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Expression analysis of MicroRNAs and their target genes in *Cucumis metuliferus* infected by the root-knot nematode *Meloidogyne incognita*

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ABSTRACT

MicroRNAs (miRNAs) are types of transcriptional and post-transcriptional modulators of gene expression that play crucial roles in response to diverse stresses. Although significant progress on the miRNA-mediated gene regulation has been made recently in plant-nematode interactions, none has been reported on root-knot nematode (RKN, *Meloidogyne* spp.) infection in *Cucumis metuliferus*, which is a relative of cucumber with resistance to *M. incognita*. To gain insights into the regulatory roles of miRNAs for resistance to RKN in *C. metuliferus*, it is necessary to create expression profiles for miRNAs and their targets. In this study, ten miRNAs were identified from our miRNAs sequencing data of *C. metuliferus* for expression analysis through quantitative reverse transcription-PCR (qRT-PCR). The results showed that RKN infection had a significant effect on both miRNAs expression and their corresponding targets in either resistant or susceptible plants but with differential expression. Moreover, four out of ten selected miRNA-target pairs, miR156-SBP, miR390-ARF3, ath-miR159a-MYB104 and aly-miR827-3p-PTI, exhibited inverse expression patterns between miRNAs and their targets, which laid a foundation for investigating resistance mechanisms induced in *C. metuliferus* by *M. incognita* infection. Additionally, the possible roles of these miRNAs have been discussed during *C. metuliferus*-RKN interactions.

1. Introduction

Root-knot nematodes (RKN, *Meloidogyne* spp.) are most economically important plant-parasitic nematodes in cucumber (*Cucumis sativus* L.) and cause serious threat to cucumber production throughout the world [1,2]. Current control measures rely heavily on nematicides but alternative strategies are required as effective chemicals has been phased out or decreased with limited application due to toxicity. The application of natural host resistance has been the most effective and environmentally friendly strategy for pathogen or pest control [3,4]. Much research effort has been directed towards identifying RKN-resistant germplasms in cultivated cucumber, but so far no commercial cucumber cultivars resistant to *M. incognita* are available [5,6]. Fortunately, natural host resistance against *M. incognita* was identified in several wild *Cucumis* species, e.g. *C. metuliferus*, which is a relative of cucumber endemic to Africa [7]. However, early attempts to incorporate this resistance into cultivated *Cucumis* species have been unsuccessful [8,9]. The conventional plant breeding by transferring the resistance to susceptible lines through the hybridization, such as tomato [10] and pepper [11], is limited for RKN resistance improvement in cucumber. The RKN-resistant genes involved in *C. metuliferus* have not been identified [12,13]. Therefore, it is necessary to characterize the molecular basis for the observed resistant phenotypes.

The development of next generation sequencing approach facilitates the study of crop resistance mechanisms against nematodes. In recent years, significant progress has been made in transcriptome research and important information on incompatible interaction between *Cucumis* and *M. incognita* have been obtained [12–14], but the evidence on functional genomics is still extremely insufficient. MicroRNAs (miRNAs) are a major class of small non-coding RNAs. Various miRNAs are transcriptional and post-transcriptional modulators of gene expression that play crucial roles in the responses to diverse stresses [15]. Increasing evidence indicates that miRNAs-mediated gene regulation is a fundamental mechanism in plant-pathogen interactions including nematodes. Posttranscriptional miRNA-mediated mRNA cleavage appears to be an important mechanism of gene regulation triggered by plant parasitic nematodes [16]. It has been reported that the *Arabidopsis* miRNA396-GRF1/3 regulatory module acts as a developmental regulator in the reprogramming of root cells during cyst nematode infection and is involved in the control of syncytium size and development

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[17,18]. It also has been shown that miR858 plays a role in syncytium formation by regulating the expression of its target transcription factor MYB83 [19]. Zhao et al. [20] reported that the miR319/TCP4 module acts as a regulator of jasmonic acid levels upon RKN infection in tomato and affects the nature of host resistance. The regulatory module miR390/TAS3 is necessary for proper gall formation possibly through auxin-responsive factors [21]. As RKN induces similar galls and giant cells in a wide range of plant species, miRNAs are of particular interest as potential regulators of the gene networks underlying gall formation. Although substantial progress on the miRNA-mediated gene regulation has been made recently in other plant-nematode interactions, none has yet been reported in *C. metuliferus* following RKN infection, and the underlying regulatory networks that control the high level of resistance to *M. incognita* remain poorly understood.

In our recent study, a total of 212 miRNAs with differential expression was obtained in *C. metuliferus* following RKN infection using Solexa sequencing technology (unpublished data). To gain insights into the regulatory roles of miRNAs, ten out of the 212 differentially expressed miRNAs involved in plant-parasitic nematode interactions in other plant species were chosen and confirmed with qRT-PCR. Three out of the ten miRNAs, miR156, miR164 and miR172 are involved in plant-parasitic nematode interactions [22], and others are reported to contribute to transcriptome reprogramming during the formation of syncytia and giant cells, such as miR396 [18], miR319 [20], miR827 [23], miR390 [21], miR858 [19] and miR159 [24]. Together, these studies make it clear that host miRNAs pathways are powerful targets for nematodes to modulate large scale changes in gene expression inside their feeding site. Understanding miRNAs regulatory mechanisms of the response to RKN infection in *C. metuliferus* will aid in the identification of potential targets resistant to RKN for cucumber improvement, and provide fundamental knowledge for designing better strategies for breeding new cucumber cultivars with high resistance to RKN. Further, the interaction between *C. metuliferus* and *M. incognita* will be considered as a model to study the role of plant miRNAs during RKN infection.

2. Materials and methods

2.1. Plant cultivation and nematode culture and inoculation

Two *C. metuliferus* genotypes 'CmR07' and 'CmS12', which were resistant and susceptible to *M. incognita*, respectively, were used in this study [12,25]. Sterilized seeds were planted in 11-cm-diam. × 9-cm-deep plastic pots filled with sandy soil (sand: soil = 2:1). The plants were grown under controlled greenhouse conditions at 22–28 ˚C and 16 h daylight. *Meloidogyne incognita* isolated from cucumber was identified with molecular markers and nematodes were multiplied on tomato plant ('Zhongshu 4'). Nematode eggs were extracted from tomato roots with NaOCl [26]. Four-day hatched second-stage juveniles (J2) from eggs were used for inoculum. Plants at the two-true-leaf stage were inoculated with 2000 J2 in 2 ml of deionized water per seedling around the roots, and the control mock-inoculated replicates received the same amount of deionized water. The treatments were arranged in a completed randomized block design with three replications. The roots were harvested and cleaned up with high pressure tap water at 4 and 30 days post inoculation (dpi) respectively, and root samples of each treatment were pooled from three individual plants per replicate. The harvested roots were immediately put into liquid nitrogen and then stored at −80 ˚C until further RNA extraction.

2.2. RNA extraction and cDNA synthesis

Approximately 100 mg of roots for each sample were ground to powder with liquid nitrogen. Total RNA from each replicate was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (TaKaRa) to remove genomic DNA. RNA quantity and purity were assessed with Bioanalyzer 2100 (Agilent, CA, USA). For miRNAs, total RNA was reverse-transcribed into cDNA using the SuperScript first-strand synthesis system (Invitrogen, USA) according to the manufacturer's instructions. For the targets of miRNAs, reverse transcription was performed using TransScript First-Strand cDNA Synthesis SuperMix (TransGen). The cDNA was used as a template to perform qRT-PCR with gene-specific primers and SYBR Green Mix (TaKaRa).

2.3. Expression analysis of selected miRNAs and their targets through qRT-PCR

Expression of these miRNAs and their target genes was determined by qRT-PCR using 2 × SYBR Green Fast qPCR Master Mix (High Rox, B639273, BBI) on an StepOne Plus Real time PCR (ABI, Foster, CA, USA). The reaction mixture are as follows: 2 μL template cDNA, 10 μL 2 × SYBR Green Fast qPCR Master Mix, 0.4 μL forward and reverse primers respectively and 7.2 μL RNase-free water. Reaction conditions for thermal cycling were: 95 ˚C for 3 min, 45 cycles of 95 ˚C for 5 s, 60 ˚C for 30 s and 72 ˚C for 30 s. In each qRT-PCR experiment, each gene was run in triplicate with different cDNAs synthesized from three biological replicates. Relative fold changes of gene expression were calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Expression levels of miRNAs and their targets were normalized by using cucumber *U6* and *EF1α* as an internal reference, respectively. The primers for reverse transcriptase of miRNAs and qRT-PCR are listed in Table 1.

2.4. Statistical analysis

All the data were subjected to the analysis of variance (ANOVA) using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL). Data are the means and standard error of three independent experiments, with three biological and three technical sample replicates at each time point for each genotype within an experiment. The means were compared by Fisher's Protected Least Significant Difference Test at 5%, and $P < 0.05$ was considered as significantly different.

3. Results

3.1. Expression of selected miRNAs in C. metuliferus roots upon RKN infection

The analysis of differential expressed miRNAs indicated that nine miRNAs except miR172 (aly-miR172b-5p) was downregulated at 4 dpi during resistant response to RKN (Fig. 1). Among these miRNAs, the lowest expression at 4 dpi was observed for miR396 (smo-miR396) followed by miR156 (smo-miR156b). The expression of other miRNAs was only slightly downregulated at 4 dpi and there was no significant difference between the RKN-infected treatment and non-infected control. At 30 dpi, eight miRNAs except miR159 (ath-miR159a) and miR858 (cme-miR858) was significantly upregulated, the miR156 had the highest expression, followed by miR396. During susceptible response to RKN, two miRNAs, miR164 (cme-miR164b) and miR827 (aly-miR827-3p) was upregulated at 4 dpi, but only miR827 changed its expression significantly (Fig. 1). At 4 dpi, the miR319 (cpa-miR319) showed the lowest downregulation in the expression, followed by miR165 (aly-miR165a-3p). A slight upregulation in the expression of miRNAs was observed in the susceptible response compared with the resistant one, although most of the miRNAs except miR165 and miR858 were upregulated at 30 dpi and two miRNAs, miR319 and miR390 (stu-miR390-3p) were upregulated in a significantly greatest level compared with others.

Table 1
Primers used in this study.

miRNAs	Primers	
smo-miR156b	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGTGCTCT	
	F: ACACTCCAGCTGGGCTGACAGAAGATAG	R: TGGTGTCGTGGAGTCG
ath-miR159a	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTAGAGCT	
	F: ACACTCCAGCTGGGTTTGGATTGAAGGG	R: TGGTGTCGTGGAGTCG
cme-miR164b	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGCATGT	
	F: ACACTCCAGCTGGGTGGAGAGGCAGGGC	R: TGGTGTCGTGGAGTCG
aly-miR165a-3p	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGGATG	
	F: ACACTCCAGCTGGGTCGGACCAGGCTT	R: TGGTGTCGTGGAGTCG
aly-miR172b-5p	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGTGAATC	
	F: ACACTCCAGCTGGGGCAGCACCATCAA	R: TGGTGTCGTGGAGTCG
cpa-miR319	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGAGCT	
	F: ACACTCCAGCTGGGATTGGACTGAAGGG	R: TGGTGTCGTGGAGTCG
stu-miR390-3p	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATATGATC	
	F: CACATTCGTTATCTATTTTTTGGCGC	R: TGGTGTCGTGGAGTCG
smo-miR396	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTTCAA	
	F: ACACTCCAGCTGGGTTCCACGGCTTTC	R: TGGTGTCGTGGAGTCG
aly-miR827-3p	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCGTTTGTT	
	F: ACACTCCAGCTGGGTTAGATGACCATC	R: TGGTGTCGTGGAGTCG
cme-miR858	RT: CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCCACCTT	
	F: TTTCGTTGTCTGTTCGACCTTAA	R: TGGTGTCGTGGAGTCG
U6	F: ACATCCGATAAAATTGGAACG	R: TTTGTGCGTGTCATCCTTGCG

miRNAs	Target genes	Forward primer	Reverse primer
smo-miR156b	SBP (Csa_1G001450)	ACGGTTGATTTGAAACTCGG	CCTCTTGCCCTCTTAGATGGT
ath-miR159a	MYB104(Csa_7G043580)	AAACAAAGGATGGACCAAGGA	GAGATGATGAATGGGAGGAGC
cme-miR164b	NAC (Csa_1G009870)	AAATACCCAACTGGATTGAGGAC	GGGAAGGTTTAGAGCAGAGAGTT
aly-miR165a-3p	ATHB14(Csa_6G525430)	TGGATTTAGCCTCTACCCTTGA	AACAGAGCCCACAACACTACG
aly-miR172b-5p	RAP2 (Csa_5G175970)	ATATGCTCACCAGACAACTT	AATGTAACGCCAACGATACT
cpa-miR319	TCP4 (Csa_4G088720)	CTCGGACTCCATTGCTGATAC	TCTTGGTTCTGGCTACTCGTC
stu-miR390-3p	ARF3 (Csa_6G518210)	CAACACTTGTTCGGATGGTG	CCCACACCAAATGTTCCTCT
smo-miR396	GRF3 (Csa_3G751470)	ACATTTCCCTCATTATCCCACT	GCTTTCTTGAACGGTTACGG
aly-miR827-3p	PTI (Csa_6G041190)	TTACCATGCTCCAGAATACGC	TGCTTTACTTTGTCCTCGCTTA
cme-miR858	MYB86 (Csa_5G152790)	CTCATTCGGCTCTTCCTT	ATTACCAGATGTCTCGTTCC
	EF1α	AGACCTTCTCTACATACCCACCATT	CTATTTCTTCTTCACAGCGGACTT

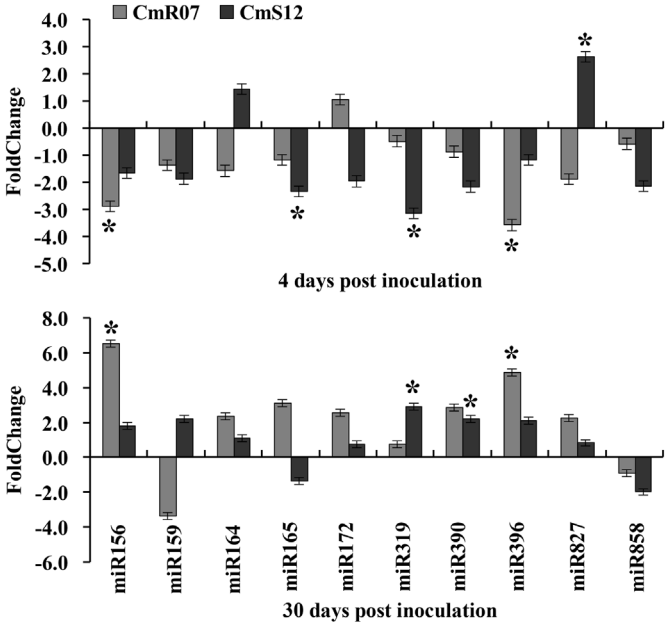

Fig. 1. Expression analysis of miRNAs in roots of CmR07 and CmS12 infected with RKN at 4 and 30 dpi. Asterisks indicate significant differences in expression of miRNAs between two genotypes at each time point as determined by Fisher's Protected Least Significant Difference Test.

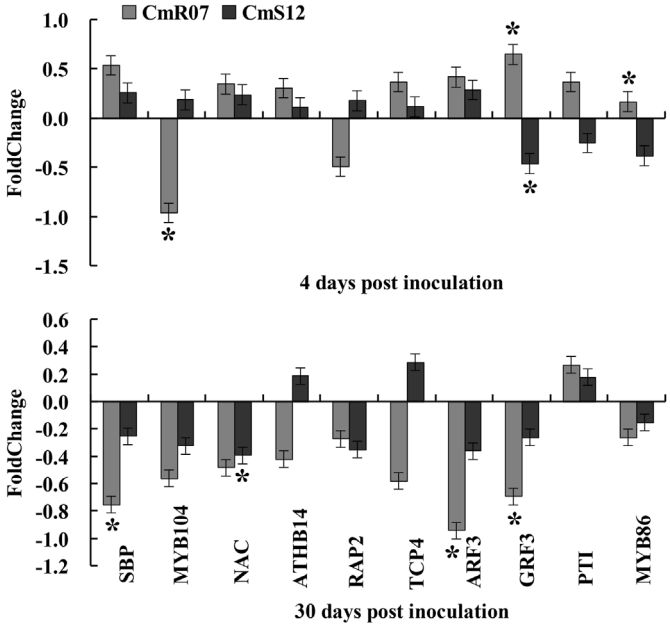

Fig. 2. Expression analysis of miRNA targets in roots of CmR07 and CmS12 infected with RKN at 4 and 30 dpi. Asterisks indicate significant differences in expression of targets between two genotypes at each time point as determined by Fisher's Protected Least Significant Difference Test.

3.2. Changes of target gene expression in C. metuliferus roots following RKN infection

RKN infection also altered the expression of miRNAs targets in both resistant and susceptible roots of *C. metuliferus* (Fig. 2). During resistant response to RKN, eight targets were upregulated at 4 dpi except MYB104 (Csa_7G043580) and RAP2 (Csa_5G175970), which were the targets of ath-miR159a and aly-miR172b-5p, respectively. The GRF3 (Csa_3G751470), the target of smo-miR396, was expressed in the highest level with fold changes of 0.65, and MYB86 (Csa_5G152790),

the target of cme-miR858, was expressed the least with fold changes in expression of 0.17. Nine targets were downregulated and one target, PTI (Csa_6G041190) targeted by aly-miR827-3p, was upregulated at 30 dpi. The target of stu-miR390-3p, ARF3 (Csa_6G518210), had the greatest expression level with fold changes of −0.94, following SBP (Csa_1G001450, the target of smo-miR156b) and GRF3 with fold changes of −0.75 and −0.69, respectively. During the susceptible response at 4 dpi, three targets, GRF3, PTI and MYB86 were downregulated. There was no significant difference in expression levels among other seven upregulated targets either in resistant or susceptible response. Most of the targets were downregulated at 30 dpi except ATHB14 (Csa_6G525430, targeted by aly-miR165a-3p), TCP4 (Csa_4G088720, targeted by cpa-miR319) and PTI. NAC (Csa_1G009870), targeted by cme-miR164b, was downregulated in the greatest level among the remaining target genes at this time point, and following with ARF3 and RAP2.

3.3. Correlation of the expression levels between miRNAs and their targets

The correlation in the expression pattern of miRNAs and their potential targets was determined through qRT-PCR. The results indicated four pairs of negative correlation between miRNAs and their corresponding target genes (Fig. 3). For example, two expressed miRNAs smo-miR156b and stu-miR390-3p were upregulated whereas their corresponding target genes, SBP and ARF3 exhibited downregulation, regardless of resistant or susceptible response to RKN. In a reverse case, ath-miR159a was downregulated and the target MYB104 was upregulated during the resistant response but with upregulated miRNA and downregulated target during the susceptible one. An opposite expression pattern was also found in the pairs of aly-miR827-3p-PTI with upregulated miRNA and downregulated target PTI in the resistant response but with downregulated miRNA and upregulated target in the susceptible one. Interestingly, a similar trend in expression change between miRNAs and its target genes was identified in aly-miR172b-5p/RAP2 and cme-miR858/MYB86, in which the expression of both aly-miR172b-5p and RAP2 was upregulated, cme-miR858 and MYB86 was downregulated during the resistant response whereas both of them upregulated during the susceptible one.

4. Discussion

Plant miRNAs are transcriptional and post-transcriptional modulators of gene expression in response to biotic or abiotic stresses [15]. Despite the large number of miRNAs were identified under various stress conditions, only few of these miRNAs have been functionally characterized. In recent years, roles of plant miRNAs in regulation of genes involved in plant-cyst nematode interactions have been reported, e.g. GRF1/3 regulated by miR396 [18], NLA regulated by miR827 [23] and MYB83 modulated by miR858 [19]. Three regulatory modules of miRNAs during RKN-plant interaction were identified as miR319/TCP4 [20], miR390/TAS3 [21] and miR159/MYB33 [24]. In the present study, we extended these findings by identifying miRNAs expressed in *C. metuliferus* roots at two key time points: the formation of RKN feeding site at 4 dpi and nematode reproduction at 30 dpi. The results indicated that RKN infection took a significant effect not only on miRNAs expression but also on their targets in both resistant and susceptible responses, miRNAs and their target genes were expressed in a differential pattern, suggesting miRNAs may be an adaptive mechanism improving plant resistance to nematode infection.

Plant miRNAs mediate gene expression largely by either preventing mRNAs translation or targeting mRNAs for cleavage [27]. It is generally believed that a miRNA is negatively correlated with the expression of its corresponding target gene [28,29]. The result that four miRNA-target pairs, miR156-SBP, miR390-ARF3, ath-miR159a-MYB104 and aly-miR827-3p-PTI, exhibited inverse expression patterns between the pairs are supported by recent studies of plant-RKN interactions [20,22], suggesting that these miRNAs may play an important role during *C. metuliferus*-RKN interactions. Plant miR156 and its target SBP are defined as a regulatory module that plays important roles in diverse aspects of plant development [30]. Zhao et al. [20] and Kaur et al. [22] reported a negative correlation in expression between different member of miR156 and SBP family during RKN pathogenesis in tomato. A role of the miR390/TAS3 regulatory module in plant responses to RKN was demonstrated in decreasing infection rate when miR390 KO mutant lines were infected with *M. javanica*. Gall formation requires miR390/TAS3 through the effects of auxin-responsive factors [21]. In addition, the miR159 family contains three different members, miR159a, miR159b and miR159c [31]. It has been shown that miR159a and miR159c were more abundant in galls at 14 dpi [24], whereas miR159b was repressed in galls at 3 dpi [21]. Following *M. incognita* infection, MYB33 in *Arabidopsis* roots was shown to be strongly expressed and translated at 3 dpi, and the concentration of MYB33 protein was decreased to an undetectable level at 14 dpi, whereas miR159 was repressed at 3 dpi and overexpressed at 14 dpi, and miR159 mutant was demonstrated to have lower susceptibility to RKN [24]. Moreover, Hewezi et al. [23] reported new functional roles of the miR827/NLA regulatory system in suppressing basal defense responses in the nematode feeding site to promote parasitism and miR827 functions as a negative regulator of plant immunity in response to *Heterodera schachtii* through suppressing the activity of NLA in the syncytium. These findings suggest that different miRNAs are likely to play specific roles during plant-nematode interactions, and their roles may vary with plant and/or nematode species [32]. Further, these studies demonstrate the complexity of miRNA-mediated gene regulation.

It is worth noting that the result that the pair of smo-miR396/GRF3 had no negative correlation during the susceptible response (Fig. 3), which was inconsistent with the findings of Zhao et al. [20] and Kaur et al. [22] with the upregulated miR396 and the downregulated GRF during RKN invasion in tomato roots. The various plant and nematode species may be responsible for this difference. Besides of these, Hewezi et al. [18] reported that miRNA396-GRF1/3 regulation is required for appropriate formation and maintenance of syncytium in *Arabidopsis* roots. One possible explanation is that cyst nematodes and RKNs feed on syncytia and giant cells, respectively, and the potential mechanisms of formation of feeding sites are distinct [23,33]. Furthermore, the miRNAs without a negative correlation with their targets (Fig. 3) suggested that these miRNAs are not essential or redundant for the *C. metuliferus*-RKN interactions even though substantial differential expression of miRNAs was observed in infected roots [20].

5. Conclusion

This study demonstrates that miRNA-mediated gene regulation was involved in *C. metuliferus*-RKN interactions. Four miRNAs were identified as negative correlation in the expression with their corresponding targets. These modules could be used as candidate regulatory system and laid a foundation for investigating resistance mechanisms induced in *C. metuliferus* by *M. incognita* infection. Further functional analyses will be required to investigate the biological significance of these regulatory modules in *C. metuliferus*-RKN interactions.

Author statement

Deyou Ye designed the experiments, performed miRNAs/targets analysis and wrote the manuscript. Ye Jiang and Congli Wang contributed to plant cultivation, nematode culture, inoculation and samples collection. Congli Wang revised the manuscript. Philip A. Roberts contributed to the supervision of the experiments and the revision of the manuscript. All authors approved the final manuscript.

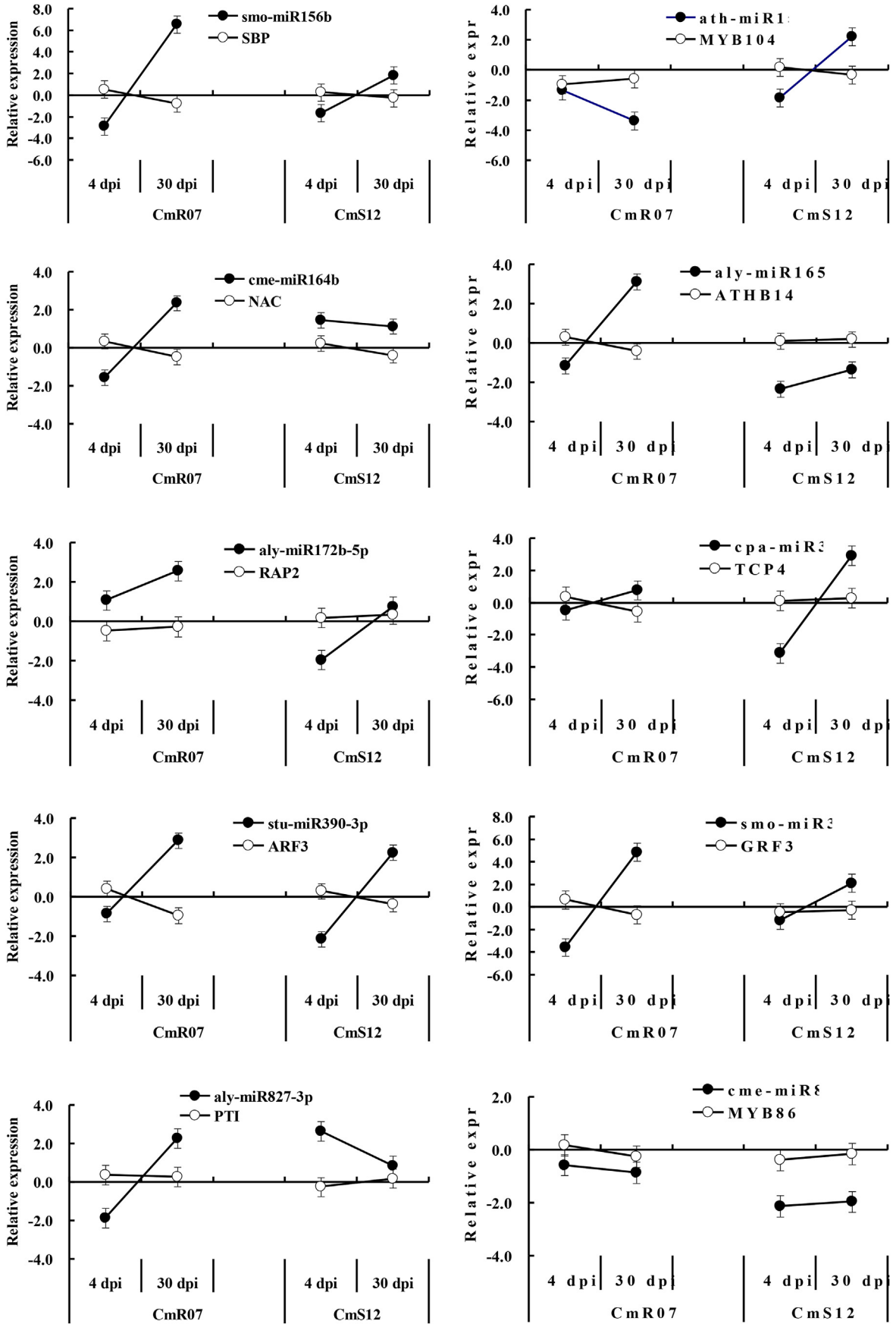

Fig. 3. Relationship between expression of miRNAs and their corresponding targets through qRT-PCR analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pmpp.2020.101491.

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