Gma-miR408 Enhances Soybean Cyst Nematode Susceptibility by Suppressing Reactive Oxygen Species Accumulation

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Abstract: Soybean cyst nematode (SCN, *Heterodera glycine*) is a serious damaging disease in soybean worldwide, thus resulting in severe yield losses. MicroRNA408 (*miR408*) is an ancient and highly conserved miRNA involved in regulating plant growth, development, biotic and abiotic stress response. Here, we analyzed the evolution of *miR408* in plants and verified four *miR408* members in *Glycine max*. In the current research, highly upregulated gma-miR408 expressing was detected during nematode migration and syncytium formation response to soybean cyst nematode infection. Overexpressing and silencing *miR408* vectors were transformed to soybean to confirm its potential role in plant and nematode interaction. Significant variations were observed in the MAPK signaling pathway with low OXI1, PR1, and wounding of the overexpressing lines. Overexpressing *miR408* could negatively regulate soybean resistance to SCN by suppressing reactive oxygen species accumulation. Conversely, silencing *miR408* positively regulates soybean resistance to SCN. Overall, gma-miR408 enhances soybean cyst nematode susceptibility by suppressing reactive oxygen species accumulation.

Keywords: *miR408*; Soybean cyst nematode; Overexpress; Short tandem target mimic; ROS-reactive oxygen species

1. Introduction

MicroRNAs are typically endogenous small non-coding RNAs of 20 to 24 nucleotides (nt) in length, which play a crucial role in negatively regulating post-transcriptional gene expression levels [1]. MicroRNA genes encoding miRNAs are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II), and then these pri-miRNAs are processed into miRNA-5p/miRNA-3p duplexes by the RNase III family enzyme DICERLIKE1 (DCL1). Subsequently, these duplexes are 2′-O-methylated by the methyltransferase HUA ENHANCER1 (HEN1) at the 3′ end. One strand of the duplex is incorporated into ARGONAUTE1 (AGO1) to form an active RNA-induced silencing complex (RISC). MicroRNA/target complementarity guides RISC to target the mRNA slicing. In plants, these small yet impactful miRNAs play an important role in orchestrating various biological processes, such as development, responses to environmental stress, transduction of phytohormone signals, and defenses against plant pathogens [2].

MicroRNA408 (*miR408*) is an ancient and highly conserved miRNA, which is involved in the regulation of plant growth, development and stress response [3]. A total 182 *miR408* precursors (*MIR408*) and 351 mature *miR408* sequences (*miR408*) have been identified from 118 different plants, which are deposited in miRBase [4] and sRNAanno [5]. Among these one hundred and eighteen plants, there are two species of bryophyte, one species of pteridophyte, three species of gymnosperms, and one hundred and twelve species of...
angiosperms (dicots vs. monocots are 86 vs. 24, respectively). Most miR408 precursors are produced separately in the two arms. For instance, the miR408 precursors produce miRNA in the two arms of Arabidopsis thaliana, Oryza sativa, Glycine max, etc. Among them, the members of miR408-5p were quite specific, while the members of miR408-3p were conservative. miR408 evolved gradually from bryophytes and played important roles in the evolution of plants to dicotyledonous plants. It may have been the emergence and change of miR408 that led to the evolution of various higher plant species [3]. miR408 has been reported to target a variety of blue copper protein members, including those in small blue proteins (phytocyanin family: plantacyanin/PLC, uclacyanin/UCL) and blue oxidases (laccase/LAC). miR408 regulates the growth and development of different plants by down-regulating its targets, encoding blue copper (Cu) proteins, affects copper homeostasis in the plant cell [6]. In addition, miR408 improves tolerance to stress by down-regulating target genes and enhancing cellular antioxidants, thereby increasing the antioxidant capacity of plants [7–10].

Expression of miR408 gene adapting to the diverse environmental stresses suggests that multiple different transcription factors may be involved in its regulation. Feng et al. [7] reported that tae-miR408 was positively correlated with the resistance of host plants to abiotic stresses and stripe rust by regulating one of its target genes, the chemocyanin-like protein gene (TaCLP1) in wheat. Upon drought stress, expression of miR408 was up-regulated in Medicago truncatula [10] and in Hordeum vulgare [11], while reduced miR408 expression was observed in Prunus persica [12] and in Oryza sativa [13]. During Puccinia striiformis f. sp. tritici (Pst) infection, the up-regulation of miR408 may trigger the lignin biosynthetic pathway as a hypersensitive response (HR) to prevent epidermis rupture. Ma et al. [14] proposed that increasing the expression of miR408 can reduce reactive oxygen species (ROS) and regulate the target genes encoding Cu-containing proteins, thereby increasing the endogenous availability of Cu for other Cu proteins involved in response to abiotic and biotic stress.

Here, we identified four miR408 members in Glycine max, which are gma-miR408a/b/c/d. Detailed expression patterns during plant development were analyzed by expressing pmiR408:GUS in soybean roots. Overexpression (OE) and short tandem target mimic (STTM) silence lines were generated to determine the regulatory role of miR408 on SCN infected response. This study aimed to understand the function of miR408 in the SCN resistance of soybeans.

2. Results
2.1. Identification of miR408 in Glycine Max

As a typical multifunctional miRNA, miR408 family members have been annotated in more than 100 different plants. In order to reveal the distribution and evolution of miR408 family members in plants, we downloaded all miR408 precursor and mature sequences of plants from the miRbase database (http://www.mirbase.org/, accessed on 2 July 2021) and sRNAanno database (http://plantsrna.org/, 2 July 2021). Taxonomic and phylogenetic analyses were performed on them, and the results were summarized in Tables S1 and S2. Phylogenetic tree was conducted with 182 precursors sequence from 118 species, as shown in Figure 1.

All phylogenetic analysis steps were conducted using PhyloSuite and its plug-in programs [15]. The miR408 precursor sequences was aligned with MAFFT. Maximum likelihood phylogenies were inferred using IQ-TREE [16] under the TVMe+R5 model for 5000 ultrafast bootstraps. Evolview-v2 was used to visualize the tree, with Physcomitrella patens (Bryophyta, Fuariales) as the root of the tree, followed by Marchantia polymorpha (Marchantiophyta, Marchantiales), which are also non-vascular plants. A step further is the vascular plant Selaginella moellendorffii (Lycopodiophyta, Selaginellales). A total 112 species of Angiosperms and 3 species of Gymnosperms clustered in a clade. Except for a few precursors, most miR408 in monocotyledonous plants are clustered in one clade, and miR408 in dicotyledonous plants are clustered in another clade. The dicotyledonous
plants are mainly divided into two clades: Asterids and Rosids. This is consistent with plant evolutionary relationships in the APG IV taxonomy of angiosperms.

Figure 1. Phylogenetic analysis of miR408 precursor from 118 plant species. The miR408 precursor sequences were aligned with MAFFT. Maximum likelihood phylogenies were inferred using IQ-TREE under the TVMe+R5 model for 5000 ultrafast bootstraps. Evolview-v2 visualized the tree. Group labels from outside to inside were division, class, clade, order according to the 4th Angiosperm Phylogeny Group (APG) classification of angiosperms.

miR408 is widely distributed in different plants, among from Bryophyta (represent the first green plants to colonize terrestrial plants) to Angiosperms. It is clear that the miR408 family is an ancient family of miRNAs; bryophytes may be the evolutionary ancestors of the miR408 family, which, since then, have been strongly conserved.
Gma-miR408 family members described in sRNAanno are gma-miR408a/b/c/d (Table 1), and the corresponding precursor sequence is pre-gma-miR408a/b/c/d (Table 2, GEO Accession: GSM1874239) [17]. The schematic stem-loop structures of pre-gma-miR408a/b/c/d were shown in sRNAanno database. Sequence alignment of the miR408 precursor revealed 80.71% identity between A. thaliana and G. max. The mature miRNA of the precursor was in the identical stem arm (Figure 2). This implied that the pre-gma-miR408 could be processed correctly to form mature gma-miR408.

Table 1. Gma-miR408 family members described in miRbase and sRNAanno.

| miRNA Name | Location | Mature Sequence-5p | Mature Sequence-3p |
|------------|----------|--------------------|--------------------|
| gma-miR408a | Chr2:837419-837550 | CAGGGCAACACCCGAGCAAPGGAGACUUAACAA | AUGACUCUCUCUCUCUCUCUGGC |
| gma-miR408b | Chr3:44626686-44626830 | CUGGCGCAACACCCGAGCAAPGGAGACUUAACAA | AUGACUCUCUCUCUCUCUCUGGC |
| gma-miR408c | Chr10:36557001-36557134 | CAGGGCAACACCCGAGCAAPGGAGACUUAACAA | AUGACUCUCUCUCUCUCUCUGGC |
| gma-miR408d | Chr19:47163684-47163821 | CUGGCGCAACACCCGAGCAAPGGAGACUUAACAA | AUGACUCUCUCUCUCUCUCUGGC |

Table 2. Precursor sequences of gma-miR408 family members described in sRNAanno.

| miRNA Name | Precursor Sequence |
|------------|--------------------|
| gma-miR408a | ACAGGACAAAGCAGGGGAACAGGCAGAGCAUGAUGGAGCUAUCAA |
| gma-miR408b | CAGGGCAAAAGCGGGGAACAGGCAGAGCAUGAUGGAGCUAUCAA |
| gma-miR408c | ACAGGACAAAGCAGGGGAACAGGCAGAGCAUGAUGGAGCUAUCAA |
| gma-miR408d | ACAGGACAAAGCAGGGGAACAGGCAGAGCAUGAUGGAGCUAUCAA |

Figure 2. Multiple alignment of miR408 sequences. (A) Consensus sequences of mature miR408-3p displayed by WebLogo. (B) Consensus sequences of mature miR408-5p displayed by WebLogo. (C) Precursor sequence analysis of miR408 between Glycine max and Arabidopsis thaliana. Sequence alignment of pre-ath-miR408 and pre-gma-miR408a/b/c/d. Boxes indicate the sequences of mature miR408-3p and miR408-5p.

The sequences of miR408-5p and miR408-3p were aligned, respectively, then displayed by WebLogo [18] (Figure 2). miR408-3p is relatively more conserved, and most mature
sequences of miR408-3p are identical except for the 1st, 5th, 17th, and 24th bases. In contrast, miR408-5p was quite specific, with differences in half the bases. The consensus mature miR408-3p sequence was 5'-AUGCUACUGCCUUCACCUG-3' and shared high identity from the 2nd to the 24th nucleotide. Mature gma-miR408-3p shared the same sequence as the consensus sequence, indicating that gma-miR408 might play a similar role as in other species. Based on the reported miR408 in other species (e.g., ath-miR408a, osa-miR408a) and its higher abundance in plants, gma-miR408-3p was used in further study.

2.2. Gma-miR408 Response to Heterodera glycines Infection

To determine the potential function of gma-miR408 in the response to H. glycines infection, the expression levels were detected by quantitative real-time PCR (qRT-PCR) among different days post-inoculation (dpi) in the susceptible soybean cultivar Williams 82 (W82), with the non-infected as control (Figure 3). The abundance of gma-miR408 did not significantly change in the non-infected plants at different time points. Compared with control, the expression level of miR408 significantly upregulated reaching a maximum of 5.7 times higher. The miR408 was significantly upregulated in susceptible Williams 82 during the infection process (1 dpi), migration stage (5 dpi) of nematode. Furthermore, the levels of miR408 revealed no differences between treatment and control at syncytium formation (10 dpi) and syncytium maintenance stage (15 dpi). The dynamic expression of miR408 during nematode infection showed that miR408 responded to H. glycines infection, indicating that miR408 may have a role in resistance to SCN migration and syncytium formation.

Figure 3. Quantitative Real-time PCR validations of soybean cyst nematode responsive miRNAs in the susceptible soybean cultivar Williams 82. Soybean cyst nematode infected Williams 82 plants were named as W82+SCN, the non-infected as W82+CK. The level of expression was normalized to the level of U6. Each bar shows the mean ± SE of triplicate assays. Multiple t-tests were applied to analyze the difference between W82+SCN with W82+CK. *** indicates a statistically significant difference as a relative at \( p < 0.001 \).

2.3. Expression Pattern of gma-miR408 in Soybean Roots

To explore the expression pattern of the miR408 family’s response to cyst nematodes infection, the plant binary expression vector pNINC2GUS was used to induce W82 to generate transgenic hairy roots. We produced Agrobacterium-mediated transient transformation of hairy roots expressing the \( \beta \)-glucuronidase (GUS) reporter gene driven by the miR408 promoter (pmiR408: GUS). Gmubi promoter ubi:GUS as a positive control. GUS activity of four independent transgenic hairy roots (pmiR408a/b/c/d) were assayed under no-infected and H. glycines infected conditions (Figure S1). In non-infected plants, GUS staining was only observed in vascular tissues of the root. Under H. glycines-infected conditions, GUS
activity was observed in the developing syncytium and the whole root (Figure 4). The expression pattern of miR408 indicated a functional role during the initiation and progression of nematode parasitism.

**Figure 4.** Histochemical staining of GUS activity driven by miR408 promoters in transgenic soybean in response to *H. glycine* infection. Soybean cyst nematode infected Williams 82 plants were named W82+SCN, the non-infected were W82+CK. Use *ubi*:GUS as a positive control. The miR408 promoter sequence after adding base arms was inserted into the linearized *pNINC2GUS* and obtained the GUS vector *pmiR408*:GUS. GUS activity of the *pmiR408*:GUS plants under non-infected conditions only showed in the vascular tissue of roots. While the whole root showed GUS activity response to nematode infection. GUS reactivity gradually weakens with the stage of nematode infestation. Bars = 100 µm.

2.4. Induction of gma-miR408 Overexpression and Silencing Transgenic Soybean Hairy Roots

To further investigate the function of gma-miR408 in the SCN responses, the plant binary expression vector *pNINC2RNA* was used to induce W82 to generate transgenic hairy
roots [19,20]. We obtained miR408-silencing transgenic plants via Agrobacterium-mediated transformation with miRNA short tandem target mimicry (STTM, these plants were named STTM-miR408) and miR408 overexpression plants with pre-miR408a (OE-miR408, Figure S2). Confirming the success of our transgenic approaches, we found that the miRNA abundance of miR408 was decreased in STTM-miR408 plants (0.3, \(p < 0.05\)) and increased in OE-miR408 plants (8.5, \(p < 0.05\)) compared with their corresponding empty-vector (EV) plants (Figure 5).

![Figure 5](image-url)

**Figure 5.** The expression level of miR408 in transgenic OE-miR408 and STTM-miR408 soybean hairy roots. The level of expression was normalized to the level of U6. Each bar shows the mean ± SE of triplicate assays. Statistical comparisons between nematodes in EV and other transgenic hairy roots were made by one-way analysis of variance (ANOVA). Different characters mean significant differences found at \(p < 0.05\).

### 2.5. Transcriptomic Analyses for OE-miR408 and STTM-miR408

To further explore the molecular basis of resistance against SCN, an RNA sequencing assay was performed using transcriptomic hair roots. The cDNA libraries were generated from 15-day-old transgenic hairy roots induced by *A. rhizogenes* K599. Empty-vector plants were used as control. Three soybean plants were pooled as one sample, which were named EV, OE, and STTM, respectively. The quality of RNA samples was detected using NanoDrop 2000 (Thermo, Waltham, MA, USA). We constructed nine small RNA libraries (3 treatment × 3 biological replicates) sequences using an Illumina novaseq6000 platform. The nine libraries provided a total of 452,070,014 sRNA raw reads, and the Q30 of all the libraries was \(\geq 90.8\%\). After data quality control, a total of 374,555,456 sRNA clean reads were obtained, and each library contained a minimum of 38,188,248 clean reads. The data were then mapped to the soybean genome. More than 89.6% reads were successfully aligned to the reference genome (Table 3).

### 2.6. Differentially Expressed Genes for OE-miR408 and STTM-miR408

The differentially expressed genes (DEGs) were filtered via DESeq with \(padj < 0.05\) (Table 4, Figure 6). Compared OE with EV, 3653 DEGs were examined including 2191 and 1462 up- and down-regulated genes, respectively (Table S5). A total of 933 DEGs were selected (598 and 335 DEGs up- and down-regulated between EV and STTM, respectively, Table S6). Compared OE with STTM, 3831 DEGs were detected including 1626 and 2205 up- and down-regulated genes, respectively (Table S7). Venn diagrams show 432 DEGs shared by EV vs OE and EV vs STTM (Table S8). There were 85 and 32 DEGs detected for the opposite regulated between them (Table S9).
Table 3. The raw reads and clean reads obtained in 9 small RNA libraries.

| ID    | Raw Reads | Clean Reads | Mapped Reads (%) | Uniquely M | Multiple M | Q30(%) |
|-------|-----------|-------------|------------------|------------|------------|--------|
| EV-1  | 46,804,494| 39,360,542  | 36,669,992 (93.01)| 35,970,124| 639,868    | 90.89  |
| EV-2  | 48,333,980| 41,035,690  | 38,123,699 (92.90)| 37,471,922| 651,777    | 91.34  |
| EV-3  | 48,613,286| 40,137,396  | 37,364,825 (93.09)| 36,697,461| 667,364    | 91.25  |
| OE-1  | 48,044,828| 38,188,248  | 35,289,502 (92.41)| 34,698,477| 591,025    | 91.20  |
| OE-2  | 56,847,288| 45,955,306  | 42,646,102 (92.80)| 41,945,756| 700,346    | 91.84  |
| OE-3  | 57,874,234| 52,788,600  | 49,278,779 (93.35)| 48,478,778| 800,001    | 92.68  |
| STTM-1| 47,124,578| 38,307,708  | 35,646,187 (93.05)| 35,042,320| 603,867    | 91.42  |
| STTM-2| 50,267,440| 40,530,212  | 37,142,081 (91.64)| 36,466,073| 676,008    | 91.55  |
| STTM-3| 48,159,886| 38,251,754  | 34,301,873 (89.67)| 33,70,2718| 599,155    | 91.13  |
| Total | 452,070,014| 374,555,456| 346,403,040       | 340,473,629| 5,929,411  |        |

Table 4. The number of differentially expressed genes in each comparison group.

| Group  | Total | Down Regulated | Up Regulate |
|--------|-------|----------------|-------------|
| EV vs OE | 3653  | 1462           | 2191        |
| EV vs STTM | 933   | 335            | 598         |
| OE vs STTM | 3831  | 2205           | 1626        |

Figure 6. Differential expressed genes analysis in each comparison group. (A): The volcano plots show the numbers of significantly differentially expressed genes in each comparison group (padj < 0.05). (B): Venn diagrams show the distribution of up and down-regulated genes between EV vs. OE and EV vs. STTM (padj < 0.05).
2.7. Functional Analysis and Clustering

KEGG pathway and GO ontology analyses were conducted for these DEGs. KEGG pathway enrichment analyses showed that DEGs were mainly mapped into Biosynthesis of secondary metabolites (ko01110), Metabolic pathways (ko01100), MAPK signaling pathway-plant (ko04016), and Plant-pathogen interaction (ko04626), and Starch and sucrose metabolism (ko00500) showed in Figure 7. MAPK signaling pathway-plant (ko04016), and Plant-pathogen interaction (ko04626) were shown in Figures S3 and S4.

The results revealed that eight GO terms were enriched commonly and significantly, including response to hypoxia (GO:0001666), response to decreased oxygen levels (GO:0036293), response to oxygen levels (GO:0070482), response to chitin (GO:0010200), cellular response to oxygen levels (GO:0071453), cellular response to decreased oxygen levels (GO:0036294), response to wounding (GO:0009611), cellular response to hypoxia (GO:0071456) showed in Figure 8.

Figure 7. KEGG pathway enrichment of induced DEGs. (A) KEGG pathway enrichment of DEGs between EV vs. OE. (B) KEGG pathway enrichment of DEGs between EV vs. STTM.

Figure 8. GO enrichment of induced DEGs. (A) GO enrichment of DEGs between EV vs. OE. (B) GO enrichment of DEGs between EV vs. STTM.
2.8. Gma-miR408 Suppressing Reactive Oxygen Species Accumulation

We next investigated ROS levels in the roots of different soybean lines by monitoring the accumulation of ROS (H\textsubscript{2}DCFDA probe labeling) and H\textsubscript{2}O\textsubscript{2} (3,3′-diaminobenzidine staining, DAB). As expected, the EV plants exhibited an accumulation of both ROS and H\textsubscript{2}O\textsubscript{2}. When compared with EV, OE-miR408 and STTM-miR408 transgenic roots had decreased ROS and H\textsubscript{2}O\textsubscript{2} levels, whereas the ROS levels (fluorescence signal intensity of H\textsubscript{2}DCFDA probe labeling and dark brown stains of DAB staining) were ordered EV > STTM > OE (Figure 9). Thus, we hypothesized that miR408 expression could inhibit reactive oxygen species accumulation. Meanwhile, miR408 is not the only factor affecting the accumulation of ROS.

![Figure 9](image)

**Figure 9.** Detection of physiological and biochemical indicators of the OE-miR408 and STTM-miR408 soybean hairy roots. H\textsubscript{2}DCFDA probe labeling and DAB staining of Agrobacterium-mediated transient transformation of hairy roots. Bar = 100 µm.

2.9. Gma-miR408 Enhances Soybean Cyst Nematode Susceptibility

The regulation of miR408 in the resistance of soybean to SCN was verified by inoculating soybean cyst nematode on transgenic plants. Three transgenic lines (EV, OE-miR408 and STTM-miR408) were used. Nematode infection and development were detected at 1 dpi and 15 dpi by a modified acid-fuchsin staining-destaining procedure [21].

At 1 dpi, the number of second-stage juveniles (J2s) invading soybean hairy roots reached 149 to 194, and there was no difference in nematode infestation between EV, OE-miR408 and STTM-miR408 (Figure 10A). At 5 dpi, obvious differences were observed in the total number of nematodes and swollen juveniles (Figure 10B). For the total number, OE-miR408 increased significantly compared with 126 nematodes in EV, at 155 (p < 0.05). In contrast, the total number of nematodes was significantly reduced, with 86 in STTM-miR408 (p < 0.05). There were 57 swollen juveniles in STTM-miR408, and 93 swollen juveniles in EV. STTM-miR408 had a significant difference with EV (p < 0.05). OE-miR408 had 120 swollen juveniles, which was significantly higher than EV.

At 15 dpi, there were obvious differences in the total number of nematodes and the average number of nematodes in each stage (Figure 10C). The total number of nematodes in OE-miR408 increased significantly compared to 110 nematodes in EV, at 139 (p < 0.05). In contrast, the total number of nematodes was significantly reduced in soybean hairy roots with 70 in STTM-miR408 (p < 0.05). At 15 dpi, the number of J2s in EV, OE-miR408 and
STTM-miR408 hairy roots was not significantly different. There were significant differences in the numbers of swollen juveniles and females in each hairy root. There were 22 swollen juveniles and 30 female juveniles in STTM-miR408, 40 swollen juveniles, and 62 female worms in EV. STTM-miR408 had significant difference with EV ($p < 0.05$). OE-miR408 had 48 swollen juveniles and 67 females, which was significantly higher than EV. These results showed that overexpressing gma-miR408 enhances soybean cyst nematode susceptibility.

Figure 10. Infection and development of soybean cyst nematode in transgenic hairy roots. (A) Number of second-stage juveniles (J2s) in soybean hairy roots examined at 1 dpi. (B) Number of different stages of nematodes in soybean hairy roots at 5 dpi. (C) Number of different stages of nematodes in soybean hairy roots at 15 dpi. Multiple statistical comparisons between nematodes in EV and other transgenic hairy roots were made by one-way ANOVA. Different characters mean significant differences found at $p < 0.05$.

3. Discussion

MicroRNAs are the second most abundant plant sRNA class [22]. Many plant miRNA families have been conserved for a long time in the evolution of land plants. Axtell and Bartel [23] demonstrated that at least eight miRNA families remained basically unchanged before the emergence of seed plants, and at least two families (miR160 and miR390) have remained unchanged since the last common ancestor of mosses and flowering plants. miR408 was widely distributed in different plants, from Bryophyta (representing the first green plants to colonize terrestrial plants) [24,25], to ferns that have differentiated vasculature, gymnosperms with uncoated ovules, and angiosperms that flowered and set seed in the end. Evolutionary analysis showed that the miR408 family was an ancient and widely distributed miRNA family [4,5]. The miR408 evolution was consistent with plant evolutionary relationships in the APG IV taxonomy of angiosperms.

Since miR408 was first discovered in Arabidopsis thaliana, the crucial roles of miR408 family members in plants have been verified in many studies [26], and miR408 has been regarded as an important regulator of plant vegetative growth and reproductive development. In recent years, an increasing number of studies have shown that, in addition to regulating plant growth and development, miR408 is also stress-responsive in many plant species. Respond to cold stress, the expression of miR408 increased in A. thaliana and O. sativa [14,27]. miR408 helps plants improve their tolerance to cold stress by regulating the
genes related to Cu homeostasis, oxidative stress, and lignin biosynthesis. Under salinity stress, the up- or down-regulation of miR408 expression in A. thaliana, Salvia miltiorrhiza, Nicotiana benthamiana, O. sativa and Triticum aestivum is inconsistent, indicating that different plants have different salt stress resistance mechanisms [7,14,28,29]. miR408 not only plays important roles in the tolerance to various abiotic stresses, but also plays vital roles in the tolerance to biotic stresses such as LPS stress (Gram-negative bacteria), Puccinia striiformis f. sp. Tritic (Pst) infection, P. graminis f. sp. tritici infection and Rhizoctonia solani infection [7,14,30–32]. Our study found that nematode infection can induce the expression of miR408 in roots. During nematode migration and syncytium formation, the expression of miR408 was significantly up-regulated, indicating miR408 may have a role in resistance to SCN migration and syncytium formation.

Among the identified roles of miR408 and its targets in response to biotic and abiotic stress, some have been verified by performing overexpression, T-DNA insertion, and RNAi experimentation. miR408 is involved in various biotic and abiotic stresses, existing studies have shown that miR408 has a great relationship with the antioxidant system. Ma et al. [14] proposed that increasing the expression of miR408 can reduce ROS and regulate the target genes encoding Cu-containing proteins. In O. Sativa, a late responsive miR408 might be involved in R. solani infection [31]. Gupta et al. [32] reported that miR408 in T. aestivum was involved in the defense response to stem rust infection. During Pst infection, up-regulation of miR408 triggers the lignin biosynthetic pathway as a hypersensitive response (HR). Here, we explore the function of gma-miR408 by overexpressing and silencing miR408 transgenic plants via Agrobacterium-mediated transformation. Transcriptomic analyses for OE-miR408 and STTM-miR408 showed DEGs were mainly involved in MAPK signaling pathway-plant, and Plant-pathogen interaction. The amount of ROS accumulation in both ways was EV > STTM > OE. We hypothesized that miR408 expression could inhibit reactive oxygen species accumulation. miR408 maybe not the only factor affecting the accumulation of ROS, exploring the factors and mechanisms that regulate the homeostasis of reactive oxygen species will be the focus of future research.

In conclusion, the results from this study clearly indicate that gma-miR408 responds to SCN infection, during nematode migration and syncytium formation. Overexpressing miR408 could negatively regulate soybean resistance to SCN by inhibiting reactive oxygen species accumulation. In reverse, silencing miR408 positively regulates soybean resistance to SCN. Overall, gma-miR408 enhances soybean cyst nematode susceptibility by suppressing reactive oxygen species accumulation.

4. Materials and Methods
4.1. Plant Materials

The cultivated soybean seeds of Glycine max (Williams 82, W82) provided by the Nematology Institute of Northeastern China (Shenyang, China) were used for our study. The seeds were surface sterilized with 1% NaClO for at least 10 min and then washed several times with distilled water. The seeds were added to PVC tubes (height × diameter = 10 × 3 cm) containing equal ratios of sterilized sand and soil in a climatic chamber (light/dark = 16/8, 23–26 °C, 50% relative humidity). Hoagland’s nutrient solution was added to soybean seedlings once every three days. Soybean germination and culturing proceeded for 10 days for nematode inoculation and 5–7 days for soybean inoculation with Agrobacterium rhizogenes K599 (cotyledons not unfolded).

4.2. Nematode

The Heterodera glycines (soybean cyst nematode, SCN) race 3 population was propagated on W82 in infected soil in a greenhouse in the Nematode Institute of Northeastern China. SCN cysts were extracted from the infected soil on a 60-mesh sieve (250 µm), and harvested cysts were then crushed on an 80-mesh sieve (180 µm). Eggs were collected on a 500-mesh sieve (25 µm) and further purified using 35% (w/v) sucrose solution. Eggs were sterilized with 0.1% NaClO for 10 min before being rinsed with sterilized water several
times to remove any traces of NaClO. The sterilized eggs were transferred to a modified Baermann pan with 3 mM ZnSO$_4$ at 25 °C in the dark for 5 days to allow them to hatch, and the freshly hatched pre-parasitic second-stage juveniles were then harvested.

4.3. Nematode Infection

Ten days after soybean seedlings were germinated, 2000 J2s were added to each root system. For hairy soybean roots induced, 500 J2s for each root system. Infections were synchronized by washing the infected roots 24 h post-inoculation.

4.4. Construction for Genetic Transformation Vectors

To construct pmiR408:GUS, the precursor sequences of soybean miR408 (gma-miR408a/b/c/d) were amplified from the genomic DNA of W82 using the primers a/b/c/dp-F and a/b/c/dp-R, respectively. All the primers used are shown in Table S3. The PCR products were further added base arm using the primers a/b/c/dp-Fi and a/b/c/dp-Ri, respectively. Then, precursor sequences of soybean miR408 were subcloned into pNINC2GUS and verified by primers JCF and JCR (Figure S1). The vector pNINC2GUS uses pCAMBIA3301 as a backbone and combined it with the enhanced CaMV 35S promoter, GusPlus, and visualization element (EGFP tag) of p3405.1. Gmubi promoter ubi:GUS as a positive control.

The vector pNINC2RNAi uses pCAMBIA3301 as a backbone and combined with the Gmubi promoter, rcbS terminator, and visualization element (EGFP tag) of pG2RNAi2. To silence miRNAs, we designed short tandem target mimics (STTM) of miR408. STTM-miR408 consists of two short identical sequences that mimic miR408 target sites with three additional nucleotides CTA bulges corresponding to positions 10 to 11 of the miR408. Two short identical sequences were linked with 48 nt linker and flanked with Asc I and Avr RII restriction enzyme sites at 5′ end and 3′ end, respectively. The promoter of miR408 and STTM-miR408 (Table S4) were synthesized in Genewiz (Wuhan, China); finally, they were cloned into pNINC2RNAi and named as pNINC2EX OE-miR408 and pNINC2EX STTM-miR408. Positive validation of vectors transfers into A. rhizogenes K599 by primers EGFG F/R and JC F/R (Figure S2).

4.5. Transformation Process of Soybean Hairy Roots

GUS, overexpressing, and silencing plasmids were transferred into A. rhizogenes K599 by the freeze-thaw method. Then, transgenic hairy roots were induced by the Agrobacterium-mediated method described as [33]. Briefly, young seedlings with unfolded cotyledons are infected with A. rhizogenes at the cotyledonary node, and the infection sites are preserved in a humidified environment. After 5–7 days, hairy roots started to sprout from the site of infection. Hairy roots were covered with sterilized vermiculite wetted by Hoagland’s nutrient solution for ten days until hairy roots could sustain the plants. Then, the hairy roots were screened with a handheld lamp (Luyor, Shanghai, China) to visualize GFP expression. Hairy roots carrying strong GFP signals were reserved and used for further tests, and the rest of non-GFP hairy roots and the main roots were removed. After 15 days, transgenic soybean hairy roots were collected to further confirm the effects of overexpressing and silencing.

4.6. Gene Expression Analysis of miR408

The samples were collected from roots of 1 dpi, 5 dpi, 10 dpi, and 15dpi in susceptible soybean cultivar Williams 82, with the no-infected as control. For soybean hairy roots, the samples were collected from hairy roots cultured for 15 d, and the effects of over-expression and silencing were confirmed by detecting the expression level of the miR408 gene by qRT-PCR (the primers used for qRT-PCR are shown in Table S4). The soybean U6 (GenBank accession LOC100819552) was used as an internal reference gene, with three parallel and three biological repeats per sample, and the results were analyzed by the $2^{-\Delta\Delta C_{T}}$ method.
4.7. RNA Isolation, Reverse Transcription, and cDNA Libraries Sequencing

The total RNA was isolated from the soybean hairy roots using Reagent RNA extraction kit (CWbio, Beijing, China). The total extracted RNA was used to generate sequencing libraries using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, Beijing, China). Library quality was assessed on the Agilent Bioanalyzer 2100 (Agilent, Waldbronn, Germany). The cDNA libraries were sequenced on the Illumina sequencing platform by Metware Biotechnology Co., Ltd. (Wuhan, China).

4.8. Differential Gene Analysis and Enrichment

DESeq2 v1.22.1 / edgeR v3.24.3 was used to analyze the differential expression between two groups, and the \( p \) value was corrected using the Benjamini and Hochberg method. The corrected \( p \) value and \( |\log_{2}\text{foldchange}| \) are used as the threshold for significant differential expression. The enrichment analysis was performed based on the hypergeometric test. For KEGG, the hypergeometric distribution test is performed with the unit of the pathway; for GO, it is performed based on the GO term.

4.9. Statistical Analyses

Statistical analyses were conducted using IBM SPSS STATISTIC v.25 (Armonk, NY, USA) and GraphPad Prism 9 software (GraphPad Inc., San Diego, CA, USA). Multiple t-tests were applied to detect the significant differences in the relative expression level of \( \text{miR408} \) at different times, and one-way analysis of variance (ANOVA) was applied to detect the significant differences in the relative expression level of \( \text{miR408} \) of different hair roots and the number of nematodes.

5. Conclusions

In conclusion, the results from this study clearly indicate that \( \text{gma-miR408} \) responds to SCN infection. During nematode migration and syncytium formation, the expression of \( \text{miR408} \) was significantly up-regulated relative to soybean roots compared with control plants. No differences were observed in the later parasitism stage. Nematode infection can induce the expression of \( \text{miR408} \) in whole roots, resulting in GUS signaling, while GUS signal was only expressed within the vasculature of no-infected control. Overexpressing and silencing \( \text{miR408} \) vectors were transformed to soybean to confirm its potential role in plant and nematode interaction. Significant variations were observed in the MAPK signaling pathway with low OXI1, PR1, and wounding of the overexpressing lines. Overexpressing \( \text{miR408} \) could negatively regulate soybean resistance to SCN by suppressing reactive oxygen species accumulation. Conversely, silencing \( \text{miR408} \) positively regulates soybean resistance to SCN. Overall, \( \text{gma-miR408} \) enhances soybean cyst nematode susceptibility by suppressing reactive oxygen species accumulation.

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Abbreviations
The following abbreviations are used in this manuscript:
W82 Williams 82
SCN soybean cyst nematode
qRT-PCR quantitative real-time PCR
dpi day post-inoculation
J2s second-stage juveniles
OE overexpressing
STM Short tandem target mimic

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