Identification of lncRNAs Responsive to Infection by *Plasmodiophora brassicae* in Clubroot-Susceptible and -Resistant *Brassica napus* Lines Carrying Resistance Introgressed from Rutabaga

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Clubroot disease, caused by *Plasmodiophora brassicae* Woronin, is a major threat to the production of *Brassica* crops. Resistance to different *P. brassicae* pathotypes has been reported in the A genome, chromosome A08; however, the molecular mechanism of this resistance, especially the involvement of long noncoding RNAs (lncRNAs), is not understood. We have used a strand-specific lncRNA-Seq approach to catalog lncRNAs from the roots of clubroot-susceptible and -resistant *Brassica napus* lines. In total, 530 differentially expressed (DE) lncRNAs were identified, including 88% of long intergenic RNAs and 11% natural antisense transcripts. Sixteen lncRNAs were identified as target mimics of the microRNAs (miRNAs) and eight were identified as the precursors of miRNAs. KEGG pathway analysis of the DE lncRNAs showed that the cis-regulated target genes mostly belong to the phenylpropanoid biosynthetic pathway (15%) and plant–pathogen interactions (15%) while the transregulated target genes mostly belong to carbon (18%) and amino acid biosynthesis pathway (19%). In all, 24 DE lncRNAs were identified from chromosome A08, which is known to harbor a quantitative trait locus conferring resistance to different *P. brassicae* pathotypes; however, eight of these lncRNAs showed expression only in the resistant plants. These results could form the basis for future studies aimed at delineating the roles of lncRNAs in plant–microbe interactions.

Keywords: canola, clubroot, genomics, long non-coding RNAs, metabolomics, *Plasmodiophora brassicae*, plant responses to pathogens, primary metabolism, proteomics, RNA-seq

Intergenic regions in the genome have been shown to be involved in transcriptional and posttranscription regulation of

gene expression (Kapranov et al. 2007). These regions are ubiquitously transcribed into RNAs and are classified based on their different sizes and their regulatory functions. These types of functional RNAs are classified as noncoding RNAs (ncRNAs) (Ariel et al. 2015; Kapranov et al. 2007; Wilusz et al. 2009). Long ncRNA (lncRNAs) are defined to be greater than 200 bp (Chen 2009; Kapranov et al. 2007) and have been implicated in various gene regulatory processes, including the reprogramming of gene expression in plants in response to a variety of biotic and abiotic stresses (Nejat and Mantri 2018).

lncRNAs have been classified into different categories based on their genomic location and orientation. These include promoter upstream transcripts, enhancer RNAs, intergenic ncRNAs (lincRNAs), intronic ncRNAs, bidirectional lncRNAs, overlapping lncRNAs, and natural antisense transcripts (NATs) (Mattick and Rinn 2015; Morris and Mattick 2014; Wu et al. 2017). Similar to the mRNA transcription process, lncRNAs are also transcribed by RNA polymerase II in plants and are modified with a 5′-cap and 3′-end poly-adenylation (Guttman et al. 2009).

Advances in the next-generation sequencing have enabled genomewide identification of lncRNAs in many eukaryotes, including several plant species. The first systematic investigation on plant lncRNAs began with the identification of 6,480 lincRNAs in *Arabidopsis thaliana* through bioinformatics analysis; however, expression profiling using microarray and RNA-seq confirmed 2,708 transcripts to be lincRNAs (Liu et al. 2012). Similarly, exploitation of expressed sequence tag databases and 30 different RNA-seq datasets resulted in the identification of 20,163 putative lncRNAs (Li et al. 2014). Among these, 1,704 were considered to be high-confidence lncRNAs in maize, which did not exhibit sequence similarity with known ncRNAs. Most (>90%) of the lncRNAs were found to be in the intergenic regions, with more than 50% of the lncRNAs observed to be expressed in a tissue-specific manner and largely affected by transgenic factors (Li et al. 2014).

Regulatory roles for the lncRNAs have also been reported in many plant species; for example, in cellulose, lignin, and gibberellin biosynthesis as well as in wood formation in *Populus tomentosa* (Chen et al. 2015). Such regulatory roles have also been observed in flower and fruit development in diploid strawberry *Fragaria vesca* (C. Y. Kang and Liu 2015) and trifoliate orange *Poncirus trifoliata* L. Raf. (Wang et al. 2017). In *Brassica campestris*, Song et al. (2013) identified an lncRNA, *BcM11*, involved in pollen development. Additional studies have supported a

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crucial role of lncRNAs in plant growth and development, disease response, and genetic or epigenetic regulation of traits (Huang and Zhang 2014; Huang et al. 2018; Nejat and Mantri 2018).

lncRNAs are also known as the class of riboregulators that are expressed in response to plant biotic stress. Zhu et al. (2014) identified 35 lncRNAs, including 15 NATs and 20 lincRNAs, involved in resistance to *Fusarium oxysporum* in *A. thaliana*. Of these, antisense transcription in approximately 20% of the annotated *A. thaliana* genes was observed. The role of lncRNAs in response to diseases has also been documented in many other crop species; for example in tomato against *Tomato yellow leaf curl virus* (Wang et al. 2015); in *B. napus* in response to *Sclerotinia sclerotiorum*, which causes stem rot disease (Joshi et al. 2016); as well as in the forest tree genus *Populus* in response to *Melampsora larici-populina* (N. Wang et al. 2017). It has also been reported that overexpression of the lncRNA16397 in tomato induces the expression of glutaredoxin, reduces the accumulation of reactive oxygen species, and, thus, confers resistance to *Phytophthora infestans* (Cui et al. 2017). Recently, L. Zhang et al. (2018) reported that silencing of lncRNAs *GhlncNAT-ANX2* and *GhlncNAT-RLP7*, which are involved in the regulation of lipoxygenase activity, results in enhanced resistance toward *Verticillium dahliae* and *Botrytis cinerea*. ELF18-induced long noncoding RNA 1 is another functionally characterized lncRNA, which enhances resistance against *Pseudomonas syringae* in *A. thaliana* by positively regulating pathogenesis-related 1 (*PR1*) gene expression (Seo et al. 2017). Taken together, all of these reports suggest a significant role for lncRNAs in response and resistance to diseases in plants and warrant continued investigation. To our knowledge, no study has been conducted thus far to understand the role of lncRNAs in resistance to other diseases such as the clubroot (CR) disease of *Brassica* crops.

CR is a soilborne disease affecting cruciferous, crops including canola, cabbage, turnip, Brussels sprout, and many other *Brassica* crops (Howard et al. 2010). It is caused by a soilborne, obligate parasite, *Plasmodiophora brassicae* Woronin, a member of the eukaryotic subgroup Rhizaria (Burki et al. 2010). *P. brassicae* is a fungus-like protist which is known to possess a complex lifecycle and is unable to grow and multiply without a living host (Hwang et al. 2012). This pathogen can stay in soil for over 15 years (Wallenhammar 1996) in the form of resting spores and germinates only in the presence of secretions from the plant roots (Macfarlane 1970). Disease development leads to the formation of galls on the roots, which negatively affect water and nutrient uptake by roots, leading to stunted growth (Dixon 2009). In the case of the most severely affected fields, CR disease can cause yield loss of approximately 30% in canola (Tewari et al. 2005). Due to the nature of the occurrence of this disease and its perpetual infestation cycle, it is difficult to control this disease with just implementation of improved agricultural practices and chemical treatments (Dobson et al. 1983; Howard et al. 2010; Kowata-Dresch and May-De Mio 2012).

Despite the existence of commercial, CR-resistant cultivars and integrated management approaches, it is still crucial to achieve a better understanding of plant responses to *P. brassicae* infection and to develop cultivars with durable resistance, in order to combat genetic shifts in this pathogen population and subsequent loss of effective resistance (Peng et al. 2014). Genome and proteome analyses of various plant species of the family Brassicaceae challenged with *P. brassicae* have provided some insights about molecular mechanisms triggering resistance in the host plants (Cao et al. 2008; Chen et al. 2016; Devos et al. 2006; Zhang et al. 2016). The availability of the *Brassica napus* genome sequence (Chalhoub et al. 2014) has now paved the way for investigation into the ncRNA and their regulatory roles in *Brassica* spp. The objective of this

research was to identify the lncRNAs involved in the regulation of resistance to *P. brassicae* by using *B. napus* canola lines carrying resistance introgressed from rutabaga (Hasan and Rahman 2016).

RESULTS

Microscopic examination of *P. brassicae* infection of *B. napus*.

Microscopic examination of the roots of the resistant (R) and susceptible (S) plants inoculated with *P. brassicae* pathotype 3 showed no evidence of infection at 6 days postinoculation (dpi) (Table 1). Infection was observed in the secondary roots at 10 dpi in both S (Fig. 1C) and R (Fig. 1D) plants. Approximately 7.3% of cells were observed to be infected in S while 2.3% of cells were observed in R (Table 1); however, there was no swelling of the roots in either S or R. At 14 dpi, 8.3% cells were observed to be infected in S compared with 2.7% in R (Table 1). A greater number of primary plasmodia were observed in the S plants as compared with the R plants (data not shown). At 18 dpi (Fig. 1E and F), approximately 51.7% of cells showed the presence of secondary plasmodium in S plants whereas 2.7% of cells in R already showed the presence of resting spores. The root cells of the S plants started to show evidence of hypertrophy at 18 dpi (Fig. 1E). As expected, there was no indication of gall formation in the inoculated R plants and they were morphologically similar to the uninoculated R and S plants. At 22 dpi (Fig. 1G and H), more than 60% of cortical cells of the S plants showed the presence of mature secondary plasmodia and resting spores (Table 1), whereas 2.7% of infected cortical cells with resting spores could be seen in the R plants, because there was no further increase in infection observed. In conclusion, this study showed that infection by *P. brassicae* can occur in both S and R plants at 10 dpi; however, the infection did not progress further in the R plants. Based on these results, we focused on the root samples collected at 10 dpi for studies on lncRNA profiling.

Identification of lncRNAs in *B. napus* following *P. brassicae* infection.

Strand-specific lncRNA-Seq libraries were prepared from the roots of the R and S plants collected at 10 dpi to identify the lncRNAs responsive to *P. brassicae* infection. In total, 461 million paired-end clean reads were obtained from six strand-specific RNA-seq libraries (Table 2). The error rate was 0.01%, with Q30 percentage exceeding 98% for all the six libraries. After removal of the low-quality sequences and the adapter sequences and artifacts, 367 million (79.8%) clean reads were mapped on to the reference *B. napus* genome; however, by use of TopHat (v2.0.9) (Trapnell et al. 2012), 73.5% of the reads could be uniquely mapped, followed by assembly and annotation of all the transcripts in *B. napus* using Cufflinks (v2.1.1) (Trapnell et al. 2010). From the total 198,553 assembled transcripts, 78.0% of transcripts were found to be mRNAs after the application of exon number and transcript length filters, which

Table 1. Percentage of infected cells observed in clubroot-susceptible (S) and -resistant (R) *Brassica napus* lines as observed microscopically at different time points^a

Time point	S lines	R lines
0 hpi	0	0
6 dpi	0.7	0
10 dpi	7.3	2.3
14 dpi	8.3	2.7
18 dpi	51.7	2.7
22 dpi	66.0	2.7

^a hpi = hours postinoculation and dpi = days postinoculation; 300 cells were examined microscopically for each time point.

Fig. 1. Different stages of *Plasmodiophora brassicae* infection observed in histopathological analysis of *Brassica napus* roots at **A** and **B**, 0 h postinoculation (hpi) and 10, 18, and 22 days postinoculation (dpi). **C** and **D**, Presence of proliferating zoosporangia in both rutabaga-derived clubroot-susceptible (S) and clubroot-resistant (R) *Brassica napus* at 10 dpi (inset shows larger magnification); **E** and **F**, matured secondary plasmodia in the cortical cells of S (black arrows) and resting spores in few cortical cells of R (white arrows) at 18 dpi; and **G** and **H**, many resting spores and secondary plasmodia in S and resting spores in very few cortical cells of R (white arrows) at 22 dpi. Bars represent the resolution of the microscopy for the particular section.

were further filtered to remove the transcripts showing an overlap with the exon regions of the annotated functional genes (as identified using Cuffcompare, v2.1.1) (Trapnell et al. 2010). Expression of these transcripts was calculated using Cuffdiff (v2.1.1) and the transcripts with fragments per kilobase of transcript per million mapped reads (FPKM) ≥ 0.5 were selected for further analysis. The Coding Potential Calculator (CPC) (Kong et al. 2007) and Pfam Scan (v1.3) (Punta et al. 2012) were used to filter the remaining transcripts for their coding potential to distinguish the protein-coding and non-coding sequences and, in total, 4,558 transcripts were deemed to be lncRNAs. These lncRNAs included 84.7% long-intergenic RNAs and 15.3% antisense RNAs. Screening of the putative lncRNAs based on q value (<0.05) identified 530 differentially expressed (DE) lncRNAs between the R and S plants, and this included 58 (10.9%) antisense lncRNAs and 472 (89.1%) lncRNAs.

Characterization and functional analysis of DE lncRNAs in response to *P. brassicae* infection.

More than 60% of the lncRNAs were observed to be 200 to 1,000 nucleotides (nt) in size and the lengths of the 530 DE lncRNAs ranged from 206 to 8,584 nt. Approximately 18% (96 of 530) lncRNAs were longer than 1.5kb nt in size (Supplementary Fig. S1). Analysis of all of the DE lncRNAs for the size of their exons indicated that 78% (663 of 853 exons) had a

length of 18 to 1,000 nt. Moreover, lncRNAs were observed to be significantly shorter compared with mRNAs. The number of transcripts for lncRNAs with a single exon constituted approximately 62% (329 of 530) of these lncRNAs, while 26% (138 of 530) consisted of two exons and only 2.6% (14 of 530) lncRNAs contained more than five exons. The DE lncRNAs were evenly distributed across the 19 *B. napus* chromosomes. Of the total 530 DE lncRNAs, 184 and 229 were observed to be located on the 10 “A” chromosomes and the nine “C” chromosomes, respectively, of the *B. napus* genome. However, 117 DE lncRNAs could not be assigned to any specific chromosome. A strong correlation (0.76) was observed between the distribution of the number of DE lncRNAs and chromosome size. When the distribution of DE lncRNAs on individual chromosomes was considered, the highest number was found on chromosome C03, followed by chromosomes C09, A09, and A03. Interestingly, chromosome A08, to which the CR resistance gene has been previously mapped, was found to harbor 24 DE lncRNAs.

Of the 530 DE lncRNAs, 345 were upregulated and 185 were downregulated in the S plants as compared with R plants (Supplementary Fig. S2). In contrast, 170 lncRNAs were upregulated and 337 were downregulated in the R plants as compared with S plants. In all, 15 lncRNAs were detected only in the R plants while eight lncRNAs were detected only in the S plants (Table 3). The top 20 upregulated and downregulated

Table 2. Mapping statistics of the sequencing reads from six-stranded *Brassica napus* libraries postinfection with *Plasmodiophora brassicae*

Replicates ^a	Number of reads (%)			
	Total	Total mapped	Multiple mapped	Unique mapped
R_1	78,145,602	62,866,707 (80.45)	4,874,861 (6.24)	57,991,846 (74.21)
R_2	77,724,002	62,072,779 (79.86)	4,964,474 (6.39)	57,108,305 (73.48)
R_3	72,187,808	57,818,092 (80.09)	4,454,507 (6.17)	53,363,585 (73.92)
S_1	85,702,896	68,181,904 (79.56)	5,206,587 (6.08)	62,975,317 (73.48)
S_2	72,543,352	57,417,259 (79.15)	4,640,617 (6.4)	52,776,642 (72.75)
S_3	74,959,046	59,616,509 (79.53)	4,735,211 (6.32)	54,881,298 (73.22)
Total	461,262,706	367,973,250 (79.77)	28,876,257 (6.26)	339,096,993 (73.51)

^a R_1, R_2, and R_3 are the biological replicates of clubroot (CR)-resistant *B. napus* and S_1, S_2, and S_3 are the biological replicates of CR-susceptible *B. napus*.

Table 3. Long noncoding RNAs (lncRNAs) uniquely expressed in clubroot disease-resistant (R) lines carrying the resistance gene of rutabaga and clubroot-susceptible (S) lines of *Brassica napus*^a

Transcript ID	Chromosome name and gene location	S (FPKM)	R (FPKM)
LNC_001161	A08: 12,183,521–12,183,982	0	0.954916
LNC_001063	A08: 3,202,637–3,204,852	0	1.02438
LNC_000600	A04: 14,816,008–14,816,758	0	1.3608
LNC_001126	A08: 3,447,903–3,448,931	0	1.7121
LNC_000450	A03: 27,099,999–27,101,445	0	1.98082
LNC_002916	C04: 47,030,126–47,030,803	0	1.98632
LNC_001146	A08: 9,573,084–9,573,548	0	2.28077
LNC_000108	A01: 19,635,847–19,636,338	0	2.30396
LNC_001065	A08: 3,788,461–3,789,000	0	2.92531
LNC_001154	A08: 10,153,693–10,154,043	0	3.7843
LNC_001089	A08: 12,365,659–12,366,252	0	5.01747
LNC_004045	C09: 43,229,826–43,230,101	0	6.3523
LNC_001851	Ann_random: 41,970,174–41,971,127	0	1.4904
LNC_001191	A08_random: 1,215,048–1,217,686	0	3.3501
LNC_001888	Ann_random: 47,682,468–47,684,525	0	5.2681
LNC_000089	A01: 12,314,342–12,314,908	2.07049	0
LNC_001341	A09: 11,766,717–11,767,907	2.1138	0
LNC_002432	C03: 26,497,021–26,497,291	5.19373	0
LNC_003616	C07: 28,269,085–28,273,791	1.93572	0
LNC_004790	Unn_random: 8,133,944–8,134,628	0.8604	0
LNC_001807	Ann_random: 35,009,658–35,010,033	1.4708	0
LNC_000887	A06_random: 299,855–300,207	1.5085	0
LNC_000736	A05_random: 1,032,000–1,032,588	0.8626	0

^a FPKM = fragments per kilobase of transcript per million mapped reads. Rows in bold indicate the lncRNAs located on chromosome A08.

lncRNAs of the R plants are presented in Supplementary Table S2. In all, 4 of the top 20 downregulated lncRNAs in R are located on chromosome A08. Recent insights from functional analysis of the lncRNAs have demonstrated that they can be involved in regulating the expression of the overlapping or neighboring genes (cis-acting) or genes located at a long distance (transacting). Genomic regions 100 kb upstream and downstream of the lncRNAs were scanned to predict the collocated target genes of cis-acting lncRNAs (Supplementary

Table S3). All 530 DE lncRNAs were found to pair with the annotated mRNAs from both upstream and downstream regions (Supplementary Fig. S3). KEGG pathway enrichment analysis revealed that the majority of the collocated target genes were from plant-pathogen interactions (15%) and the phenylpropanoid biosynthetic pathway (15%) (Fig. 2A). Similarly, 425 DE lncRNAs were predicted to have coexpressed targets based on correlation of expression between the lncRNAs and mRNAs. KEGG pathway enrichment analysis also revealed

A Pathway enrichment analysis for the co-located targets

B Pathway enrichment analysis for the co-expressed targets

Fig. 2. Functional annotation of target genes of *Brassica napus* long noncoding RNAs **A**, Pathway enrichment analysis for the collocated targets and **B**, pathway enrichment analysis for the coexpressed targets.

that the majority of the coexpressed target genes belonged to primary metabolic pathways such as biosynthesis of amino acids (19%) and carbon metabolism (18%) (Fig. 2B). Some of the most significantly expressed lncRNAs in R and S plants and their predicted target genes are shown in Figure 3. A majority of the target genes regulated by these lncRNAs belonged to the plant defense-related pathways such as plant-pathogen interactions, phenylpropanoid biosynthetic pathways, plant hormone-signaling pathways, and primary

metabolic pathways (Fig. 3). Genes involved in plant-pathogen interactions include the *calcium-dependent protein kinase*, *defensin*, *pathogen-related protein*, and *disease resistance protein (leucine-rich repeat [LRR] receptor protein)* genes (Fig. 3); and important genes from the phenylpropanoid biosynthetic pathway upregulated in R include *caffeic acid 3-O-methyltransferase (CoMT)*, *shikimate O-hydroxycinnamoyltransferase (HCT)*, and *cinnamoyl alcohol dehydrogenase 7 (CADH7)* (Fig. 3).

Fig. 3. Classification of long noncoding RNAs with the most significant differential expression and their predicted targets into the most represented functional groups. lncRNAs upregulated in R (green), lncRNAs upregulated in S (orange), and target genes with an asterisk are leucine-rich repeat protein genes.

DE genes of located on the A08 chromosome.

Of the DE lncRNAs detected at 10 dpi, 24 lncRNAs were distributed on the chromosome A08 (Fig. 4); this chromosome has been reported to carry a genomic region conferring resistance to five *P. brassicae* pathotypes (Hasan and Rahman 2016). Among these, LNC_001065, LNC_001165, and LNC_001146 target the largest number of genes involved in plant defense (Fig. 4). While comparing their expression in the R and S plants, a greater number of the 24 DE lncRNAs were found to be upregulated in the S plants as compared with the R plants. In addition, 8 of the 24 lncRNAs were detected only in the R plants; however, they showed a low and inconsistent expression among the three biological replicates (Fig. 5; Supplementary Table S4). Genes targeted by these 24 lncRNAs were found to be involved in primary metabolic pathways, plant–pathogen interactions, and secondary metabolite and phenylpropanoid biosynthetic pathways (Fig. 4).

All 24 DE lncRNAs, which were located on chromosome A08, were found to pair with the neighboring annotated target mRNAs (cis-regulation), and 21 of these were predicted to transregulate the target mRNAs. Among the 21 transregulated lncRNAs, LNC_001191, LNC_001126, LNC_001063, LNC_001117, LNC_001118, and LNC_001167 (Fig. 5) are known to be involved in the regulation of *disease resistance protein* genes distributed throughout the *B. napus* genome (Supplementary Table S5). Furthermore, LNC_001167 was predicted to be involved in the regulation of three different disease resistance genes encoding toll interleukin 1 receptor nucleotide-binding site LRR proteins, similar to those found in *A. thaliana*, including *RPS4* (resistance to *Pseudomonas syringae* 4), *RPP1* (recognition of *Peronospora parasitica* 1), and *EDR2* (enhanced disease resistance 2). Expression of LNC_001063, LNC_001126, and LNC_001191 could not be detected in the R plants whereas a 0.7- to 2.7-fold increase in expression was observed for LNC_001117, LNC_001118, and LNC_001167 in the S plants.

When considering both lncRNAs and their targets located on chromosome A08, 15 of the abovementioned 24 lncRNAs were predicted to transregulate their target genes located on this chromosome (Fig. 5; Supplementary Table S6). No expression was observed for LNC_001063, LNC_001065, LNC_001089, LNC_001126, LNC_001146, LNC_001154, or LNC_001191 in the S plants, whereas a low expression was detected in the R plants (Supplementary Table S7). Based on KEGG pathway analysis, targets of these seven lncRNAs were found to be involved in the biosynthesis of secondary metabolites and in the primary metabolic pathways, including carbon and amino acid metabolism (Fig. 4). Among all of the lncRNAs located on chromosome A08, the greatest level of expression was observed for LNC_001165 in the S plants (Fig. 5; Supplementary Table S8). This lncRNA was predicted to transregulate 76 genes in the *B. napus* genome; however, 29 of these target genes are located on chromosome A08 and are known to be involved in carbon metabolism and biosynthesis of secondary metabolites.

Quantitative real-time PCR validation for the expression of lncRNAs.

To validate our findings from lncRNA-seq analysis and to further determine whether these genes are involved in defense responsive pathways, three lncRNAs from A08, three lncRNAs that are located on different chromosomes but have at least some of their target genes located on A08, and three lncRNAs were randomly selected for quantitative real-time (qRT)-PCR validation. While selecting the lncRNAs, only the lncRNAs which were predicted to have a maximum of four targets were used. Expression patterns of these nine lncRNAs were found to be similar in lncRNA-seq and qRT-PCR analysis (Fig. 6A);

however, all of the target genes of an lncRNA did not follow the same pattern (Fig. 6B). For example, the three targets *BnaA09g52970D*, *BnaC03g59610D*, and *BnaA08g06670D* of LNC_003848 showed an expression profile similar to the results of lncRNA-seq; however, the fourth target (*BnaA07g30470D*) followed an opposite trend. It is probable that the target *BnaA07g30470D* is a false positive with *q* value > 0.05. A similar situation was also found in the case of the targets *BnaC04g09450D*, *BnaC03g27800D*, and *BnaA07g23650D* of LNC_001173. Interestingly, such anomalies were not observed in the cases where lncRNAs were predicted to have only one target, such as LNC_003279 and LNC_001192. Finally, the expression of two additional genes, *BnaA10g17370D* and *BnaC09g40660D*, showed excellent correlation between RNAseq (unpublished) and qRT-PCR results.

B. napus lncRNAs as endogenous target mimics for microRNAs.

By use of the RNA target analysis server psRNAtarget (Dai et al. 2018), with default parameters and maximum expectation of 3.5, 15 DE lncRNAs were identified which could possibly serve as target mimics of microRNAs (miRNAs) (Supplementary Table S9). Among these, LNC_001191 and LNC_001163 were found to be located on chromosome A08 and as targets of the miRNAs *bna-miRNA6028* and *bna-miRNA824*, respectively. Putative target AGL16 and the target mimic LNC_001163 of *bna-miRNA824* are shown in Figure 7A. Among these two lncRNAs, LNC_001191 was expressed only in the R plants, and a greater level of expression was found for LNC_001163 (Fig. 5). qRT-PCR analysis also confirmed the upregulation of LNC_001163, *bna-miRNA824*, and its target AGL16 in the R plants (Fig. 7B). It was found that *bna-miRNA6028* targets three lncRNAs (LNC_002844, LNC_001263, and LNC_001191); among these, the expression of LNC_002844 and LNC_001263 was downregulated in the R plants to a greater extent as compared with LNC_001191. From this data, six lncRNAs were predicted to target single miRNAs whereas four lncRNAs were predicted to target multiple miRNAs of the same family. The interaction analysis using Cytoscape v.3.7.0 (Fig. 8) depicts intricate interactions between the lncRNAs and miRNAs and mRNAs. Therefore, the abovementioned endogenous target mimics (eTMs) identified in response to *Plasmodiophora brassicae* infection were predicted to conserve the mRNA targets by disrupting the miRNA–mRNA interactions.

lncRNAs as potential miRNA precursors.

lncRNAs can also function as precursors for miRNA and siRNA, playing important roles in modulating gene expression and, thus, implicating their roles in diverse biological functions in plants. In this study, 8 of the 530 DE lncRNAs were identified to be possible precursors of miRNA (Supplementary Table S10). For instance, LNC_001710 seems to harbor 100% of the stem-loop sequence of miRNA-160a; and LNC_000265 and LNC_004360 were predicted to be the precursors for miRNA169n and miRNA166a, respectively (Fig. 9), and were expressed at higher levels in the R plants. Similarly, miRNA166a was also predicted to arise from LNC_00563; however, expression of this lncRNA was not significantly different between the R and S plants. The miRNA166 is a family of six miRNAs in *B. napus*, of which miRNA166a (LNC_004360) and miRNA166e (LNC_001636) were represented in this study.

Natural antisense transcripts.

In total, 59 natural antisense transcripts (NATs) was identified to be regulating their corresponding sense transcripts in response to infection by *P. brassicae* at 10 dpi (Supplementary

Fig. 4. Long noncoding RNAs (lncRNAs) on chromosome A08 and their interaction with target genes in *Brassica napus* genome involved in plant defense. lncRNAs not regulating any target genes involved in plant defense are shown in italics and parentheses. Yellow ovals = lncRNAs on chromosome A08, red squares = target genes involved in plant-pathogen interaction, blue squares = target genes involved in plant hormone signaling, gray squares = target genes involved in primary and secondary metabolism, and eTM = endogenous target mimic.

Table S11). All of these NATs were validated in silico by determining the thermodynamics of RNA–RNA interaction (Hofacker 2009; Lorenz et al. 2011). Based on differential expression of the lncRNAs, 38 NATs were identified. After filtering the 38 NATs based on expression of their sense transcripts in the RNA-seq data and potential pathogen defensive functions of the genes, 18 NATs were selected, of which 4 were validated using qRT-PCR (Figure. 10). The optimal secondary structure formation happens when thermodynamic free energy is minimum; such minimum free-energy structures of these four lncRNA-mRNA dimerized pairs, predicted using RNAfold (Lorenz et al. 2011), are shown in Figure 10.

DISCUSSION

CR disease is known to cause significant yield loss in *Brassica* spp., including vegetables and oilseed crops. Significant achievements have been made in combating this disease through the development of R cultivars (Piao et al. 2004; Rahman et al. 2014); however, the molecular mechanisms involved in the host plant resistance have not been well understood. Results from our study indicate the possible involvement of lncRNAs in mediating resistance in *B. napus* canola lines carrying resistance introgressed from rutabaga. We did not find evidence of infection in the roots at 6 dpi in both R and S plants; however, infection was observed in both R and S plant roots at 10 dpi. In contrast, Dobson et al. (1983) observed root hair infection in Chinese cabbage at 1 dpi. However, Asano et al. (2000) detected initial infection in turnip at 4 dpi with rapid increase up to 6 dpi; similar results have also been reported by Hwang et al. (2012) in canola. Thus, it is evident that the stage of root hair infection may be dependent on the type of plant material and the method of inoculation which, in the present study, was injection

of spore suspension into the soil (direct inoculation). A noticeable reduction in the formation of primary plasmodia was found in the R plants, with no infection in the newly emerging roots at 14 dpi, whereas gall formation had already begun in the S plants at this stage. Detection of infection at an early stage in the R plants could be related to the plant development; an influence of the developmental stage of the host plant on the pathogen's ability to cause infection has been suggested by Fei et al. (2016). Thus, our study suggests that, despite microscopically visible infection that occurs in the R plants at their early growth stage, the resistance genes restrict further development of the disease.

Most of the 530 DE lncRNAs detected in this study were from the intergenic regions (lincRNAs); these lncRNAs showed considerably greater expression as compared with the expression of the antisense lncRNAs (NATs), which is consistent with the results from other plant species (Li et al. 2014; Wang et al. 2014; J. Zhang et al. 2018). The lincRNAs regulate plant responses to CR by remodeling epigenetic and chromatin architecture, stabilization and localization of proteins and RNA molecules by tethering to them and transcription regulation involving enhancer-associated activity, and cis- or trans-mediated gene activation (Kopp and Mendell 2018; Kornienko et al. 2013; Nejat and Mantri 2018; Zaynab et al. 2018). To understand the function of DE lincRNAs under CR stress, we analyzed the protein coding genes coexpressed with these lincRNAs. This putative functional prediction relied on the development of a coexpression network. The coexpression analysis indicated that 425 lncRNAs were predicted to have transregulated targets whereas all DE 530 lncRNAs were predicted to have cis-regulated targets. KEGG pathway analysis revealed that a majority of transregulated target genes belong to carbon (18%) and amino acid biosynthesis pathways (19%); however, most of the cis-regulated target genes belong to the phenylpropanoid biosynthetic pathway (15%) and are involved in plant–pathogen interactions (15%). This suggests that the energy required to fuel the defense in R plants is probably provided by the carbon metabolism. Also, it is likely that biosynthesis of specific amino acids which are upregulated or reduced are involved in the plant defense (Rojas et al. 2014). However, more comprehensive studies are required to verify this hypothesis.

lncRNAs, like NATs, are also known to mediate the regulation of the corresponding sense transcripts in a concordant or discordant manner at a transcriptional and posttranscriptional level (Rai et al. in press; Wight and Werner 2013). In *B. napus*, 111 NATs were detected in response to *S. sclerotiorum* infection at 24 and 48 h postinoculation (hpi) (Joshi et al. 2016). In contrast, we observed very limited expression of the NATs in response to *P. brassicae* infection, which is possibly due to the fact that this type of lncRNA can be expressed in a spatial and temporal manner (Wang et al. 2014). Some of the NATs such as *COOLAIR* (Ietswaart et al. 2017), *cis NAT_{PHO1;2}* (Jabnour et al. 2013), *SHO lnc-NAT* (Zubko and Meyer 2007), and *TL-lncRNA* (Liu et al. 2018) are involved in the regulation of plant morphology and biotic or abiotic responses. In this study, we found that LNC_000418, LNC_000789, LNC_000674, and LNC_000562 are antisense to the protein-coding genes *Rac-like GTP-binding protein*, *defensin-like protein 2*, *flavone 3-dioxygenase 3-like protein*, and *WRKY transcription factor 12*, respectively; all four genes are directly or indirectly involved in plant secondary metabolism. Thus, considering the function of the predicted target genes of lncRNA, we suggest that primary and secondary metabolic pathways are also regulated by NATs and may play a role in the resistance to *P. brassicae*.

Most of the target genes identified in this study belong to the primary and secondary metabolic pathways, and include the genes involved in amino acid biosynthesis, carbon metabolism,

Fig. 5. Differentially expressed long noncoding RNAs (lncRNAs) from chromosome A08 of *Brassica napus*. Expression values are scaled from +2 (green) to –2 (red); black indicates no expression. S_1, S_2, and S_3 are the biological replicates of clubroot (CR)-susceptible *B. napus* and R_1, R_2, and R_3 are the biological replicates of CR-resistant *B. napus*. lncRNAs with asterisks are known to target genes encoding disease resistance proteins.

phenylpropanoid biosynthesis, and biosynthesis of secondary metabolites, indicating their probable association with the CR disease (Fig. 3). The importance of phenylpropanoid biosynthesis has already been demonstrated in response to biotic stress because it is known to generate an array of secondary metabolites involved in host–pathogen interactions (Caretto et al. 2015; Vogt 2010). Genes encoding proteins such as phenylalanine ammonia lyase (Vogt 2010), laccase 4, laccase 7 (Hu et al. 2018), and UDP-glycosyltransferases (Le Roy et al. 2016) have been implicated to play a significant role in phenylpropanoid biosynthetic pathways, and they have also been

identified as targets of lncRNAs in our study. It is known that *caffeoyl-CoA O-methyltransferase 1*, *CoMT*, *HCT*, and *CADH7* play critical roles in the biosynthetic pathway for lignification (Harakava 2005); among these genes, *CoMT*, *HCT*, and *CADH7* were found to be upregulated in the R plants at 10 dpi (Fig. 3). This suggests that the upregulation of this pathway prevents the spread of the *P. brassicae* in the roots of R plants and, thus, contributes to the decline in disease progression. Rojas et al. (2014) suggested that induction of primary metabolic pathways may be equally important because they can modulate signal transduction cascades influencing the plant defenses. Several

Fig. 6. A, Quantitative real-time (qRT)-PCR validation of pathogen-induced nine long noncoding RNAs (lncRNAs) detected by lncRNA-seq. **B**, qRT-PCR validation of lncRNAseq data for 10 targets predicted by correlation of expression with lncRNAs and two randomly selected genes. The expression of lncRNAs and target genes was normalized to *UBC9* (endogenous control) and lncRNAs regulating their respective targets are indicated within parentheses.

researchers have suggested that carbon skeletons synthesized during photosynthesis are used for growth (primary metabolism) or defense responses (secondary metabolism) (Caretto et al. 2015). Tayeh et al. (2014) reported that external application of the disaccharide sugar trehalose (consisting of two molecules

of glucose; it is also known as mycose or tremalose) increases the expression of *PR protein* genes in *Arabidopsis*, and external application of glucose upregulates *PR1* and *PR5* (Xiao et al. 2000). Trehalose is considered an inducer of plant defense to biotic and abiotic stresses (Tayeh et al. 2014). In this study, we

Fig. 7. Putative endogenous target mimic (eTM) LNC_001163 of *AGL16* inhibiting *bna-miR824* in response to clubroot infection in *Brassica napus*. **A**, Predicted base-pairing interactions between *bna-miR824* and *AGL16* and eTM long noncoding RNA LNC_001163 and *bna-miR824*. **B**, Quantitative real-time PCR results for LNC_001163, *AGL16*, and *bna-miR824*.

Fig. 8. **A**, **B**, and **C**, Interaction networks of a single long noncoding RNAs (lncRNAs), microRNA (miRNA) family, and the mRNAs; and **D**, interaction network of multiple lncRNAs with miRNA and mRNAs.

found an increased expression of LNC_002915, which is predicted to regulate an α,α -trehalose phosphate synthase 10 (*TPS10*), and a considerable decrease in the expression of LNC_00675 and its targets regulating the synthesis of trehalose phosphate phosphatase A. In addition, alkaline or neutral invertase and other similar proteins are involved in carbon metabolism and are known to cleave sucrose into glucose and fructose for energy production (Ehness et al. 1997) and we observed upregulation of these and similar genes in our study (Fig. 2). Functional research with these lncRNAs can provide further insight into the regulatory mechanism involved in primary metabolism and, eventually, in pathogen defense.

Hasan and Rahman (2016) mapped the CR resistance of rutabaga, which was introgressed into oilseed *B. napus*, on chromosome A08. By use of this resistance, we identified eight lncRNAs showing expression only in the R plants; however, expression these lncRNAs was low and not very consistent. Kopp and Mendell (2018) suggested that many lncRNAs can exhibit low expression in plants but still play an important role. Taking this into account, it can be assumed that these lncRNAs might play an important role in CR resistance through regulating protein coding genes or proteins by tethering to them (Fig. 4). Coexpression analysis of LNC_001063, LNC_001191, and LNC_001126 had predicted 8, 46, and 47 target genes, respectively, in the *B. napus* genome, and each lncRNA was found to regulate a different resistance (*R*) gene as well as genes

directly or indirectly modulating primary metabolism (Fig. 4). Another lncRNA (LNC_001167) was also predicted to regulate three *R* genes and 35 other genes modulating primary metabolism and phenylpropanoid biosynthesis (Fig. 4).

We further validated the relative expression of the selected lncRNAs and their target genes based on their direct or indirect association with chromosome A08. For instance, relative expression exhibits a positive correlation and LNC_003848 was upregulated in the R plants and exhibits a positive correlation with the expression of the target gene *Transparent Testa 12* (BnaA08g06670D) located on chromosome A08. Genes *transparent testa 12* as well as *gibberellin 2- β -dioxygenase 2* (BnaC03g59610D) are known to be involved in flavonoid and plant hormone biosynthesis and were found to be transregulated by LNC_003848. Flavonoids are known to play important roles such as protecting the plants from UV radiation, pathogen attack, and photo-oxidative stress (Falcone Ferreyra et al. 2012; Treutter 2006). The specific roles of these lncRNAs, their target genes, as well as the regulatory mechanism remain to be validated.

lncRNAs have also been hypothesized to form functional networks with miRNAs and mRNAs by functioning as either target mimics of miRNAs or precursors to miRNAs (Meng et al. 2012; Wu et al. 2013). Results from our study indicate that LNC_001163 and LNC_001191, located on chromosome A08, could serve as an eTM of bna-miR824 and bna-miR6028, respectively. Kutter et al. (2007) reported that bna-miR824

Fig. 9. Representative predicted long noncoding RNA functioning as microRNA (miRNA) precursor sequences.

targets the MIKC-type MADS box protein agamous-like 16 (AGL16), and our results indicated that the expression of *AGL16* and the predicted target mimic LNC_001163 of *bnamiR824* were upregulated and positively related to each other in response to CR infection (Fig. 7B). Interaction analysis among the mRNAs, miRNAs, and lncRNAs suggested multiple

and complex sets of interactions, including instances where a single lncRNA interacts with an miRNA family (Fig. 8A, B, and C) and a single miRNA interacts with multiple lncRNAs and mRNAs (Fig. 8D). Shumayla et al. (2017) also reported interaction of 1,047 lncRNAs with 222 miRNAs, which further interacted with 209 mRNA transcripts with various roles in

Fig. 10. Minimum free energy (MFE) structures for representative long noncoding RNA-mRNA pairs. MFE for each secondary structure is given in the bracket. Quantitative real-time PCR results for each pair are shown on the right side.

Triticum aestivum. We also detected eight lncRNAs in the present study to be the precursors of five miRNAs. For instance, LNC_000265 was observed to be upregulated in the R plants, and this seems to be the precursor for bna-miR169 in *B. napus* (bna-miR69n) (Fig. 9). bna-miR169 has been reported to be upregulated in response to drought in tomato (Zhang et al. 2011) and salt and drought stress in maize (Luan et al. 2014). Additionally, LNC_004360, a putative precursor of miRNA166a, was significantly expressed in the R plants (Fig. 9). The expression of miRNA166 has also been associated in response to *Botryosphaeria dothidea* in *Populus trichocarpa* (Zhao et al. 2012). Recently, J. Zhang et al. (2018) reported seven lncRNAs from *Brassica oleracea* and 15 lncRNAs from *B. rapa*, which are precursors of nine and 19 miRNAs belonging to eight and 15 miRNA families, respectively. Xin et al. (2011) and Joshi et al. (2016) demonstrated that lncRNAs can serve as precursors of miRNAs in plants and are involved in the regulation of plant responses to biotic stress. Several researchers such as Chauhan et al. (2017), Koroban et al. (2016), and Kumar (2014) also have summarized the functions of miRNAs in biotic and abiotic stresses, and results from our interaction analyses also lend credence to additional layers of gene regulation which may result from interactions of lncRNAs–miRNA and lncRNAs–target miRNAs.

Conclusion.

Using lncRNA-seq analysis, we have, for the first time, identified 530 DE lncRNAs in *B. napus* in response to *Plasmodiophora brassicae*. By comparing the response of the lncRNAs in the R and S plants, we have suggested a potentially important role in mediating resistance to *P. brassicae*. qRT-PCR analyses confirmed the expression trend of the lncRNAs and NATs with that of lncRNA-seq. Of the 530 lncRNAs, 16 DE lncRNAs were predicted to function as eTMs of miRNAs and eight DE lncRNAs as the precursor of miRNAs. Of the 24 DE lncRNAs from chromosome A08, eight showed low expression only in the R plants. Pathway analyses showed that the majority of the target genes regulated by these lncRNAs belong to the pathways involved in plant–pathogen interaction, plant hormone signaling, and primary-secondary metabolism. In this regard, the lncRNAs and their targets from A08 provide valuable information because this chromosome is associated with resistance to multiple pathotypes. Moreover, the knowledge of the *P. brassicae*-responsive lncRNAs gained from this research can be used for knock-down or overexpression studies to understand their molecular mechanisms and regulatory functions and may be useful in the development of markers in a breeding program for CR resistance.

MATERIALS AND METHODS

Plant material.

A doubled-haploid (DH) population derived from F₁ generation of Rutabaga-BF × UA AlfaGold was used in this study. The parent Rutabaga-BF is a CR-R inbred line derived from the rutabaga (*B. napus* var. *napobrassica*) cultivar Brookfield through self pollination of single plants (Hasan and Rahman 2016), while UA AlfaGold is an open-pollinated CR-S spring-type *B. napus* canola cultivar (Rahman 2016). The details of the development of the DH lines and phenotyping for resistance to CR disease have been described previously (Hasan and Rahman 2016).

Microscopy analysis for primary infection by *P. brassicae*.

To determine the time of primary infection by *P. brassicae* and the development of disease symptoms, 12 CR-R and 12

CR-S DH lines were grown in a greenhouse at 22 and 15°C (day and night, respectively) with a 16-h photoperiod. In total, eight plants of these lines were grown in 72-cell trays (52 by 26 by 5 cm, length by width by depth, respectively) filled with Sunshine Professional Growing Mix (Sun Gro Horticulture Canada Ltd.). Ten-day-old seedlings were inoculated with *P. brassicae* single-spore isolate SACAN-ss1 of pathotype 3 (Williams 1966) (obtained from Dr. Stephen Strelkov, University of Alberta) following a direct inoculation method. The details of inoculum preparation and inoculation procedure have been described in a previous study (Hasan and Rahman 2016). Briefly, the inoculum was prepared by crushing the galls using sterile water and the homogenized spore suspension was filtered through multilayered cheesecloth. The spore concentration was adjusted to 1×10^7 spores/ml and inoculation was done by injecting 1 ml of the spore suspension into the soil near the root of the seedlings (Voorrips and Visser 1993). Root samples of the inoculated seedlings were collected at 0 hpi and 6, 10, 14, 18, and 22 dpi. Uninoculated controls were also included in the study.

The roots were gently washed with water, cut into 10- to 15-mm segments, and fixed in a solution of formalin, acetic acid, and alcohol comprising 5 ml of commercial formalin (40% formaldehyde solution), 5 ml of glacial acetic acid, and 90 ml of 50% ethyl alcohol (Yeung and Saxena 2005) at room temperature for 24 h (Verma et al. 2014). Following this, samples were dehydrated in a series of ethanol dilutions, gradually followed by the treatment with the toluene (clearing agent) using Leica TP1020 tissue processor (Leica Biosystems). The tissues were then infiltrated with ParaplastH (Leica Biosystems) and cast into rectangular blocks. Longitudinal sections of roots, 10 mm thick, were prepared using A0 Rotary microtome (Leica Biosystems) and affixed to glass slides. The samples on the glass slides were deparaffinated with toluene, rehydrated using a graded series of ethanol, stained with Harris Hematoxylin, and counterstained with Eosin Y (Leica Biosystems). After dehydrating again with 100% ethanol and toluene, the slides were mounted with DPX mounting media (Sigma-Aldrich). The sections were viewed with a Zeiss Axioscope, photographed with Zeiss AxioCam, and analyzed using AxioVision software (Verma et al. 2014).

Plant materials for lncRNA-seq analysis.

In all, 12 R and 12 S DH lines were used for this study. In total, 192 seedlings of the 24 DH (12 each of R and S) lines ($24 \times 8 = 192$) were grown in the abovementioned 72-cell trays filled with Sunshine Professional Growing Mix. Seedlings were inoculated with *P. brassicae* single-spore isolate SACAN-ss1 pathotype 3, as described above. The inoculated plants were maintained in a greenhouse at 22 and 15°C (day and night, respectively) with a 16-h photoperiod, and the soil was kept saturated for successful infection and disease development. The experiment was repeated three times as biological replicates.

RNA isolation, library preparation, and sequencing.

For RNA isolation, roots of three seedlings from each of the 12 R and 12 S DH lines were harvested at 10 dpi as bulk of R and S samples, respectively; frozen in liquid nitrogen; and stored at –80°C until further use. The remaining plants of the R and S DH lines were grown to maturity and phenotyped for resistance in order to confirm the resistance or susceptibility of the DH lines.

Total RNA from the R and S bulks was extracted using TRIzol reagent (Invitrogen) and the Qiagen RNeasy Plant Mini Kit (Qiagen) as per the manufacturer's instructions. Root

samples were homogenized into a fine powder and 100 mg of this tissue powder was treated with 1 ml of TRIzol in an Eppendorf tube. The mixture was centrifuged at 9,425 relative centrifugal force (RCF) for 15 min at 4°C and the supernatant was collected in a new tube. To achieve phase separation, 200 µl of chloroform was added to the supernatant, mixed vigorously, incubated at room temperature for 5 min, and centrifuged at 9,425 RCF. The aqueous layer was collected in a new tube without disturbing the interface layer containing DNA and 0.5 ml of chilled 95% ethanol was added and mixed. The sample was then transferred to an RNeasy minispin column (pink) of the Qiagen RNeasy Plant Mini Kit and the next steps were carried out as per the manufacturer's instructions. The quality and quantity of total RNA was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). The integrity of the total RNA was determined on an Agilent Tapestation using RNA Screen Tape assay following the manufacturer's instructions (Agilent Technologies). The RNA samples with RNA integrity number greater than nine were used for further analysis.

In total, six libraries—three R (each sample with bulks of 12 plants) and three S (each sample with bulks of 12 plants)—were prepared and 4 µg of RNA of each sample was used for library preparation. Ribosomal RNA (rRNA) was removed from the samples using an Epicentre Ribo-zero rRNA Removal Kit (Epicentre) and strand-specific libraries were prepared using a NEB Next Ultra Directional RNA Library Prep Kit (NEB) following the manufacturer's instructions. RNA library sequencing was carried out using the Illumina HiSeq platform and 2 × 150-bp paired-end reads were generated. The library preparation and sequencing were conducted by Novogene.

Mapping to the reference genome and transcriptome assembly.

The lncRNAseq raw data in FASTQ format was processed using Novogene's in-house perl scripts and the reads containing adaptors, unrecognized bases, and low-quality reads were removed. Based on the clean reads, percentage of the total number of bases where the Phred score is greater than 20 (Q20) and 30 (Q30) was calculated. This indicated the base call accuracy and percentage of GC content in clean data. To align the reads, reference genome annotation files were downloaded from *B. napus* reference genome v5.0 (Chalhoub et al. 2014) and indexed using Bowtie v2.0.6 (Langmead and Salzberg 2012). The paired-end clean reads were aligned to this reference genome using TopHat v2.0.9 (Trapnell et al. 2012) and the mapped reads from each sample were assembled using the statistical segmentation model Scripture β2 (Guttman et al. 2010) and Cufflinks v2.2.1 (Trapnell et al. 2010). A combination of these enabled us to distinguish the expressed loci from experimental noise. The spliced reads were then used to assemble the expressed segments and a probabilistic model to simultaneously assemble and provide a maximum-likelihood explanation of the expression at a specific locus. The program Scripture was run with the default parameters, while 'min-frags-per-transfrag = 0' and '-library-type' were set for Cufflinks, with other parameters set to default. Sorting and removal of the duplicated reads and merging of the bam alignment file for each sample was accomplished by Picard- tools v1.41 and Samtools v0.1.18.

lncRNA detection and target prediction.

The Cufflink assemblies generated from the samples were merged using the software Cuffmerge (Trapnell et al. 2010) to remove the replicates and artifacts. The remaining transcripts were subjected to the exon number filter (exon ≥ 2) and transcript length filter (>200 nt) to remove the other known

noncoding RNA (rRNA, tRNA, snRNA, snoRNA, premiRNA, and pseudogenes) using Cuffcompare (Trapnell et al. 2010). Subsequently, the transcript sequences were classified and class-coded into candidate lincRNAs, intronic lncRNAs, and antisense lncRNAs. Four different software tools, including Coding Noncoding Index v2 (Sun et al. 2013); CPC 0.9-r2 (Kong et al. 2007), Pfam scan v1.3 (Punta et al. 2012), and phylogenic codon substitution frequency (Lin et al. 2011), were used to filter out the transcripts with coding potential. Only the transcripts without coding potential were considered as the candidate set of lncRNAs. The software program Cuffdiff v2.1.1 (Trapnell et al. 2010) was used to calculate the FPKMs in each sample. Fold changes for the DE lncRNAs were calculated as $\log_2(\text{fold change}) = \log_2(\text{FPKM susceptible}/\text{FPKM resistant})$. A *P* value was assigned to each lncRNA and adjusted by the Benjamin and Hochberg approach (Benjamini and Hochberg 1995) for controlling the false-discovery rate. DE lncRNAs were considered significant when the absolute value of $\log_2(\text{fold change}) > 1$, with a *q* value < 0.05. The targets, where the lncRNA plays a cis role, were predicted by searching for genes at 100,000 bp upstream and downstream of the lncRNAs. To predict the trans role, correlation between expression of lncRNAs and coding genes was calculated using the Pearson correlation coefficient, and genes having absolute correlation coefficient > 0.95 were considered for further functional enrichment analysis.

Classification and characterization of lncRNA functions.

The lncRNAs acting as precursor of the known miRNAs were identified by BLASTn analysis (e-value = 1e-5) (J. Zhang et al. 2018). For this, all of the stem loop sequences of known *B. napus* miRNA were downloaded from the miRbase database and aligned with the lncRNA sequences showing significant differential expression. The hairpin loop formation in the lncRNAs was first checked using the miRNAfold server (Tav et al. 2016), and the secondary structure was plotted using the Vienna RNAfold web server (Gruber et al. 2008) and visualized using Forna (Kerpedjiev et al. 2015).

lncRNAs mimicking the endogenous miRNA targets were predicted by psRNA-Target V2.2017 (Dai et al. 2018) by subjecting them to stringent parameters of maximum expectation set to 3.5 and target accessibility set to 25.0. The interaction between the lncRNAs, miRNAs, and miRNA gene targets were visualized using Cytoscape v3.6.1 (Shannon et al. 2003).

NATs were subjected to RNAup (ViennaRNA Package v2.4.9) (Hofacker 2009; Lorenz et al. 2011) to calculate the thermodynamics of their interaction with their corresponding sense mRNAs and, therefore, determine the potential candidates of lncRNA-mRNA interaction. The potential dimerized secondary structures were calculated using RNAcofold (ViennaRNA Package v2.4.9) (Lorenz et al. 2011).

qRT-PCR analysis.

The lncRNAs responsive to the *P. brassicae* infection and their respective targets from *B. napus* were validated using qRT-PCR. This included the lncRNAs from chromosome A08, lncRNAs which are found to be regulating the targets on chromosome A08 and elsewhere in the genome, as well as some random lncRNAs which regulate target genes predicted to play a role in plant hormone signaling. Sequences of the primers used in qRT-PCR are listed in Supplementary Table S1. All qRT-PCR analyses were performed on QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific) using PowerUP SYBR Green Master Mix from Applied Biosystems (Thermo Fisher Scientific). RNA isolated from roots of R and S DH lines inoculated with *P. brassicae* were treated with RNase-free DNase I. The total

RNA was reverse transcribed using random hexamers and the MiRNA-X miRNA First-Strand Synthesis Kit (Clontech Laboratories). Cycle threshold (Ct) values were determined for each sample based on three biological replicates and two technical replicates from each of the biological replicate. Constitutively expressed gene *UBC9* (*Ubiquitin-conjugating enzyme 9*) was used as an endogenous control. Comparison of gene expression was performed in the two samples, R and S, each normalized using an endogenous control with S as the calibrator. Fold change was calculated using the formula $2^{-\Delta\Delta Ct} = [(Ct \text{ gene of interest} - Ct \text{ endogenous control})_R - (Ct \text{ gene of interest} - Ct \text{ endogenous control})_S]$ (Schmittgen and Livak 2008).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

psRNAtarget (version updated in 2017):
<https://plantgrn.noble.org/psRNAtarget/>
B. napus reference genome v5.0:
<http://www.genoscope.cns.fr/brassicapapus/data/>
 miRbase database: <http://www.mirbase.org/>
 miRNAfold server <https://evryrna.ibisc.univ-evry.fr/miRNAfold>
 Vienna RNAfold web server:
<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>
 Forna: <http://rna.tbi.univie.ac.at/forna/>

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