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RNAi of *MiASB* caused high mortality of *Meloidogyne incognita* juveniles and inhibited the nematode disease

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ABSTRACT

The southern root-knot nematode, *Meloidogyne incognita*, is one of the most prevalent and damaging plant-parasitic nematodes in the world and causes serious damages to agricultural production. We cloned a mitochondrial ATP synthase b subunit gene fragment of *M. incognita* (*MiASB*) based on the nematode genomics prediction. By soaking in the *MiASB* dsRNA solution, the hatching of RNAi treated eggs was reduced by 60% compared to negative control and by 64% compared to untreated control. Mortality of RNAi treated second stage juvenile (J2) was 8.6 times higher than that of negative control and 26 times higher than the untreated control. Inoculating the RNAi treated egg masses and J2 to tomato seedlings showed the pathogencity was significantly reduced. For the RNAi treated egg masses, the amount of root galls on silence treated seedlings was reduced by 92% compared to that on the negative control seedlings, and reduced by 93% compared to that on untreated control seedlings. For the treated J2, the amount of root galls on silence treated seedlings was reduced by 83% and 86% compared to negative and untreated control seedlings, respectively. The study revealed the *MiASB* silence had a positive effect on prevention and control of root-knot nematode disease, and also showed that the *MiASB* may be involved in the pathogenesis of nematode, which provided new ideas and ways to the research of nematode pathology and nematode disease control.

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Keywords: Root-knot Nematode; *Meloidogyne* Spp.; Mitochondrial ATP Synthase; RNA Interference; dsRNA

1. INTRODUCTION

Root-knot nematodes (RKN), genus *Meloidogyne*, are root endoparasites that can develop on a wide range of plant species. The infective second stage juvenile (J2) penetrates the root tip and migrates intercellularly until it reaches the differentiating vascular cylinder [1]. It induces root-knot, stunts nutrient deficiency and disrupts the physiology of the host plants through their reproduction and feeding within plant roots, leading to a signify-cant reduction in crop yield and deterioration of product quality [2,3]. Crop infestation by RKN causes 70 billion U.S. dollars of crop losses annually in fruit and vegetable production [4]. The RKN is being controlled in the fields mainly based on cultural practices, host-plant resistance and the application of synthetic pesticides. Nematode control has become difficult in recent years owning to the withdrawal or restricted use of effective synthetic pesticides for environmental problems and human and animal health concerns [3,5-7], which urges the need for alternative control methods. Strategies based on the specific blocking of parasitism gene products involving in the success of infection would offer attractive alternatives to reduce nematode populations in the field [8].

RNA interference (RNAi) is a mechanism for post-transcriptional gene silencing. This technique uses the fact that exposure of an organism to double-stranded RNA (dsRNA) from a gene of interest causes posttranscriptional silencing of the endogenous gene and allows a null phenotype to be mimicked [9]. Since it was firstly

discovered over two decade ago in *Caenorhabditis elegans* [10], RNAi have been proved to be a widespread phenomenon shared by many phyla [11]. In *C. elegans*, targeting the dsRNA into the nematode intestine by microinjection or ingestion allows the silencing of genes expressed in distal tissues. In *Nippostrongylus brasiliensis*, dsRNA uptake into the nematode body was induced by the cationic lipid polymer lipofectin. In *Brugia malayi*, a low-volume culture system was developed to expose females to dsRNA [1]. Nowadays, RNAi has been developed as a powerful tool for studying gene function in a variety of organisms [12].

Mitochondria and chloroplasts serve as power stations of living cells. By generating the biological energy currency ATP, ATP synthases play a decisive role in this process [13]. Mitochondrial ATP synthase (also named F1F0-ATP synthase or complex V) is located in the inner mitochondrial membrane together with respiratory chain complexes I-IV [14] and is a rotating nanomotor in prokaryotic and eukaryotic cells that uses a transmembrane electrochemical ion gradient as an energy source to convert ADP and Pi to ATP [15]. F1F0-ATP synthase consists of up to 18 different subunits [16], each of which is the essential components for ATP synthase to generate ATP.

In preliminary studies, based on the genomic data of *C. elegans*, *M. hapla*, *M. incognita* and RNAi data in Wormbase, we predicted some functional genes in *Meloidogyne* nematode, one of which was *ASB* encoding the b subunit of the F0 proton channel portion of F1F0-ATP synthase. In this study, we cloned a 650 bp *ASB* fragment from *M. incognita* (*MiASB*). Using RNAi by soaking it in the dsRNA solution of *MiASB*, we investigated the effects of *MiASB* on the hatching of the egg masses and the mortality of *Meloidogyne* nematode juveniles, and also studied its inhibitory effect on nematode disease caused by *M. incognita*.

2. MATERIALS AND METHODS

2.1. Nematode Collection

Nematode *M. incognita* was extracted from the pure culture that was previously initiated by egg masses and propagated on tomato (*Solanum lycopersicum*) in the glasshouse. Egg masses were handpicked using sterilized forceps from heavily infected roots 45 days after incubation. The egg masses were surface-sterilized (1% NaOCl) for 1 min followed by washing with sterile distilled water. Some of the egg masses was placed on 15 mesh sieves (8 cm in diameter) containing crossed layers of tissue paper in Petri-dishes with sterile distilled water just deep enough to contact the egg masses and incubated 25˚C to 26˚C to obtain freshly hatched J2. Emerged J2 were collected daily and stored at 4˚C. J2 up to 4 days old were used in experiments [17].

2.2. Plant Material

Tomato (*Solanum lycopersicum* cv. Lichun) was used in the experiment. Seeds of tomato were soaked in warm water for 6 h, then were placed on the sterile filter paper in a Petri dish for germinating at 25˚C in dark. The germinated seeds were sown in pots containing culture substrate composed of peat and vermiculite at 2 to 1 ratio in greenhouse. Seedlings with 3 - 4 true leaves were used for further experiments.

2.3. Molecular Cloning of *MiASB* Fragment

Total RNA was isolated from *M. incognita* J2 using Trizol (Invitrogen), following the manufacturer's protocol. The first strand was synthesized using SuperScript TM III First-Strand Synthesis System for RT-PCR (Invitrogen) according to manufacturer's instructions. A 650 bp fragment of *MiASB* was amplified by PCR using *MiASB*-specific primers including *MiASB*-F (5'-TGT TGG ACA AAC TTT TGC TT-3') and *MiASB*-R (5'-AAA TTT TCT TGG GTT GCT TTG-3') designed according to the *MiASB* (GeneBank Accession: JQ247982), a gene we cloned in the preliminary studies based on the predicted *MiASB* according to the nematode genomics using bioinformatics methods.

Polymerase chain reaction (PCR) was performed using 2 μl of the first-strand reaction as a template. Amplifications were performed with Taq DNA polymerase (2.5 U, Promega) using 1 mM of each of the primers and 200 mM of each of the deoxy nucleotide triphosphates (dNTP). The conditions were as follows: 94˚C, 3 min; followed by 35 cycles of 94˚C, 30 s; 53˚C, 30 s; 72˚C, 60 sand a final cycle of 72˚C, 10 min. The resulting PCR products were analyzed by agarose gel electrophoresis. Bands with the correct size were excised from the gel, purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 and cloned into pGM-T Vector (TA Cloning Kit, Tiangen) according to the manufacturer's instructions, forming the vector pGM-*MiASB*. The pGM-*MiASB* was transformed into the competent cells of *E. coli* DH5*α* and incubated at 37˚C overnight. The white clones grown on the LB medium containing Ampicillin/X-gal/IPTG were picked and the plasmids were extracted and sent to Invitrogen (Beijing) Co., Ltd., Beijing, China for sequencing.

2.4. Plasmid Recombinant and *in Vitro* Transcription to Produce dsRNA

The pGM-*MiASB* was digested with restriction endonuclease *Eco* RI (TaKaRa), the resulting products of which were analyzed by agarose gel electrophoresis, bands with the small size were excised from the gel, pu-

rified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0, ligated to the LITMUS 28 i plasmid (NEB, USA)digested with the exactly same restriction endonuclease using T4 DNA ligase (Invitrogen), resulting in the plasmid LITMUS-*MiASB*. and then transformed into competent cells of *E. Coli* DH5*α* and incubated at 37˚C overnight. LITMUS-*MiASB* plasmid from a single positive colony was confirmed by sequencing. To insert T7 RNA polymerase site into the two ends of the *MiASB*, 1 μl of the identified LITMUS-*MiASB* plasmid were used as a template to perform another PCR. Amplifications were performed with Taq DNA polymerase (2.5 U, Promega) using 2 mM of T7 primer (5'-TAA TAC GAC TCA CTA TAG G-3') and 10 mM of each of the deoxy nucleotide triphosphates (dNTP), with an initial denaturation step of 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 s, 55˚C for 30 s, 72˚C for 50 s, and a final elongation step of 72˚C for 10 min. After amplification, the PCR products (T7-*MiASB*-T7) were separated by electrophoresis on agarose gel and purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0. Then the purified T7-*MiASB*-T7 fragment was used to generatedds RNAs using *in vitro* Transcription T7 Kit (TaKaRa) following the manufacturer's instructions. The synthesized dsRNAs was stored at −70˚C for further experiments.

2.5. RNAi of *MiASB* by Soaking

RNAi experiment was performed in a 24-microwell plate according to the previous studies [1,9,18] with slight modification. Uniform single egg mass or approximately 2000 freshly J2 were deposited in each well and socked in M9 buffer solution (43.6 mM Na_2HPO_4, 22 mM KH_2PO_4, 18.7 mM NH_4Cl, and 8.6 mM NaCl) containing 1% resorcinol, 50 mM octopamine, 3 mM spermidine, 0.05% gelatin, and dsRNA at 2 mg/ml in the dark at room temperature on a rotator (RNAi treatment). One control samples were incubated in the same solution but without dsRNA (Negative control). And the other control samples were incubated in the sterile water and also no dsRNA was added (Untreated control). For the egg RNAi treatment, half of the egg masses was treated just for 1 day and the total cumulative number of juvenile hatched from the egg masses were counted one days later (1-day-treatment), and the other half of the egg masses were treated for 3 days and total cumulative number of juvenile from these egg masses were counted 3 days later (3-day-treatment) to estimate the RNAi effect on the hatching of the egg masses. The cumulative numbers of dead juvenile in 1-day- and 3-day-treatments were also recorded at the corresponding time point, respectively, to essay the RNAi effect on the mortality of juveniles.

For the J2 RNAi treatment, Total J2 and the dead J2 was counted 6 h after the treatmentto determine RNAi effect on the mortality of J2. The juvenile that was straight and did not move even after mechanical prodding were defined as dead.

2.6. Infection of Plants

The RNAi treated egg masses (1-day-treatment and 3-day-treatment) and RNAi treated J2 were applied close to the roots of seedlings using a sterilized micropiette. 3 RNAi treated egg masses or approximately 6000 RNAi treated J2 were inoculated to each seedling. The two corresponding controls (negative control and untreated control) were also inoculated according to the RNAi treatment. These treated seedlings were cultured in the greenhouse. The seedlings were uprooted 45 days later, and root galls were recorded to evaluate the effects of the *MiASB* RNAi on the disease caused by *M. incognita*.

3. RESULTS

3.1. Generation of *MiASB* dsRNA

A 650 bp fragment of *MiASB* was obtained by PCR using total RNA of *M. incognita* J2 as template. The identity of the cloned *MiASB* fragment with the corresponding parts of the *MiASB* (GeneBank) accession: JQ247982) was as high as 100%.To generate the dsRNA of *MiASB* (**Figure 1**), the recombinant pLITMUS-*MiASB* was firstly constructed by linearizing pGM-*MiASB* and pLITMUS-28i with *Eco* RI, respectively, and ligated with T4 DNA ligase. Then pLITMUS-*MiASB* were used to performed PCR as a template with T7 Primer and the T7 polymerase site was inserted into two ends of the *MiASB* and formed the fragment T7-*MiASB*-T7. After that, *in vitro* transcription was catalyzed by T7 polymerase with fragment T7-*MiASB*-T7 as template andds RNA of *MiASB* was finally generated.

3.2. *MiASB* RNAi Inhibited Hatching of *M. incognita* Egg Masses

On the first day, juveniles were hatched from the egg masses, the amounts of which were slightly but not significantly different ($F = 0.98$, $P = 0.3975$). On the third-days, the cumulative juvenilea mounts in the RNAi treatment was significantly lower than those in negative and untreated controls ($F = 14.9$, $P = 0.003$). The average amountin *MiASB* treatment was 63, which was reduced by 60% and 64% compared to negative and untreated controls, respectively (**Figure 2**). The juvenile amount innegative control was slightly, but not significantly, higher than untreated control treatment, which showed the reagents supplemented in the RNAi solution hardly influenced the hatching of the egg masses, but RNAi of *MiASB* significantly inhibited the hatching of egg masses

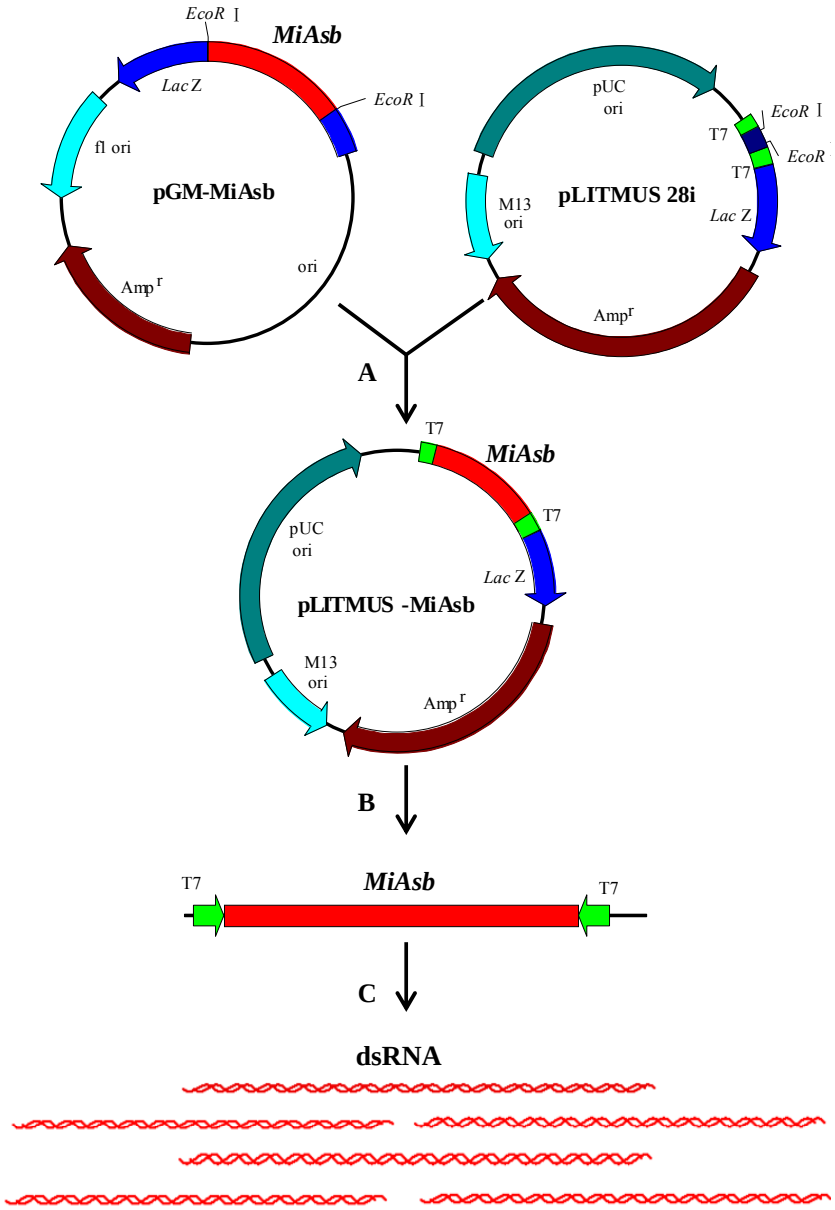

Figure 1. Generation of the dsRNA: A: pGM-*MiASB* and pLITMUS-28i were linearized with *Eco* RI and the respective resulting production were ligated with T4 DNA ligase to construct pLITMUS-*MiASB*; B: The pLITMUS-*MiASB* were used to perform PCR as a template with T7 primer, the result of which was T7 polymerase site was inserted into two ends of the *MiASB* and forming the fragment T7-*MiASB*-T7. C: *In vitro* transcription was catalyzed by T7 polymerase with fragment T7-*MiASB*-T7 as template and the dsRNA of *MiASB* was generated.

of *M. incognita*.

3.3. *MiASB* RNAi Caused High Mortality of *M. incognita* Juveniles Hatched from the Egg Masses

RNAi of *MiASB* significantly inhibited the hatching of *M. incognita* egg masses, and also caused high mortality of the juveniles (**Figure 3**). On the first day, the mortality of hatched juveniles in the RNAi treatment was 5.9 and 25.5 times higher than the negative and untreated controls ($F = 15.01$, $P = 0.0003$). On the third day, 62.8 of the 63.3, 6.8 of the 158.4 and 5.3 of the 176.7 hatched juvenilesdied in the RNAi treatment, negative and untreated controls, respectively. The cumulative percentage of juvenile mortality in the *MiASB* RNAi treatment was 23 times higher than negative control and 33 times higher than the untreated control, which were significantly dif-

ferent (F = 6417.04, P < 0.0001).

3.4. *MiASB* RNAi Caused High Mortality of *M. incognita* J2

M. incognita J2 was soaked in the dsRNA solution directly and were died. 6 h after treatment, about 434 of the 2000 J2 was found dead in the RNAi treatment, but 50 of the 2000 J2 in the negative control and 17 of 2000 J2 in untreated control were dead, respectively. The J2 mortality of RNAi treatment was 8.6 and 26 times higher than the negative and untreated controls, respectively (**Figure 4**), and were significantly different (F = 157.34, P < 0.0001).

3.5. *MiASB* RNAi Treated Egg Masses Reduced Root Galls on Tomato Seedlings

RNAi treated egg masses were inoculated to the tomato seedlings. 45 days later, the root galls were induced on all the tomato seedlings. For the 1-day-treatment, the number of root galls were between 18 and 30 in the RNAi treated seedlings, and those ranged from 48 to 112 for the two controls. The average number of root galls on RNAi treated seedling sreduced by 70.6% and 74.7% compared to the negative and untreated control seedlings, respectively (**Figure 5**) and were significantly different (F = 37.1, P < 0.0001). For the 3-day-treatment, the root gall amounts on the RNAi treated seedling were between 0 and 10, but those on the two controls ranged from 30 to 79. The average number of root galls on negative control and untreated control seedlings were 51.7 and 59, respectively, and that on the silenced seedlings was only 3.8 (**Figure 5**). Statistical analysis showed a significant difference existed among the number of root galls on the three differently treated seedlings (F = 38.1, P < 0.0001). The amount of root galls on the RNAi treated seedlings reduced by 92% and 93% compared to the negative and untreated control seedlings, respectively.

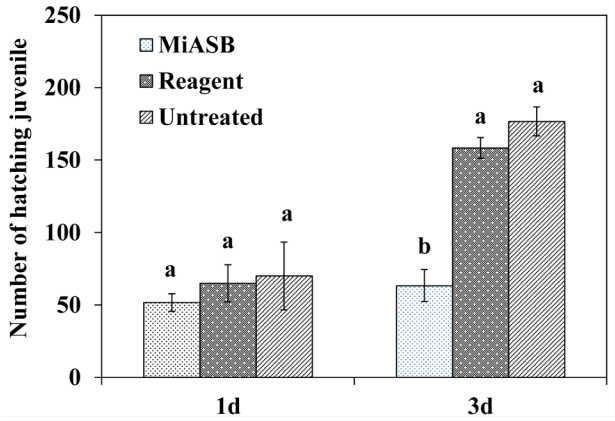

Figure 2. The number of juveniles hatched from *MiASB* RNAi treated egg masses of *M. incognita* was significantly lower than those hatched from the negative and untreated controls 3 days later.

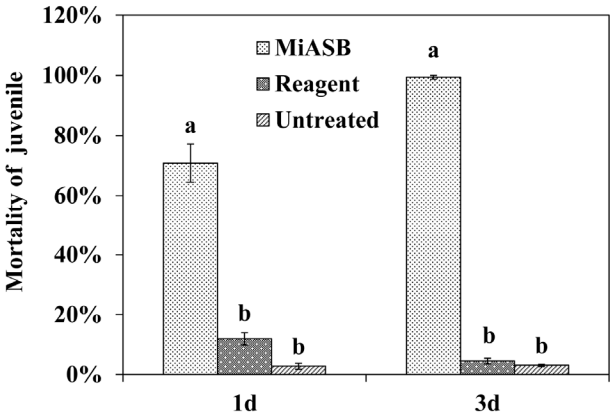

Figure 3. The mortality of juveniles hatched from *MiASB* RNAi treated egg masses was significantly higher than those hatched from the negative and untreated controls.

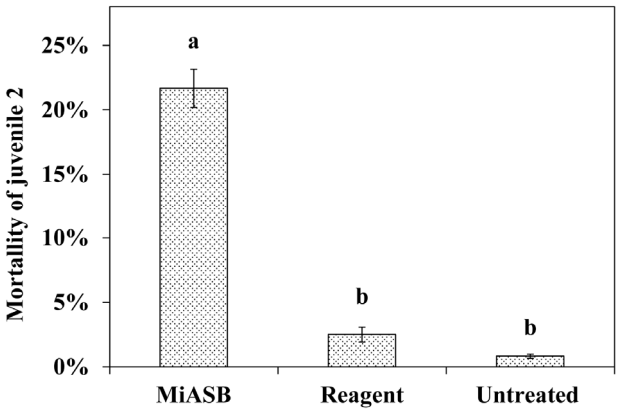

Figure 4. The mortality of J2 in the *MiASB* RNAi treatment was significantly higher than those of the negative and untreated controls 6 h later.

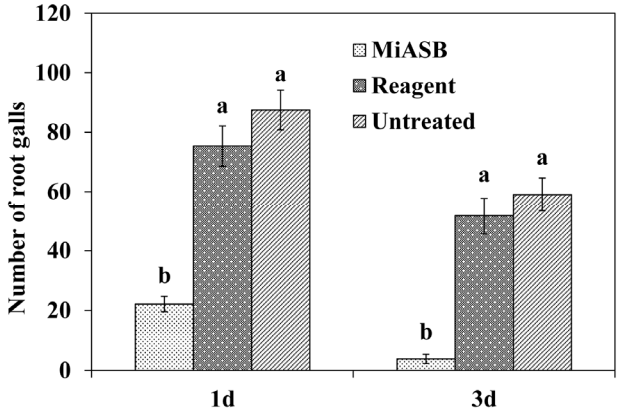

Figure 5. The root galls induced on the tomato seedlings inoculated with *MiASB* RNAi treated egg masses were significantly reduced compared to those inoculated with the negative and untreated control egg masses 45 days later.

3.6. *MiASB* RNAi Treated *M. incognita* J2 Reduced Root Galls on Tomato Seedlings

MiASB RNAi treated J2 were inoculated to the tomato seedlings. 45 days later, the root galls were induced on all the seedlings. But the number of root galls grown on negative control and untreated control seedlings was larger and the sizes were bigger compared to those on the *MiASB* RNAi treated seedlings (**Figure 6**). The root gall amounts on RNAi treated seedlings were between 38 and 57, but those on the two controls ranged from 240 to 368. The average number of root galls on negative control and untreated control seedlings was 273 and 321, respectively, and that on the RNAi treated seedlings was only 46. Statistical analysis showed a significant difference existed among the number of root galls on the three differently treated seedlings ($F = 30.64$, $P = 0.0007$) (**Figure 7**). The amount of root galls on RNAi treated seedlings reduced by 83% and 86% compared to that on the negative and untreated control seedling, respectively.

4. DISCUSSION

Plant parasitic nematodes have been so far refractory to transformation or mutagenesis, and no reverse genetic tool is available to analyze the roles of genes in parasitism [1]. With the discovery of gene expression control via small interfering RNA (siRNA) and micro RNA (miRNA) molecules, biologists are exploring genes and developmentfrom a new perspective [19]. Understanding of this ubiquitous phenomenon has revealed RNAi as a powerful tool to manipulate gene expression and analyze gene function [10]. Induction of RNAi by delivering double-stranded RNA (dsRNA) has been very successful in the model nonparasitic nematode *C. elegans*, and RNAi has been used to systematically analyze 90% of the predicted genes on chromosome I [20], 96% of the predicted genes on chromosome III [21], approximately 2500 nonredundant cDNAs [22], and the function of 350 oocyte cDNAs [23], 86% of the 19,427 predicted genes

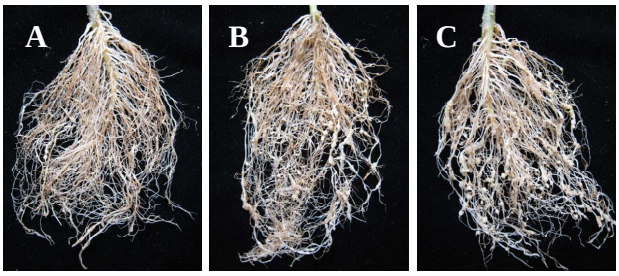

Figure 6. Phenotype of underground parts of the differently treated tomato seedlings. The number of root galls grown on the negative control seedlings (B) and untreated seedlings (C) were larger and the sizes were bigger compared to those on *MiASB* RNAi treated seedlings (A).

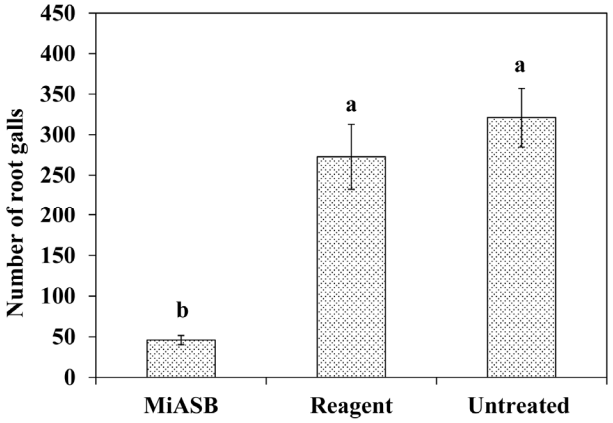

Figure 7. The root galls induced on the tomato seedlings inoculated with *MiASB* RNAi treated J2 were significantly reduced compared to those inoculated with the negative and untreated control J2 45 days later.

of *C. elegans* and identified mutant phenotypes for 1722 genes [24], 98% of all genes predicted in the *C. elegans* genome and developed a phenotypic profiling system [25]. These projects have rapidly advanced the understanding of gene function in *C. elegans*. Subsequently, RNAi has been applied successfully to animal parasitic nematodessuch as root-knot nematode [1,26,27], cyst nematodes [9,18,19,28] and *Haemonchus contortus* [29]. In the present research, based on the previously researches, by soaking the egg masses and J2 of *M. incognita* in dsRNA of *MiASB*, we found RNAi treatment significantly inhibited the hatching of the eggs and caused high morality of the juveniles, which showed we successfully triggered RNAi in parasitic nematode *M. incognita*.

The silencing of root-knot nematode genes by soaking in dsRNA highly depends on dsRNA uptake by nonfeeding J2 [1]. No *in vitro* culture system is yet available for plant parasitic nematodes, and the only free-living stage is the infective J2 [9]. The invasive J2 did not take up dsRNA from solution, so a major challenge in applying RNAi to plant parasitic nematodes is getting the dsRNA ingested by the nonfeeding J2. Urwin *et al*. [18] (2002) useda neurotransmitter octopamine to induce feeding in invasive parasitic cyst nematode J2, allowing uptake of dsRNA from solution, and used this method to knock out several genes. Rosso *et al*. [1] successfully induced uptake by adding resorcinol. In our study, based on the previous studies, we supplemented 1% resorcinol and 50 mM octopamine to solve the problem. Incubating J2 in 1% resorcinol for 4 h resulted in active pharyngeal uptake and did not show detrimental effects on nematode infectivity and development, but long incubation times showed a deleterious effect on the nematodes [1]. In the present research, we incubated the egg masses and juvenilesin 1% resorcinol for 1 day, even for 3 days which

were far longer than 4 h. To avoid the experiment deviation and improve experiment accuracy, we designed two controls, one was negative control to counteract the effect of the reagent including resorcinol and other elements supplemented in the solution. The other was sterile water to replace M9 buffer solution and the supplements. But the results showed no significant difference in the hatching inhibition and juveniles mortality between the two controls.

In F1F0-ATP synthase, the static peripheral stalk (δb2) do not appear to be a part of the main stalk, but it has been suggested that they form a second connection, a stator that fixes the catalytic $\alpha 3\beta 3$ subcomplex to the subunit to allow rotation of central stalk and c-ring (c_n) [30]. And synthesis of ATP depends upon rotation of the central stalk ($\gamma\varepsilon$) and c-ring (c_n) [15]. In the present research, the nematode egg masses and juveniles were soaked in the dsRNA solution of *MiASB*, which triggered RNAi in the eggs and the juvenile bodies, resulting in the inhibition of egg hatching and high morality of juveniles, and further reduced the pathogencity. In the process of growth and development, nematodes need ATP as energy to perform various activities including infection. Once the RNAi of *MiASB* was triggered in the nematode, it caused the reduction of b subunit in ATP synthases, further impaired the formation of proton channel portion of ATP synthase, finally impacted the production of ATP. In return, the reduced ATP production slowed down the metabolism of the nematode, leading to the low hatching ability, high mortality and reduced infection, which finally resulted in the low nematode disease.

5. CONCLUSION

In conclusion, we successfully triggered RNAi in the *M. incognita*, and RNAi of *MiASB* significantly reduced the nematode disease. The finding of this research suggested *MiASB* may be associated with the pathogencity of the nematode, and it provided new ideas and ways to further study on nematodes pathogenic mechanisms and prevention and control of nematodes.

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