropriately.

Although the gene-for-gene resistance model has guided research on plant–pathogen interactions in the past, it is becoming clear that plant immunity steps beyond a single gene and is orchestrated by diverse sets of receptors and downstream regulators. Currently, blackleg disease is primarily controlled through the effective application of *R* genes that operate through yet-to-be-uncovered mechanisms. In this article, we describe the earliest changes to gene expression that reprogram cells following *Rlm2* recognition of *L. maculans*. This, together with physiological data, strongly suggest that calcium and JA play a central role in *Rlm2*-mediated immune signaling. Understanding the processes that enable immunity in *Brassica napus* will provide the necessary information to tailor plant defense against biotic stress. Further, it is still unclear whether or not all *R* genes effective against *L. maculans* converge on similar immune pathways to prevent pathogen attack or through distinct gene regulator networks. Answering this question may provide a “silver bullet” to control this pathogen and help direct future research for crop improvement.

MATERIALS AND METHODS

Plant growth and inoculation.

Susceptible Topas DH16516 and the *Rlm2* transgenic line NLA51-1 (Larkan et al. 2015) were grown in Sunshine mix number 4 soil (Sun-Gro Horticulture) in controlled environments with a 16-h photoperiod (21°C in light and 16°C in darkness). Seven-day old cotyledons were point inoculated with 10 µl of an *L. maculans* isolate 00-100 (*AvrLm2*) (Ghanbarnia et al. 2015) pycnidia suspension (10⁶) or sterile water (mock). All inoculations and tissue collections were performed between 11:00 A.M. and 2:00 P.M. to minimize time-of-day effects. To explore the role of calcium in resistance, 10 µl of 5 mM EGTA was added to the inoculation site as an additional treatment and allowed to dry before inoculation.

Tissue processing.

Inoculated cotyledons were processed for LMD at 1 and 3 dpi per the methods of Becker et al. (2017b). Inoculation sites were excised by making two cuts parallel to the cotyledon petiole-like structure. At minimum, 30 inoculation sites per biological replicate (three biological replicates per treatment) were

collected and fixed overnight in 3:1 (vol/vol) ethanol-acetic acid at 4°C. Tissues were then dehydrated in a graded-ethanol series (30 min each of 75, 85, 95, 100, and 100%) followed by xylenes infiltration (1 h each of 3:1, 1:1, and 1:3 ethanol-xylene [vol/vol] and 100% xylene) at 4°C. Ten paraffin chips were added to the xylenes and kept at 4°C overnight. Following, samples were brought to room temperature before incubating at 42°C for 30 min and then at 58°C for 1 h. The mixture was replaced with 100% melted paraffin every hour for 4 h before embedding.

Sectioning and LMD.

Embedded tissues were sectioned at 10 µm using a Leica RM2125 rotary microtome in RNase-free conditions and mounted on PEN membrane slides as per Millar et al. (2015). Slides were washed for 1 min in xylenes to deparaffinize before proceeding to LMD on a Leica LMD6000. Sections of 100 µm proximal to the inoculation site were collected in 60 µl of lysis buffer. RNA was isolated from at least 20,000,000 µm² of collected tissue per biological replicate. RNA extractions were performed exactly as reported in Belmonte et al. (2013). Following this, RNA quality was assessed using the Agilent 2100 Bioanalyzer and was of sufficient quality for quantitative reverse-transcription PCR and RNA-seq, as described by Millar et al. (2015).

Library construction.

First- and second-strand cDNA synthesis was performed per the alternative HTR (C2) described by Kumar et al. (2012), with the following modifications: input RNA decreased to 50 to 75 ng, no poly(A) bead isolation was performed, anchored oligo(dT₂₀) primers were used during first-strand synthesis instead of random hexamers, half reaction volumes for all steps, and cDNA sheared via sonication instead of enzymatic digestion. Libraries were prepared using the NEBNext Ultra II kit per the manufacturer's instructions. Quality and distribution of libraries was assessed with the Agilent 2100 Bioanalyzer, and libraries were quantified with the NEBNext Library Quant Kit for Illumina and the NanoDrop 3300 fluorospectrometer. Library size selection of the 300- to 500-bp region was performed with the E-gel size select system. Sequencing was performed on the HiSeq 2500 at Genome Prairie, SK, Canada.

Data analyses.

RNA sequencing of libraries produced approximately 423 million-paired end reads, with an average of 17.6 million reads/sample (PRJNA477556). Adapter sequences, low-quality bases (Q < 25), and low-quality reads (avg Q < 30) were removed using the Trimmomatic v0.36 software (Bolger et al. 2014). Paired-end reads passing quality control were aligned to the *B. napus* genome v4.1 (Chalhoub et al. 2014) with HISAT2 in cufflinks compatibility mode, allowing no more than two mismatches. Output SAM files were converted to BAM and sorted with Samtools. Raw counts were obtained from BAM files using the HTSeq Python Framework (Anders et al. 2015). Dendrograms and similarity matrices were generated from raw counts using DESeq (Love et al. 2014). From BAM files, Cuffquant, Cuffnorm, and Cuffdiff (Trapnell et al. 2012) were used to generate normalized counts in FPKM and to significantly identify DEGs (*P* < 0.05; false discovery rate = 0.05). Differentially expressed gene sets were input into SeqEnrich (Becker et al. 2017a) to identify enriched metabolic pathways, gene ontology terms, promoter TF binding motifs, and predictive transcription factor networks. Networks were visualized in Cytoscape 3.6.0.

L. maculans transcriptome analyses were performed per Haddadi et al. (2016) and all reads were trimmed as above and

mapped to the *L. maculans* isolate v23.1.3 genome (Rouxel et al. 2011). Transcript abundance was measured as log₂ reads per kilobase of exon per million mapped reads. DEGs (*P* < 0.05; false discovery rate = 0.05) were identified with DESeq2 (Love et al. 2014). Identification of secretory protein signals was performed according to Haddadi et al. (2016).

Real-time qPCR experiments.

RNA was converted to cDNA with the qScript cDNA Supermix (Quantabio). Following this, qPCR was performed using the CFX Connect Real-Time System (Bio-Rad) and PerfeCTa SYBR-Green Supermix (Quantabio) as per the manufacturer's instructions in a 15-µl reaction volume. A list of primer sequences is supplied in Supplementary Table S1. The ΔΔCt method (Rieu and Powers 2009) was used to compare relative mRNA abundance, normalized to the endogenous housekeeping gene *actin* (GenBank AF111812.1). Statistics were performed as described by McLoughlin et al. (2018).

JA measurements.

Inoculation sites (30 per bioreplicate, and five bioreplicates per treatment) were hand dissected, immediately flash frozen, and pulverized in liquid nitrogen. Following this, 5 ml of extraction solvent (2-propanol-H₂O-HCl, 2:1:0.002, pH 2.4) and 25 ng of dihydrojasmonic acid internal standard (number D454265; Toronto Research Chemicals) were added to each sample, which was then shaken at 4°C for 30 min. Next, 7 ml of dichloromethane was added, and samples were shaken again at 4°C for 30 min. After centrifugation at 5,300 rpm for 5 min, the lower organic phase was transferred and dried on ice in a nitrogen evaporator system. The dried sample was then dissolved in methanol, mixed aggressively, and filtered through 0.2 µm polytetrafluoroethylene before ultraperformance liquid chromatography (UPLC). JA standards were prepared with internal standards to generate calibration curves and response factors. Samples were analyzed on a Waters Acquity UPLC/mass spectrometry with Micromass Quattro micro API, as described by Henriquez et al. (2016).

AUTHOR-RECOMMENDED INTERNET RESOURCE

Cytoscape 3.6.0: <https://cytoscape.org>

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