Identification of Differentially Methylated miRNA Genes During Compatible and Incompatible Interactions Between Soybean and Soybean Cyst Nematode

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DNA methylation is a widespread epigenetic mark that affects gene expression and transposon mobility during plant development and stress responses. However, the role of DNA methylation in regulating the expression of microRNA (miRNA) genes remains largely unexplored. Here, we analyzed DNA methylation changes of miRNA genes using a pair of soybean (*Glycine max*) near-isogenic lines (NILs) differing in their response to soybean cyst nematode (SCN; *Heterodera glycines*). Differences in global DNA methylation levels over miRNA genes in response to SCN infection were observed between the isogenic lines. miRNA genes with significant changes in DNA methylation levels in the promoter and primary transcript-coding regions were detected in both lines. In the susceptible isogenic line (NIL-S), 82 differentially methylated miRNAs were identified in response to SCN infection whereas, in the resistant isogenic line (NIL-R), only 16 differentially methylated miRNAs were identified. Interestingly, *gma-miR5032*, *gma-miR5043*, *gma-miR1520b*, and *gma-2107-ch16* showed opposite methylation patterns in the isogenic lines. In addition, the miRNA paralogs *gma-miR5770a* and *gma-miR5770b* showed hypermethylation and hypomethylation in NIL-S and NIL-R, respectively. Gene expression quantification of *gma-miR5032*, *gma-miR5043*, *gma-miR1520b*, and *gma-miR5770a/b* and their confirmed targets indicated a role of DNA methylation in regulating miRNA expression and, thus, their targets upon SCN infection. Furthermore, overexpression of these four miRNAs in NIL-S using transgenic hairy root system enhanced plant resistance to SCN to various degrees with a key role observed for *miR5032*. Together, our results provide new insights into the role of epigenetic mechanisms in controlling miRNA regulatory function during SCN–soybean interactions.

Keywords: DNA methylation, gene expression, isogenic lines, miRNA, nematode–plant interactions, soybean, soybean cyst nematode

Methylation of cytosine residues in DNA is a well-defined epigenetic mark that affects gene expression, transposon mobility, and genome integrity (He et al. 2011). In the plant genome, methylated cytosines occur in three different contexts (CG, CHG, and CHH), where H denotes any base other than guanine (G). After the advent of a bisulfite sequencing method that allows the detection of methylated cytosines with high precision at genome scale, the importance of this epigenetic mark in various aspects of gene expression and genome organization and evolution was realized (Choi et al. 2020; Pei et al. 2019). The regulatory function of cytosine methylation is context and region dependent (Feng et al. 2010; Suzuki and Bird 2008). For example, DNA methylation of gene promoters can affect gene expression by affecting the recruitment of regulatory proteins. Also, DNA methylation of transposable elements (TEs) can affect their mobility and the expression of their adjacent genes (Dowen et al. 2012; Wang et al. 2013). Furthermore, DNA methylation of gene body regions can influence gene transcription and alternative splicing (Attwood et al. 2002; Choi et al. 2020; Shayevitch et al. 2018).

Environmental stress factors are known to induce transient nonheritable changes in DNA methylation patterns (Boyko et al. 2010; Secco et al. 2015; Wibowo et al. 2016; Yong-Villalobos et al. 2015), which can alter gene expression levels at a global scale to activate adaptive response. Also, plant pathogens have been shown to induce dramatic changes in plant methylomes (Hewezi et al. 2018). Methylome changes induced by phytopathogens share a common feature of DNA hypomethylation patterns, which were found to affect specific genes involved in various biological processes and plant defense (Hewezi et al. 2018). However, microRNA (miRNA) genes have been frequently ignored in methylome analysis. The observation that miRNA genes are tightly controlled and frequently respond to pathogen infection raises the possibility of miRNAs being epigenetically modified by DNA methylation.

miRNAs are a class of noncoding small RNAs that play central roles in the regulation of gene expression at mostly posttranscriptional level. miRNAs are 21 to 22 nucleotides long and formed by processing of long primary transcripts (pri-miRNAs) folded into hairpin loop-like structures (Rogers and Chen 2013). The miRNA genes are transcribed by RNA

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polymerase II (Pol II) and the primary transcript is stabilized by addition of poly A tail to the 3' end. The hairpin-like structures of the pri-miRNAs are recognized by Dicer-like RNase III endonuclease 1 (DCL1) and processed into miRNA/miRNA* duplex. The miRNA/miRNA* duplex is methylated by HUA ENHANCER (HEN1) and exported to cytoplasm (Yu et al. 2005). In the cytoplasm, the functional strand of the mature miRNA is loaded together with Argonaute 1 (AGO1) protein into the RNA-induced silencing complex (RISC) to target mRNA transcripts containing perfect or near-perfect complementary sequence for degradation or translational repression, causing posttranscriptional gene silencing (Bartel 2004).

In animal systems, it has been shown that DNA methylation of miRNA genes affects their biogenesis and expression and that methylation over miRNA genomic regions is enhanced in stress- and disease-related phenotypes (Glaich et al. 2019; Han et al. 2007; Ju et al. 2020; Mo et al. 2019; Shen et al. 2012; Varghese et al. 2018). In plants, recent reports have also established the biological significance of DNA methylation of miRNAs. For example, Song et al. (2015) reported a negative correlation between DNA methylation at the 5' flanking region and miRNA expression level during bisexual flower development in andromonoecious poplar (*Populus tomentosa*). This led to the identification of key miRNA genes regulated by DNA methylation during flower development. In addition, in *P. simonii*, various abiotic stress treatments were found to induce DNA methylation changes of miRNA genes with substantial influence on their abundance (Ci et al. 2015; Song et al. 2016). However, no similar study has been conducted thus far in *Arabidopsis*.

Plant-parasitic cyst nematodes (*Heterodera* spp.) are obligate biotrophs that infect the root system of host plants and establish a permeant feeding site, called the syncytium, from which the nematode feeds and completes its life cycle. Recent studies have revealed that soybean cyst nematodes (SCN; *Heterodera glycines*) and beet cyst nematodes (BCN; *H. schachtii*) induce DNA methylation changes in significant numbers of protein-coding genes and TEs in a context-specific fashion (Hewezi et al. 2017; Rambani et al. 2015, 2020). Also, it has been recently shown that the root-knot nematode *Meloidogyne graminicola* triggers hypomethylation in early-developing galls formed on rice roots (Atighi et al. 2020). Functional analysis of differentially methylated genes revealed their biological importance in plant–nematode interactions (Hewezi et al. 2017; Rambani et al. 2020). For example, *Arabidopsis* T-DNA insertional mutants of differentially methylated genes, including *NRPD1a*, *Glu synthase2*, *fatty acid reductase4*, and β -galactosidase3, was found to enhance plant susceptibility to BCN (Hewezi et al. 2017). Also, overexpression of a *receptor-like protein kinase* and a *dirigent-like protein* gene in transgenic soybean hairy roots dramatically reduced plant susceptibility to cyst nematodes (Rambani et al. 2020). However, information about epigenetic control of miRNA gene expression remains elusive despite several recent studies, which have shown that altered miRNA levels can exert substantial effects on plant susceptibility to cyst and root-knot nematodes (Hewezi 2020).

Considering the key role that miRNAs play in plant–nematode interactions, together with the increasing evidence of widespread DNA methylation changes in host plants in response to nematode infection, we hypothesized that SCN may induce DNA methylation changes in miRNA genes, affecting their expression and function. In this study, we analyzed DNA methylation changes of miRNA genes using SCN-resistant and -susceptible near-isogenic lines (NILs). We identified significant changes in DNA methylation levels in the promoter and primary transcript-coding regions of a significant number of miRNA genes. We show that differentially methylated miRNAs

are specific to each line, affect gene expression, and modulate soybean susceptibility to SCN.

RESULTS

Identification of differentially methylated miRNA genes during compatible and incompatible soybean–SCN interactions.

To examine the extent to which miRNA genes are subjected to DNA methylation changes during the susceptible and resistant interactions of soybean with SCN, we first compared global DNA methylation levels over all annotated miRNA genes between two soybean (*Glycine max*) NILs that differ in their response to SCN infection (race 3). Whole-genome bisulfite sequence reads of SCN-infected and noninfected root tissues of susceptible and resistant NILs at 5 days postinfection (dpi) (Rambani et al. 2020) were mapped to the promoter and primary transcript-coding regions of all annotated soybean miRNA genes in miRBase (release 21). The promoter region was determined as 2 kb upstream of miRNA primary transcript (Monteys et al. 2010). Under noninfected conditions, the susceptible isogenic line (NIL-S) showed higher levels of DNA methylation over miRNA genes compared with the resistant isogenic line (NIL-R) in the CG, CHG, and CHG contexts (Supplementary Fig. S1A to C). Boxplot analysis of absolute methylation levels across miRNA genes revealed significant increases in CG and CHG methylation levels in NIL-S compared with NIL-R under noninfected conditions (Supplementary Fig. S1D).

We next compared global DNA methylation levels over miRNA genes in each line under noninfected and SCN-infected conditions. In NIL-S, a trend of global DNA hypermethylation of miRNA genes was observed in the SCN-infected root samples compared with the noninfected control, particularly in the CHG and CHH sequence contexts (Fig. 1A to C). However, in NIL-R, we detected a slight increase in global DNA methylation in the infected samples compared with noninfected samples, particularly in the CG and CHG sequence contexts (Fig. 1D to F). Furthermore, boxplot analysis of absolute methylation levels across miRNA genes in both lines upon SCN infection revealed significant increases in methylation levels in all sequence contexts in both lines, with the exception of CHH methylation in NIL-R (Fig. 1G and H).

The differences in global DNA methylation of miRNA genes between NIL-S and NIL-R prompted us to identify miRNA genes that experience significant changes in DNA methylation patterns in both lines in response to SCN infection. To this end, we identified differentially methylated regions (DMRs) by dividing the miRNA promoter region into nonoverlapping windows of 500 bp and used them to compare level and direction of DNA methylation between control and SCN-infected samples. Primary transcript regions of miRNAs were also compared between noninfected and SCN-infected samples. Regions were considered significantly differentially methylated if the methylation difference was greater than 25% with adjusted *P* value < 0.01. In NIL-S, we identified 82 unique miRNA genes overlapping with 95 unique DMRs (Fig. 2A; Supplementary Table S1). Of these 82 miRNAs, 34 were hypermethylated and 53 were hypomethylated (Fig. 2A; Supplementary Table S1). The remaining five miRNAs overlapped with both hyper- and hypomethylated regions. In NIL-R, we identified 16 differentially methylated miRNA genes overlapping with 18 DMRs. Of these 16 miRNA, 10 were hypermethylated and 6 were hypomethylated (Fig. 2B; Supplementary Table S2). The 95 DMRs identified in NIL-S were found in the CG (28%), CHG (48%), and CHH (24%) sequence contexts (Fig. 2C; Supplementary Table S1). The 18 DMRs identified in NIL-R were found in the

Fig. 1. Global DNA methylation levels in three sequence contexts across microRNA (miRNA) genes in the isogenic lines under soybean cyst nematode (SCN)-infected and noninfected conditions. Comparison of global DNA methylation levels between SCN-infected (green) and noninfected conditions (red) in the susceptible near-isogenic line (NIL-S) over miRNA genes in the **A**, CG; **B**, CHG; and **C**, CHH sequence contexts. Comparison of global DNA methylation levels between SCN-infected (green) and noninfected conditions (red) in the resistant NIL (NIL-R) over miRNA genes in the **D**, CG; **E**, CHG; and **F**, CHH sequence contexts. Boxplots for absolute methylation levels across miRNA genes in **G**, NIL-S and **H**, NIL-R under noninfected and SCN-infected conditions in various sequence contexts. Asterisks (**) indicate P value < 0.01 with t test and NS = not significant. Global methylation is calculated at base pair level by dividing number of methylated cytosines by total coverage. A promoter region of 2 kb is divided into 60 bins and methylation levels are averaged across each bin and plotted as a data point on a line graph. The y-axis represents methylation level within the range of 0 to 1.

CG (22%), CHG (50%), and CHH (28%) contexts (Fig. 2D; Supplementary Table S2).

SCN-induced differential methylation in miRNA genes was detected in the promoter and primary transcript-coding regions

(Fig. 2E and F). Differential DNA methylation in NIL-R occurred mainly in the promoter region, with only gma-miR5032 showing hypermethylation in the primary transcript-coding region (Fig. 2F). In NIL-S, however, 56 miRNAs showed

Fig. 2. Heatmap presentations of differentially methylated microRNAs (miRNAs) identified in the isogenic lines. **A**, Methylation direction and sequence contexts of 82 differentially methylated miRNAs identified in the susceptible near-isogenic line (NIL-S). **B**, Methylation direction and sequence contexts of 16 differentially methylated miRNAs identified in the resistant NIL (NIL-R). miRNAs showing opposite methylation patterns in the isogenic lines are highlighted in blue. Red and green in the color bar represent hypermethylation and hypomethylation, respectively. DNA methylation contexts of differentially methylated regions identified in **C**, NIL-S and **D**, NIL-R. Distribution of differentially methylated promoters and primary transcript-coding regions identified in **E**, NIL-S and **F**, NIL-R. Pri-miRNA = long primary transcript miRNA.

differential DNA methylation in the promoter, 20 in the primary transcript-coding region, and 6 in both the promoter and primary transcript-coding regions (Fig. 2E). These results suggest that the miRNA primary transcript-coding region is less vulnerable to DNA methylation changes compared with promoter regions

regardless of the size difference between these regions. We also measured the distance between the DMRs and the transcriptional start site (TSS) of miRNA genes. The average distances of the DMRs to the miRNA TSS were 556 and 647 bp for NIL-S and NIL-R, respectively (Supplementary Tables S1 and S2).

Fig. 3. Gene ontology (GO) term enrichment analysis of the target genes of differentially methylated microRNAs (miRNAs) identified in the isogenic lines. Significantly enriched biological process GO terms for target genes of differentially methylated miRNAs identified in **A**, the susceptible near-isogenic line (NIL-S) and **B**, the resistant NIL (NIL-R). GO terms are clustered based on semantic similarity to other GO terms in the Uniprot database. The size of the circle is proportional to the abundance of the GO terms in our dataset. The color bar represents log₁₀ of GO term *P* value obtained from soybase GO enrichment tool.

TEs in miRNA gene promoters may enhance miRNA vulnerability to DNA methylation changes upon SCN infection.

We analyzed the promoter of miRNA genes for the presence of TEs to examine their potential involvement in increasing miRNA susceptibility to DNA methylation changes upon SCN infection. Interestingly, the promoters of 37 differentially methylated miRNA genes identified in NIL-S contained 92 TEs, which belong mainly to the Copia and Gypsy families of the long terminal repeat retrotransposons (Supplementary Table S3). Similarly, the promoters of five differentially methylated miRNAs identified in NIL-R contained nine TEs of the Copia and Gypsy families (Supplementary Table S4). It is noteworthy that 74 of these 99 unique TEs are among the previously identified differentially methylated TEs in these isogenic lines in response to SCN infection (Rambani et al. 2020). Therefore, the presence of TEs in the promoter of differentially methylated miRNAs may make miRNA genes prone to DNA methylation changes in response to SCN infection.

Identification and functional classification of target genes of differentially methylated miRNA during compatible and incompatible interactions.

To gain insight into the biological processes in which the differentially miRNAs function, we scanned publicly available degradome datasets to identify target genes of these miRNAs. For the differentially methylated miRNAs detected in NIL-S, we identified 165 target genes from previously published degradome datasets (Arikait et al. 2014; Ding et al. 2016; Fang et al. 2013; Hu et al. 2013; Shamimuzzaman and Vodkin 2012; Song et al. 2011; Xu et al. 2014; Xu et al. 2016; M. Zhao et al. 2015) (Supplementary Table S5). In addition a set of 167 targets were predicted using psRNATarget (Dai and Zhao 2011) (Supplementary Table S5) by applying high-stringency parameters that allow only one nucleotide mismatch between the mature miRNA sequence and its binding sites. For the differentially methylated miRNAs detected in NIL-R, 28 target genes were identified from the degradome datasets in addition to 49 predicted targets (Supplementary Table S6).

Gene ontology (GO) term enrichment analysis of the target genes of differentially methylated miRNAs identified in NIL-S revealed significant enrichment (P value < 0.01) for 17 terms, including cell division, response to hormone stimulus, developmental growth, meristem maintenance, organ morphogenesis, sulfate transport, and regulation of cell differentiation, for instance (Fig. 3A). The identified target genes of the differentially methylated miRNAs detected in NIL-R revealed significant enrichment for five GO terms involved in amine metabolic process, maintenance of apical meristem identity, root hair cell tip growth, microgametogenesis, and somatic embryogenesis (Fig. 3B).

DNA methylation affect the expression of differentially methylated miRNAs.

When the lists of differentially-methylated miRNAs in NIL-R and NIL-S were compared, a set of five miRNAs (gma-miR9743, gma-miR1520b, gma-miR5032, gma-miR5043, and gma-miR2107-ch16) was common to both lines. gma-miR9743 was hypermethylated in both NIL-R and NIL-S in response to SCN infection. In contrast, gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR2107-ch16 had methylation change in the opposite direction in both lines (Fig. 2A and B). gma-miR5032, gma-miR5043, and gma-miR2107-ch16 were hypermethylated in NIL-R and hypomethylated in NIL-S in response to SCN infection. A DMR overlapping with the gma-miR1520b promoter was hypomethylated in NIL-R and hypermethylated in NIL-S at 5 dpi

(Fig. 2A and B; Supplementary Tables S1 and S2). In addition, the miRNA paralogs gma-miR5770a and gma-miR5770b showed opposite methylation patterns in the isogenic lines. More specifically, gma-miR5770a was hypermethylated only in NIL-S, whereas gma-miR5770b was hypomethylated only in NIL-R (Fig. 2A and B; Supplementary Tables S1 and S2). Together, the opposite methylation patterns of these four miRNAs (gma-miR1520b, gma-miR5032, gma-miR5043, and gma-miR2107-ch16) as well as gma-miR5770a and gma-miR5770b suggest a role in soybean-SCN interactions.

To examine this hypothesis, we first quantified the expression of gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770a/b in both lines in response to SCN infection at 3, 5, and 10 dpi using three independent biological samples of infected and noninfected root tissues. At 3 dpi, gma-miR5043 was significantly downregulated in both lines, whereas gma-miR5032, gma-miR1520b, and gma-miR5770a/b were significantly upregulated only in infected NIL-S root samples compared with noninfected samples (Fig. 4A and B). No significant changes in the expression of these miRNAs were detected in NIL-R in response to SCN at this time point (Fig. 4B). At 5 dpi, the expression patterns of the four miRNAs became very distinct in both lines. gma-miR5032 and gma-miR1520b were upregulated in NIL-S and downregulated in NIL-R (Fig. 4A and B). gma-miR5770a/b showed downregulation in both lines, whereas gma-miR5043 showed downregulation only in NIL-R. At 10 dpi, the four miRNA genes showed slight or no significant changes in expression in both lines (Fig. 4A and B). Taken together, these data suggest that SCN-induced differential DNA methylation in the promoter of these miRNAs at 5 dpi would be the driving force for their gene expression changes in the isogenic lines.

Furthermore, we tested whether the miRNA gene expression changes observed upon SCN infection indirectly affect the expression of their target genes at the same time point. We quantified uncleaved transcript levels of six confirmed targets of these four miRNAs in the same RNA samples using quantitative real-time reverse-transcription PCR (qRT-PCR). These genes included *Glyma.05G180700* and *Glyma.08G138300* (confirmed targets of gma-miR5032), *Glyma.13G270900* (confirmed target of gma-miR5043), *Glyma.19G182000* (confirmed target of gma-miR1520b), and *Glyma.17G019300* and *Glyma.03G005400* (confirmed targets of gma-miR5770a/b) (Arikait et al. 2014; Ding et al. 2016; Fang et al. 2013; M. Zhao et al. 2015). In NIL-S, we found that significant upregulation of gma-miR5032 and gma-miR1520b at 5 dpi was associated with significant downregulation of the target genes *Glyma.05G180700* and *Glyma.19G182000*, respectively (Fig. 4A and C). Similarly, a slight nonsignificant upregulation of gma-miR5043 at 5 dpi was correlated with significant downregulation of the *Glyma.13G270900* target gene. Also, a significant increase in the expression of *Glyma.08G138300* at 10 dpi may be related to the small downregulation of its negative regulator (gma-miR5032) at the same time point. In NIL-R, the expression of *Glyma.13G270900* and *Glyma.05G180700* at 3 or 5 dpi seems to be posttranscriptionally regulated by gma-miR5043 and gma-miR5032, respectively (Fig. 4B and D). Of note is that the remarkable downregulation of gma-miR5770a/b in both lines at 5 dpi was associated with upregulation of its target genes (*Glyma.17G019300* and *Glyma.03G005400*) only in NIL-S, suggesting that additional genetic or epigenetic regulatory mechanism may be involved in the regulation of these target genes in NIL-R.

When we compared the expression of the six target genes between the isogenic lines in response to nematode infection we observed a distinct patterns of regulation. At 3 dpi, we observed significant differences in the expression of *Glyma.05G180700*,

Glyma.08G138300, *Glyma.13G270900*, and *Glyma.03G005400* between the isogenic lines in response to SCN infection (Fig. 4C and D). At 5 dpi, the expression of the six target genes was oppositely regulated in NIL-R and NIL-S (Fig. 4C and D). More specifically, whereas these target genes showed significant downregulation of approximately twofold in NIL-S, these genes showed no changes (*Glyma.08G138300*, *Glyma.13G270900*, and *Glyma.19G182000*) or upregulation (*Glyma.05G180700*, *Glyma.17G019300*, and *Glyma.03G005400*) in NIL-R (Fig. 4C and D). Similarly, at 10 dpi, differences in the expression of two target genes (*Glyma.05G180700* and *Glyma.08G138300*) were detected between NIL-R and NIL-S (Fig. 4C and D). Together, the expression differences of these four miRNAs and their targets in NIL-S and NIL-R, particularly at 5 dpi, are consistent with the opposite

methylation patterns detected in the promoters of these miRNA genes at the same time point.

gma-miR5032, gma-miR5043, gma-miR1520b, and miR5770a/b are more highly expressed in NIL-R than NIL-S under noninfected conditions.

We compared the basal expression levels of gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770a/b in non-infected NIL-S and NIL-R control samples corresponding to 3 and 5 dpi, where differences in the expression of these miRNAs in the isogenic lines were observed upon SCN infection. Interestingly, gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770a/b exhibited higher expression in NIL-R relative to NIL-S at both time points, with an increase ranging between

Fig. 4. Quantitative real-time PCR quantification of the expression levels of four differentially methylated microRNAs (miRNAs) and their confirmed targets in the isogenic lines in response to soybean cyst nematode (SCN) infection. Expression levels of gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770a/b in **A**, the susceptible near-isogenic line (NIL-S) and **B**, the resistant NIL (NIL-R) at 3, 5, and 10 days postinfection (dpi). Expression levels of six confirmed targets of gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770a/b in **C**, NIL-S and **D**, NIL-R at 3, 5, and 10 dpi. Expression levels of *Glyma.05G180700* and *Glyma.08G138300* (confirmed targets of gma-miR5032), *Glyma.13G270900* (confirmed target of gma-miR5043), *Glyma.19G182000* (confirmed target of gma-miR1520b), and *Glyma.17G019300* and *Glyma.03G005400* (confirmed targets of gma-miR5770a/b) were quantified in the same samples used in A and B. miRNA and target genes are indicated with the same color. Fold-change values represent expression levels in SCN-infected root samples relative to noninfected control samples, which were set to 1. Shown are averages of three biologically independent samples $\pm$ standard error. Statistically significant differences from noninfected controls were determined using *t* tests with *P* value < 0.05 and indicated by an asterisk (*).

two- and 13-fold (Supplementary Fig. S2). gma-miR5043 showed approximately twofold increase in the expression in NIL-R relative to NIL-S at both time points, whereas gma-miR5032 showed more than 10-fold increase at both time points (Supplementary Fig. S2). gma-miR1520b and gma-miR5770a/b showed between 2.6- and 6.4-fold increase in the expression level in NIL-R compared with NIL-S at both time points (Supplementary Fig. S2).

Functional validation of differentially methylated miRNAs.

We examined the potential involvement of differentially methylated miRNAs in establishing the compatibility of the interaction between soybean and SCN. To this end, the four miRNAs (gma-miR1520b, gma-miR5043, gma-miR5032, and gma-miR5770b), which showed opposite methylation patterns in the isogenic lines, were overexpressed in NIL-S using transgenic hairy root system. The primary transcript sequences of these four miRNAs were overexpressed under the control of a soybean ubiquitin promoter and the RuBisCO small subunit terminator. Transgenic hairy roots expressing the empty vector were also generated in NIL-S and used as negative controls. The transgenic hairy roots were selected with the aid of the green fluorescent protein (GFP) reporter. qPCR analysis of the mature miRNA expression in three biological samples of the transgenic hairy roots revealed at least twofold increase in the abundance of these miRNAs in the transgenic hairy roots relative to the GFP control roots (Fig. 5A). Because miRNAs function through base-pairing with their target genes leading to mRNA degradation and low transcript abundance, we quantified the uncleaved transcript levels of the six aforementioned confirmed targets (*Glyma.05G180700*, *Glyma.08G138300*, *Glyma.13G270900*, *Glyma.19G182000*, *Glyma.17G019300*, and *Glyma.03G005400*) of these four miRNAs in the same transgenic hairy root samples using qPCR. The levels of uncleaved transcripts of the target genes were reduced in the miRNA overexpressed roots compared with GFP control roots (Fig. 5B), thereby validating the functionality of overexpression constructs.

We then assayed the transgenic hairy root plants to SCN susceptibility. In these experiments, each composite plant with three transgenic hairy roots was inoculated with approximately 3,000 eggs of SCN race 3 (HG type 0) and, 5 weeks after inoculation, the number of cysts per plant was counted and used to determine plant susceptibility. Overexpression of gma-miR1520b and gma-miR5043 showed approximately 30% reduction in cyst number, although this reduction is statistically insignificant (Fig. 5C). However, gma-miR5770b-overexpressing plants showed approximately 63% decrease in susceptibility to SCN. Importantly, overexpression of gma-miR5032 strongly enhanced plant resistance to SCN, with an average of 5.1 cysts per plant compared with 280.6 cysts in the susceptible control line expressing the empty vector (Fig. 5C). These results indicate that gma-miR5032 and gma-miR5770b function as positive regulators of SCN resistance.

DISCUSSION

In this study, we quantified differential DNA methylation of miRNA genes during compatible and incompatible interactions of soybean with SCN using two NILs. The use of NILs allowed us to determine the methylation differences with minimum genetic background effects. The analysis provided several insights into the differences between the resistant and susceptible responses to SCN. (i) The susceptible line is more vulnerable to DNA methylation than the resistant line because 82 miRNAs were identified as differentially methylated in NIL-S, whereas only 16 were found to be differentially methylated in NIL-R. This finding is in agreement with a previous study showing that DNA methylation changes are more dynamic in the susceptible

Fig. 5. Nematode susceptibility assays of microRNA (miRNA) overexpression lines. Expression levels of **A**, gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770b and **B**, six target genes in overexpression hairy roots samples. Fold-change values represent expression levels in the overexpression lines relative to control plants expressing the empty vector. Shown are averages of three biologically independent samples $\pm$ standard error (SE). Statistically significant differences from control samples were determined using *t* tests with *P* value < 0.05 . **C**, Nematode susceptibility levels of miRNA overexpression hairy root lines. Transgenic hairy roots overexpressing gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR5770b were generated in the susceptible near-isogenic line (NIL-S). Each composite plant was inoculated with approximately 3,000 eggs of soybean cyst nematode (SCN) race 3 per plant and, 5 weeks postinoculation, the number of cysts per plant were counted. Composite NIL-S plants expressing the empty vector were used as susceptible control. Data are averages of three replicates, each with at least three plants, $\pm$ SE. Statistically significant differences between miRNA overexpressing plants and the control were determined using analysis of variance. Bars with different letters are statistically significantly different ($P < 0.05$) from control plants expressing the empty vector.

interaction than the resistant interaction (Rambani et al. 2020). (ii) DMRs overlapping with miRNA genes showed opposite trends of regulation during resistant and susceptible interactions (Fig. 2) despite both lines showed increased global DNA methylation over miRNA genes in response to SCN infection (Fig. 1). Whereas, in NIL-S, hypo-DMRs overlapping with the miRNA gene promoter are more prevalent than hypermethylation, the opposite trend was observed in NIL-R (Fig. 2). (iii) Differential DNA methylation occurs more predominantly in miRNA promoter than primary transcript-coding regions. This is the opposite of protein-coding genes, where methylation changes in gene body regions occur more predominantly than gene promoters during plant interactions with cyst nematodes (Hewezi et al. 2017; Rambani et al. 2015, 2020). Although DNA methylation in gene promoters is known to contribute to the control of gene transcription by affecting transcription factor accessibility, the biological significance of DNA methylation of miRNA primary transcript-coding regions remains largely unknown. However, DNA methylation of miRNA primary transcript-coding regions may have a role in miRNA biogenesis, as recently reported in mammalian cells (Glaich et al. 2019). In this scenario, DNA methylation in the primary miRNA transcript-coding region may slow the elongation rate of PolII, thereby providing the necessary time for newly synthesized primary miRNA transcripts to be processed into mature molecules (Glaich et al. 2019). (iv) Two-thirds of DMRs overlapping with miRNA genes were found in non-CG sequence contexts, consistent with the predominance of CHG and CHH methylation in gene promoters (Lindroth et al. 2001). (v) Methylation changes in miRNA genes were unique between the isogenic lines. Only five miRNA genes were found to be differentially methylated in both lines. However, four (gma-miR5032, gma-miR5043, gma-miR1520b, and gma-miR2107-ch16) of these five miRNAs showed opposite methylation patterns in the isogenic lines.

One obvious question raised from our analysis is whether DNA methylation levels across miRNAs are equivalent to those observed for protein-coding genes and TEs. Under noninfected conditions, the global methylation levels over miRNAs were higher in NIL-S compared with NIL-R (Supplementary Fig. S1) and follow the same patterns previously reported in the isogenic lines for protein-coding genes and TEs, particularly in non-CG contexts (Rambani et al. 2020). Under SCN-infected conditions, NIL-R exhibited increased global methylation levels across miRNA genes, similar to what was previously reported for protein-coding genes and TEs in NIL-R upon SCN infection (Rambani et al. 2020). SCN-infected NIL-S also exhibited increased global methylation levels over miRNAs, even though the same line showed global hypomethylation over protein-coding genes and TEs upon SCN infection (Rambani et al. 2020). Despite our finding that global methylation levels were increased over miRNAs in SCN-infected NIL-S, the majority of DMRs were hypomethylated. Similarly, hypo-DMRs overlapping with genes and TEs in NIL-S were more prevalent than hyper-DMRs in response to SCN infection (Rambani et al. 2020).

Compared with *Arabidopsis*, the genomes of crop plants such as soybean contain higher content of TEs, many of which are located close to genes, making these genes more vulnerable to DNA methylation changes (Zhang et al. 2018). The presence of 99 TEs in the promoter of 41 differentially methylated miRNA genes may have contributed to the increased level of DNA methylation over miRNAs and their vulnerability to DNA methylation changes upon SCN infection. This suggestion is consistent with our finding that 74 of these 99 TEs have been previously identified as differentially methylated in these isogenic lines upon SCN infection (Rambani et al. 2020). In addition, our finding that the large majority of the TEs located in the promoter of differentially methylated miRNA genes belongs to

the Copia and Gypsy families is in line with our recent findings that retrotransposons tend to be more vulnerable to DNA methylation change upon cyst nematode infection than other types of TEs (Hewezi et al. 2017; Piya et al. 2017a; Rambani et al. 2020).

Positive and negative associations between DNA methylation of TEs and the expression of nearby genes were reported in several studies (Dowen et al. 2012; Hewezi et al. 2017; Le et al. 2014; Wang et al. 2013). DNA methylation of TEs located in miRNA gene promoters may affect the expression of miRNA genes by inhibiting TE-generated cryptic transcripts, which may interfere with miRNA transcription. TE methylation may also alter chromatin structure of miRNA gene promoters, directly affecting miRNA transcription. In addition, in some cases, TEs can function as *cis*-regulatory elements (Makarevitch et al. 2015), and their methylation status may alter the binding ability of transcriptional activators or repressors, thereby affecting miRNA transcription.

The expression levels of gma-miR5032 and gma-miR5043 in the isogenic lines are consistent with the anticipated negative association between DNA methylation and gene expression (Zhang et al. 2018). However, we detected a positive association between DNA methylation and gene expression for gma-miR1520b. This finding may be related the characteristics of the DMRs, including methylation level and sequence context, as well as the presence of regulatory elements of negative regulators and repressive chromatin marks where hypermethylation may result in gene upregulation and hypomethylation may result in gene downregulation (Gent et al. 2013; Hewezi et al. 2017; Rambani et al. 2015; Zhang et al. 2018). Another possibility is that upregulation of gma-miR1520b in NIL-S at 5 dpi could be due to recognition of the hypermethylated CHG region in the promoter by specific transcription factors that possess specific binding activity to methylated DNA-binding elements, as reported for human transcription factors (Yin et al. 2017). The association between DNA methylation and the expression of gma-miR5770a/b in the isogenic lines can be explained by the possibility that partial hypomethylation of gma-miR5770b in a 500-bp region in response to SCN was not sufficient for gma-miR5770b upregulation in NIL-R. In addition, increased hypermethylation of gma-miR5770a in NIL-S in response to SCN infection only increased the level of gma-miR5770a downregulation. Though our data point to a possible role of DNA methylation in regulating the expression of these miRNAs and, indirectly, their targets, we don't rule out the possibility that other genetic or epigenetic regulatory mechanisms may also play a role.

Several lines of evidence implicate the identified differentially methylated miRNAs between the isogenic lines upon SCN infection in plant–SCN interactions. Many of the identified differentially methylated miRNAs have been shown to change their abundance in response to SCN infection (Lei et al. 2019; Li et al. 2012; Tian et al. 2017). In addition, several of miRNA targeted genes were previously reported as SCN responsive and include genes encoding functions important for nematode infection. It is remarkable that the identified target genes are enriched in functions related to cell differentiation, organ morphogenesis, and cell fate and specification (Fig. 3A). Because these functions are vital for syncytium formation and development (Hewezi 2015), it is tempting to speculate on the importance of these differentially methylated miRNAs in establishing nematode feeding sites and, hence, the compatibility of soybean–SCN interactions. Furthermore, we noted that a significant number of the miRNA target genes are transcription factors belonging to various families. This includes auxin response, MYB, bHLH, GRAS, scarecrow, NAC, and nuclear factor Y family transcription factors, for instance (Supplementary Tables S5 and S6). This observation holds true for both

isogenic lines, and suggests a role of differentially methylated miRNAs in transcriptome reprogramming during nematode infection of soybean plants. Previous reports have established miRNAs as key mediators of global transcriptome change during nematode infection by targeting plant transcription factors (Hewezi et al. 2008, 2012; Li et al. 2012; Medina et al. 2017; Piya et al. 2017b). Among the differentially methylated miRNAs, we found miR393 (Wong et al. 2014), miR172 (Q. Zhao et al. 2015), miR319 (Zhang et al. 2016; W. Zhao et al. 2015), and miR160 (Natarajan et al. 2018), whose roles in modulating plant defense response are well established.

Our functional assays provided evidence for the involvement of differentially methylated miRNAs in soybean-SCN interactions. Overexpression of *gma-miR5770b* and *gma-miR5032* significantly enhanced plant resistance. In this context, it may be relevant to mention that the basal expression levels of these miRNAs were much higher in the NIL-R compared with NIL-S under noninfected conditions. Thus, it is reasonable to expect that constitutive overexpression of *gma-miR5032* and *gma-miR5770b* in NIL-S would surpass the slight increases in their expression observed in this line at 3 or 5 dpi, leading to plant resistance. *gma-miR5770b* targets two copper amine oxidase family proteins (Glyma.17 G019300 and Glyma.02G120600) (Ding et al. 2016; Fang et al. 2013), among other genes. Copper amine oxidase (CuAO; EC 1.4.3.6) catalyzes the oxidative deamination of polyamines. It has been shown that cyst nematodes modulate polyamine metabolism and signaling via the 10A06 effector protein (Hewezi et al. 2010), and the potential involvement of *gma-miR5770b* in modulating polyamine concentration suggests a role of DNA methylation of *gma-miR5770b* in this modulation. Oxidative deamination of biologically active amines by copper amine oxidases results in the production of hydrogen peroxide, a signaling molecule known to be involved in various aspects of plant growth and defense responses (Neill et al. 2002; Smirnov and Arnaud 2019). The generated hydrogen peroxide at a low concentration consequent to amine degradation may enhance antioxidant machinery in the syncytial cells, leading to successful nematode parasitism (Hewezi et al. 2010; Siddique et al. 2014). Thus, it is reasonable to speculate that down-regulation of copper amine oxidase genes in transgenic hairy roots overexpressing *gma-miR5770b* eliminates this protective function, leading to syncytium degradation and plant resistance. It may be important to mention that previous studies have also linked amine oxidases to various aspects of plant defenses, including cell death, cell wall lignification, and oxidative burst (Cona et al. 2006). However, the reduced nematode susceptibility of *gma-miR5770b*-overexpressing plants where *Glyma.17G019300* and *Glyma.03G005400* were down-regulated argues against a role of these copper amine oxidases in positively regulating defense responses.

The effect of *gma-miR5032* overexpression on providing robust resistance against SCN is remarkable. Two genes (*Glyma.05G180700* and *Glyma.08G138300*) encoding glycosyl hydrolases have been confirmed as targets of *gma-miR5032* (M. Zhao et al. 2015). This enzyme is involved in the breakdown of sucrose and other complex carbohydrate molecules into smaller molecules such as glucose, which is considered the major source of carbohydrate input in the syncytium (Cabello et al. 2014; Hofmann et al. 2009). Therefore, one can speculate that constitutive downregulation of these enzymes in *gma-miR5032*-overexpressing plants may affect sugar pools in the syncytium, leading to nematode starvation and plant resistance.

In conclusion, our study demonstrates that SCN can induce hyper- and hypomethylation in miRNA genes, particularly those with functions related to plant response to nematode infection. DNA methylation can regulate miRNA expression in a manner that provides cell-type-specific and infection-stage-specific

expression. Another possibility is that DNA methylation may regulate the steady-state expression of tightly regulated miRNAs, preventing aberrant induction or repression during the infection. Therefore, modulating and fine tuning the expression levels of miRNA genes in the nematode feeding sites through DNA methylation may facilitate or hinder nematode infection. Because several miRNAs have been established as major regulators of plant-nematode interactions, further studies are needed to better understand the underlying mechanisms of epigenetic regulation of miRNA expression and how interference with this regulation may enhance plant resistance.

MATERIALS AND METHODS

Plant materials.

Two soybean NILs (TN09-16 and TN09-29) were previously described (Rambani et al. 2020). TN09-16 is susceptible to SCN (race 3), whereas TN09-29 is resistant. As early as 5 dpi, the differences between the NILs were observed because NIL-R showed a Peking-type resistance of strong syncytium degradation (Rambani et al. 2020).

Differential DNA methylation analysis of miRNA genes.

Trimmed and high-quality bisulfite sequencing reads of the near isogenic lines under SCN infected and noninfected conditions (Rambani et al. 2020) were mapped to *Glycine max* assembly version 1 (JGI Phytozome database) using Bismark (Krueger and Andrews 2011). Annotation of miRNA genes in soybean genome was downloaded from miRBase (release 21) to obtain coordinates of the primary miRNA gene body in the soybean genome assembly. The promoter of miRNA was defined 2 kb upstream of primary miRNA start site. The R package methylKit (Akalin et al. 2012) was used to identify DMRs in miRNA promoter and primary transcript-coding regions. The 2-kb promoter region was further split into four bins of 500 bp for accurate detection of differential methylation. Regions were considered significantly differentially methylated if the methylation difference was greater than 25% with adjusted *P* value < 0.01. BEDTOOLS CLOSEST (Quinlan and Hall 2010) was used to identify TEs that overlap with the promoters of differentially methylated miRNAs and calculate their distance from the miRNA transcriptional start site. Metaplots representing global DNA levels over miRNA genes in various sequence contexts were generated using the ViewBS package (Huang et al. 2018) and the MethOverRegion function. Absolute methylation data extracted from ViewBS using the MethOverRegion function were used to generate boxplots representing global DNA methylation levels over miRNA genes using the R package ggplot2.

Identification of miRNA targets and their functional analysis.

The psRNA Target server (Dai and Zhao 2011) was used to identify targets of differentially methylated miRNAs. Mature miRNA sequences of differentially methylated miRNAs were used to search for complementarity sequences in all annotated soybean protein-coding genes. The alignment was performed with default parameters allowing only one mismatch in the binding region. An alignment score was calculated considering miRNA length, penalizing for G:U pairing, and opening and extending a gap. Genes with an alignment score of less than 3.0 were considered as potential miRNA targets. Targets of differential miRNAs were also identified from the sequencing degradome dataset. We compiled data from various publicly available soybean degradome datasets (Arik et al. 2014; Ding et al. 2016; Fang et al. 2013; Hu et al. 2013; Shamimuzzaman and Vodkin 2012; Song

et al. 2011; Xu et al. 2014; Xu et al. 2016; M. Zhao et al. 2015) and searched for targets of the differentially methylated miRNAs. GO of target genes was determined using the SoyBase toolshed and was visualized using REVIGO (Supek et al. 2011).

Construction of miRNA overexpression vectors.

The primary transcripts of gma-miR1520b, gma-miR5043, gma-miR5032, and gma-miR5770b were amplified from genomic DNA using a primer pair containing the *Bam*HI and *Asc*I restriction sites (Supplementary Table S7). The PCR conditions used were 94°C for 2 min; followed by 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were digested, purified, and ligated into the binary vector pG2RNAi2 under control of the Gmubi promoter. The pG2RNAi2 vector contains the GFP as a selectable marker. All sequences were confirmed by sequencing. The binary vectors containing the miRNA primary transcripts as well as the empty pG2RNAi2 vector were introduced into *Agrobacterium rhizogenes* strain K599 for generating transgenic hairy roots.

Generation of transgenic soybean hairy roots.

Soybean seed of NIL-S were surface sterilized with bleach and placed evenly spaced on a sheet of wet germination paper. Healthy germinated seedlings were transferred to an 18-cell germination tray containing sterile autoclaved vermiculite. Five-day-old seedlings were injected with strain K599 of *A. rhizogenes* containing the binary vectors. A bacterial cell suspension in distilled water was injected into the hypocotyl of soybean seedlings using a 3-ml syringe needle. The inoculated seedlings were grown in a growth chamber at 26°C with a cycle of 16 h of light and 8 h of darkness. Approximately 4 to 5 weeks after inoculation, the transgenic hairy roots were selected using GFP with the aid of an epifluorescent microscope (Olympus stereomicroscope, model SZX12; Olympus America, Center Valley, PA, U.S.A.). Soybean plants with at least three transgenic hairy roots were used for SCN susceptibility assays. Transgenic roots were also collected in three biological replicates for RNA isolation and qPCR quantification of the expression levels of miRNAs and their target genes.

SCN susceptibility assay.

The nematode susceptibility assays were conducted as described by Rambani et al. (2020). In brief, the transgenic hairy roots overexpressing gma-miR1520b, gma-miR5043, gma-miR5032, and gma-miR5770b as well as the empty vector were planted in 656-cm³ cone-tainers (6.4 by 25.4 cm) filled with steam-sterilized sand and top soil at a 3:1 ratio. The plants were arranged in a randomized complete block design with three replicates, each with at least three plants. Each plant was inoculated with approximately 3,000 eggs of SCN race 3 (HG type 0). Approximately 5 weeks postinoculation, the cysts were extracted from each plant separately and used to determine susceptibility level in miRNA overexpression plants relative to negative control plants expressing the empty vector. Statistically significant differences between miRNA overexpression plants and control were determined using analysis of variance.

Nematode inoculation, RNA isolation, and qPCR quantifications.

Soybean seed were surface sterilized and then germinated on germination paper at 26°C in darkness. After 3 days, healthy seedlings were inoculated with approximately 3,000 second-stage juvenile of SCN HG type 0 (race 3) per seedling. The plants were transferred to a plant growth chamber at 26°C with 16 h of light and 8 h of darkness. Three biological samples of root tissues were collected at 3, 5, and 10 days after nematode

inoculation from SCN-infected plants and the corresponding noninfected controls. The tissues were flash frozen, ground to fine powder, and then used for RNA isolation.

To quantify the mature miRNA, total RNA was isolated from 500 mg root tissues using TRIzol reagent (Invitrogen) following the manufacturer's instructions. RNA was reversely transcribed into cDNA using Mir-X miRNA First-Strand Synthesis Kit (Clontech) according to the manufacturer's instructions. For each assay of qRT-PCR, approximately 25 ng of synthesized cDNA was used as a template. qRT-PCR assays were performed in QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using TB Green Advantage qPCR Premix (Clontech) following the manufacturer's instructions. The PCR was performed as follows: 95°C for 3 min and 40 cycles of 95°C for 30 s and 60°C for 30 s. Dissociation curves were generated to determine amplification specificity. The dissociation program was 95°C for 15 s and 50°C for 15 s, followed by a slow gradient from 50 to 95°C.

To quantify the targets of miRNAs, total RNA was isolated from 500 mg of root tissue according to Verwoerd et al. (1989). Total RNA was treated with DNase I (Invitrogen). RT-PCR assays were performed in the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using the Verso 1-step RT-qPCR Low ROX Kit (Thermo Scientific) according to the manufacturer's protocol. The PCR program was 50°C for 15 min, followed by 95°C for 15 min and then 40 cycles of 95°C for 15 s, 60°C for 90 s, and 72°C for 30 s. The dissociation curves were generated using the following program: 95°C for 15 s and 60°C for 75 s, followed by a slow gradient from 60 to 95°C. Small nuclear RNA U6 and 60S were used to normalize the expression levels of miRNAs, whereas soybean ubiquitin (Glyma.20G141600) and β -actin (Glyma.15G050200) were used to normalize the expression levels of target genes (Rambani et al. 2015; Wan et al. 2015). Gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Primer sequences used in qPCR assays are provided in Supplementary Table S7.

Data availability.

MethylC-Seq data are available the National Center for Biotechnology Information database under accession number GSE130134.

AUTHOR-RECOMMENDED INTERNET RESOURCES

miRBase database: <http://www.mirbase.org>
JGI Phytozome database: <https://phytozome.jgi.doe.gov/pz/portal.html>
The psRNA Target server: <http://plantgrn.noble.org/psRNATarget>
SoyBase toolshed: https://soybase.org/goslimgraphic_v2/dashboard.php

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