Synthetic and Systems Biotechnology 6 (2021) 437–445

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology

Study on RNAi-based herbicide for *Mikania micrantha*

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ARTICLE INFO

Keywords:
Invasive plant
Mikania micrantha
RNA interference
Spray-induced gene silencing
Chlorophyll *a*/b-binding protein
Nucleic acid bioherbicide

ABSTRACT

The invasive plant *Mikania micrantha* Kunth (*M. micrantha*) from South America poses a significant threat to the stability and biodiversity of ecosystems. However, an effective and economical method to control *M. micrantha* is still lacking. RNA interference (RNAi) has been widely studied and applied in agriculture for trait improvement. Spray-induced gene silencing (SIGS) can produce RNAi silencing effects without introducing heritable modifications to the plant genome and is becoming a novel nontransformation strategy for plant protection. In this study, the genes encoding chlorophyll a/b-binding proteins were selected as targets of RNAi, based on high-throughput sequencing of *M. micrantha* transcriptome and bioinformatic analyses of sequence specificity. Three types of RNAi molecules, double-stranded RNA, RNAi nanomicrosphere, and short hairpin RNA (shRNA), with their corresponding short interfering RNA sequences were designed and synthesized for SIGS vector construction, from which each RNAi molecule was transcribed and extracted to be sprayed on *M. micrantha* leaves. Whereas water-treated control leaves remained green, leaves treated with RNAi molecules turned yellow and eventually wilted. Quantitative real-time PCR showed that the expression levels of target genes were significantly reduced in the RNAi-treated groups compared with those of the control, suggesting that all three types of RNAi herbicides effectively silenced the endogenous target genes, which are essential for the growth of *M. micrantha*. We also found that shRNA showed better silencing efficiency than the other two molecules. Taken together, our study successfully designed three types of RNAi-based herbicides that specifically silenced endogenous target genes and controlled the growth of *M. micrantha*. Moreover, we identified a gene family encoding chlorophyll a/b-binding proteins that is important for the growth and development of *M. micrantha* and could serve as potential targets for controlling the spread of *M. micrantha*.

1. Introduction

Mikania micrantha Kunth (*M. micrantha*) is an invasive alien plant belonging to the Asteraceae family, native to Central and South America, with a growth pattern of creeping or climbing onto other species. As a fast-growing perennial herbaceous species, *M. micrantha* possesses advantages of rapid growth, breeding, and spreading at both the vegetative and reproductive stages; it also exhibits solid competitive responses and stress tolerance [1]. Notably, *M. micrantha* is listed in the top 100 most harmful alien species worldwide by the International Union for Conservation of Nature, as it can invade and deteriorate the terrestrial environment by creeping, twining, and smothering other

Abbreviations: RNAi, RNA interference; SIGS, Spray-induced gene silencing; dsRNA, double-stranded RNA; RNP, RNAi nanomicrosphere; shRNA, short hairpin RNA; siRNA, short interfering RNA; HIGS, host-induced gene silencing; GMOs, genetically modified organisms; qRT-PCR, Quantitative real-time PCR; SEM, scanning electron microscope; LHCs, light-harvesting complexes.

Peer review under responsibility of KeAi Communications Co., Ltd.

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https://doi.org/10.1016/j.synbio.2021.11.005
Received 29 June 2021; Received in revised form 10 October 2021; Accepted 9 November 2021

plants, simultaneously releasing detrimental allelochemicals to seriously inhibit their growth, thus leading to a dramatic plunge in local biodiversity. In addition, as a pest in cash crops and commercial forests, *M. micrantha* poses a significant threat to agriculture and forestry, resulting in tremendous economic losses and hindering social development [2,3]. Therefore, it is urgent to prevent and control the rapid growth and spread of *M. micrantha*. Current control methods against *M. micrantha* mainly include alien plant monitoring, manual or mechanical eradication, chemical herbicide application, and integrated ecological strategies, such as natural enemy control and nearby biocommunity structure optimization [4,5]. Although these traditional methods have offered some progress in preventing and controlling the damage caused by *M. micrantha*, they have many disadvantages, including enormous economic costs, low efficiency, and environmental hazards [6–8]. Meanwhile, with the high technical threshold required to study its utilization value [9], more efforts should be put into the research to develop a feasible control method for biological invasion. (see Table 1)

RNA interference (RNAi), also known as post-transcriptional gene silencing, is a prevalent defense mechanism that uses homologous double-stranded RNA (dsRNA) against the invasion of exogenous genes in eukaryotic organisms to stabilize their genome [10]. Nowadays, owing to their high specificity and efficiency [11–14], RNAi-based techniques, mainly including host-induced gene silencing (HIGS) and spray-induced gene silencing (SIGS), have been widely applied in agriculture for plant disease control and trait improvement and have been proposed as a substitute for agricultural chemicals [15,16]. In particular, HIGS involves the expression of small RNAs in the host, such as small interfering RNA (siRNA) or short hairpin RNA (shRNA) and silencing target genes in interacting plant pathogens. The SIGS technique has been widely applied in agricultural research since its first utilization in 2016 [17]. More specifically, SIGS is a novel non-transformative strategy for suppressing pathogen genes via the sprinkling of in vitro-produced RNAi molecules in plant tissues. Although the molecular mechanism is unclear, current studies have reported that the sprayed RNAi molecules are absorbed into cells through the stomata or trabeculae and move systemically within the body via intercellular hyphae or plant siliques and sieve tubes, resulting in relevant gene silencing phenomena and subsequent gene silencing-induced mortality in nontransgenic ways [17–19]. Thus, apart from plant disease control, SIGS can generate RNAi silencing effects within plants without creating genetically modified organisms (GMOs).

So far, few studies have extended the use of the SIGS technique to address issues of invasion by exotic alien species. To prevent the further dispersal and environmental detriment by *M. micrantha*, we selected target genes encoding chlorophyll a/b-binding proteins based on transcriptome analyses and sequence specificity and designed and synthesized three types of corresponding RNAi molecules, dsRNA, RNAi nanomicrosphere (RNP), and shRNA, to investigate the effect of SIGS on the growth of *M. micrantha*. The morphological phenotype of leaves and reduced level of expression of target genes suggested that all three types of RNAi molecules effectively and specifically silenced genes encoding chlorophyll a/b-binding proteins, which are essential for the growth of *M. micrantha*. These findings highlighted a better way to eliminate *M. micrantha* and shed light on the improved control of other invasive alien species.

Table 1
The RCT reaction system of RNPs.

Chemicals	Dosage (μL)
ssDNA template	50
rNTP	10
RNAase inhibitor	3
DTT	1
10 × T7 RNA buffer	10
T7 RNA polymerase	5
RNase-free water	Up to 100

2. Materials and methods

2.1. Plant materials

Wild-type *M. micrantha* plants were collected from a lychee forest on the Lihu campus of the Shenzhen University; the plants with a suitable growth period and medium-sized leaves were selected as experimental samples and cut into several branches with two stem nodes and a leaf on the apical side. Then, the base end of the morphology was cultured in water under low light conditions for approximately 1 week to root. Plants with root lengths of approximately 4–5 cm were transferred to Hoagland liquid medium and grown in a light homoeothermic chamber until the 5–6 leaf stage. Plants were then transferred to soil to grow for another 3–4 d before their use in experiments. *Arabidopsis thaliana* and tobacco plants were grown in the growth room with daily cycles of 16 h/light and 8 h/dark at 23 °C.

2.2. Transcriptome sequencing

Total RNAs were extracted using TRIzol® Reagent (Invitrogen, USA). The quality of total RNA (RNA concentration >250 ng/μL; OD260/280 between 2.0 and 2.2) was measured using NanoPhotometer (IMPLEN, Germany). The qualified RNA samples were constructed to cDNA library following the Illumina Hiseq platform protocol (Illumina Inc. San Diego, CA, USA), as well as pair end sequencing was performed on the Illumina Hiseq platform.

2.3. Plasmid construction

The HT115 (DH3) and DH5α *E. coli* strains were purchased from Huinuo Biotechnology Co. (Shenzhen, China). The L4440 plasmid was provided by the Plant Epigenetics laboratory of Shenzhen University. DNA sequences of siRNAs and shRNAs were designed based on the sequences provided by Lee et al. [20] and constructed by GeneScript Co. (Nanjing, China). All primers were synthesized by Sangon Biotech Co. (Shanghai, China).

2.4. Reagents

RNAiso Plus reagent (Code No. 9109), T4 DNA ligase (Code No. 2011A), and Premix Taq™ (Ex Taq™ Version 2.0 plus dye) (Code No. RR003A) were purchased from Takara Biomedical Technology Co. (Beijing, China). Evo M-MLV II reverse transcriptase (Code No. AG11616) and SYBR® Green Premix Pro Taq HS qPCR Kit II (Code No. AG11719) were purchased from Accurate Biotechnology Co. (Hunan, China). The T4 DNA ligase reaction buffer (Code No. B0202S) and ribonucleotide solution mix (Code No. N0466L) were purchased from New England Biolabs (MA, USA). Qubit RNA BR assay kit-100 assays was purchased from Thermo Fisher (Code No. Q33223; USA) and RNase inhibitor was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Code No. S10087–1KU; Shanghai, China).

2.5. Design of RNAi molecules

The essential component for the success of RNAi is the structure of siRNAs; thus, silencing efficiency is determined according to the siRNA sequence. To obtain an efficient silencing effect while reducing the off-target rate, siRNAs were designed according to the following principles [21].

First, starting from 50 to 100 bp downstream of the start codon, while evading the site of UTR at the 5′ and 3′ ends, and going as far as possible to obtain the 21-base sequence starting with a pair of adenine (A-A); second, ensuring the presence of at least three adenine (A) or uracil (U) molecules at the site between 15 and 19 bp, or at least five

adenine (A) or uracil (U) molecules at the site between 13 and 19 bp, in the sense strand. In addition, to avoid multiple guanine (G) or cytosine (C) molecules in the sequence, the GC content must be between 30 and 52%, preferably with a cytosine (C) as base at 16, and a non-guanine (G) as base at 13 bp site to prevent the energy distribution of siRNAs from affecting the silencing efficiency, and ulteriorly restraining the formation of secondary structures.

Finally, to minimize the off-target effects, we conducted comparative tests of mRNA sequences using BLAST, with further screening performed with the employment of mRNA similarity and off-target control algorithms. After redacting the corresponding screening program, gene sequences were retrieved and compared using the NCBI NT database. The screened targeted genes with high specificity are shown in Appendix Table 5 (5′→3′).

2.6. Synthesis of dsRNAs

2.6.1. Construction and transformation of target plasmids

Total RNA from *M. micrantha* leaves was extracted and reverse transcribed to obtain cDNA using the SYBR ®Green qPCR kit. PCR amplification was then performed using specific primers to obtain the 500 bp target fragments, which were purified by gel extraction (primer sequences are shown in Appendix Table 1).

The recombinant plasmid was obtained by mixing the digested L4440 plasmid with a targeted fragment. The plasmid was then transformed into HT115 *E. coli* cells (DE3). Cells were then incubated in LB solid media supplemented with 50 mg L^{-1} ampicillin at 37 °C overnight. Single colonies were subjected to a resistance screen to select positive colonies and obtain the bacteria containing the recombinant plasmids; subsequently, colony samples we shifted for sequencing. Positive colonies were sequenced by IGE Biotechnology Co., Ltd. (Guangzhou, China).

The recombinant plasmid construction reaction included the following: purified target fragment (6 μL), L4440 plasmid digested with KpnI-SacI (2 μL), T4 ligase (1 μL), and 10 × T4 ligase reaction buffer (1 μL). The mixture was placed in a refrigerator at 4 °C overnight.

2.6.2. Synthesis of dsRNAs

Following IPTG induction, 1 mL fermentation broth was transferred to a new centrifuge tube and centrifuged at 9000 rpm and 4 °C for 5 min. The media was removed using a pipette. Then, 1 mL TRIzol reagent was added to the precipitate, which was redissolved by vigorous shaking and placed at 25 °C for 5 min to fully lyse the bacteria. Subsequently, 200 μL chloroform was added to the tube and gently shaken for 15 s; the supernatant was transferred to another clean tube and an equal volume of isopropanol was added, gently mixed, and placed at 25 °C for 10 min; the sample was centrifuged at 9000 rpm and 4 °C for 15 min; in highly concentrated samples, a jelly-like precipitate would be visible at the bottom of the tube. Following removal of the supernatant, 75% ethanol was poured along the tube wall, and the precipitate was washed by shaking. The mixture was centrifuged at 9000 rpm and 4 °C for 5 min, followed by the removal of all the supernatant; this step was repeated two more times. The tube's cap was opened in a fume hood or ultra-clean bench and left for 5 min to eradicate the ethanol. This would result in the jelly-like precipitate becoming transparent. Finally, 20 μL DEPC-treated water or deionized water was added to completely dissolve the precipitate. RNA concentration and purity were assessed using a NanoDrop™ ONE system, and both purity and integrity were further verified via gel electrophoresis.

2.6.3. dsRNA treatment and detection of gene expression

For this assay, 60 μg dsRNA was sprayed on *M. micrantha* leaves for 14 d continuously. Water-treated leaves served as the negative control. Following treatment, the morphological phenotypes were observed and recorded over time. Total RNA was extracted from leaves to examine the expression levels of target genes using quantitative real-time PCR (qRT-PCR). EIF(ISO)4G1 (eukaryotic translation initiation factor isoform 4G-1, AT5G57870) was selected as the internal control for qRT-PCR. Gene0004429 (ribulose bisphosphate carboxylase small chain 4) with a high RPKM value (RPKM = 17072.1) was selected as the negative control to evaluate the targeting specificity of RNAi molecules. The primers sequences used for qRT-PCR are listed in Appendix Table 2.

2.7. Synthesis and test of RNAi nanomicrospheres

The ssDNA template was mixed with T7 primers at a ratio of 1:1. After annealing, the ends of the ssDNA were complementarily paired with the T7 promoter and subsequently ligated with T4 DNA ligase to close the incision and form a gap-free circular template. A large number of transcribed sequences were then generated by the transcription reaction of a rolling loop involving T7 polymerase and then self-assembled into multiple tandem repeats of short hairpin RNA polymers with the basic unit of target sequences, that is, the RNAi nanosphere (RNP). The obtained structures were observed under a scanning electron microscope (SEM). Furthermore, the effect of nanospheres on *M. micrantha* leaves was tested.

Sequences of DNA templates are shown in Table 4 in the Appendix. The synthesis procedure was as follows [22]:

1 Initially, 10 μL of 10p ssDNA and 10p T7 promotor were mixed together with 4 μL ATP-containing buffer and 376 μL deionized H_2O and the mixture was centrifuged for homogeneous mixing. Then, it was placed in a qRT-PCR instrument at 95 °C for 3 min, 60 °C for 1 min, and finally at 25 °C for 2–3 h;
2 Subsequently, 4 μL T4 DNA ligase was added to the reaction system, which was placed at 25 °C for 4 h;
3 A 2 μL cirDNA sample was used on integrity testing in an 8-well 3% agarose gel (0.9 g/30 mL);
4 Then, 0.3 μM of the above-obtained cirDNA was mixed with T7 RNA polymerase (5 units/μL) and T7 RNA polymerase buffer consisting of 8 mM Tris-HCl, 0.4 mM spermidine, 1.2 mM $MgCl_2$, and 2 mM DTT or mercaptoethanol. Finally, 2 mM rNTP was added with homogeneous mixing, followed by incubation at 37 °C for 20 h.
5 The sample was mixed by repeated purging with a pipette, sonicated for 5 min, centrifuged at 6000 rpm for 6 min, and the supernatant was discarded.
6 The sample was washed with an appropriate amount of RNase-free water and then sonicated for 1 min; steps ② and ③ were repeated thrice.
7 The sample was dissolved in RNase-free water at the desired concentration and stored at 4 °C for future use.

The RCT reaction system for RNAi nanospheres.

The protocols for confirming the size of RNAi nanospheres by SEM were as followed.

After centrifugation and purification, 10 μL sample was added dropwise to the silicon wafer surface. After another short drying process of the silicon wafer, the surface of the sample was sprayed with gold. The sample was then placed on the sample stage and observed using scanning electron microscopy.

Testing the effect of RNP on *M. micrantha* was similar to that of dsRNA. Briefly, 60 μg RNP was sprayed on *M. micrantha* leaves for 10 d continuously. Water-treated leaves served as the negative control. We also selected another six leaf samples with different sizes and morphologies in order to avoid individual variations in leaves, which would introduce errors to the results. We then recorded and analyzed the results of the morphological phenotype and relative transcript levels to verify the silencing efficiency of RNPs on *M. micrantha*.

2.8. Design and synthesis of shRNAs

We designed specific sequences to synthesize short single-stranded

RNAs, each of which could form a hairpin structure containing targeted siRNA fragments within the stem, to target the genes numbered gene0004558, gene0004560, gene0025307, gene0029128, and gene0026534 (shRNAs sequences are shown in Table 3 in the Appendix). Following PCR amplification and digestion with XbaI and BlpI, the fragments were inserted into the pET-28a (+) vector, and the obtained recombinant plasmid was transferred into *E. coli* HT115 (DE3). The production of shRNAs was carried out according to the protocol for the production of dsRNAs.

The pET-28a (+)-shRNA vector is shown in Appendix Fig. 1. The reaction system for the enzymatic cleavage of PCR products and the recombinant plasmid were as follows:

Enzymatic cleavage of the targeted sequences: We mixed the PCR product (35 μL), XbaI and BlpI enzymes (1 μL each), 10 × loading buffer (5 μL), and deionized water (8 μL) were mixed and the reaction system was incubated at 37 °C for 3 h.

Plasmid digestion: Similarly, plasmid DNA (<1 μg, 10–12 μL), XbaI and BlpI enzymes (1 μL each), 10 × loading buffer (2 μL), and distilled water (4–6 μL) were mixed and the reaction system was incubated at 37 °C for 1.5 h shRNAs (60 μg) were sprayed to each leaf sample for 7 d continuously.

3. Results

3.1. Members of the chlorophyll a/b-binding protein family were selected as target genes in M. micrantha

Photosynthesis is a crucial process that significantly influences the growth and development of plants. To examine whether photosynthesis-related genes, which could serve as potential targets, are highly expressed in *M. micrantha*, we analyzed the transcriptome of three different tissues, stem, leaf, and flower. As shown in Fig. 1, the levels of expression of genes varied among the three tissues. Interestingly, genes belonging in the chlorophyll a/b-binding protein family were widely expressed in all three tissues, with the highest expression being found in the leaf. Therefore, we selected five genes from the chlorophyll a/b binding protein family, namely gene0004458, gene0004560, gene0025307, gene0026534, and gene0029128 as targets, and designed the corresponding RNAi molecules.

3.2. dsRNA molecules silenced the endogenous members of the chlorophyll a/b-binding protein family

To determine whether all dsRNAs were effectively expressed and further evaluate their expression, we subjected them to 1% agarose gel electrophoresis. We accordingly observed the presence of strong bands with an expected size of 500 bp for all target genes, suggesting the successful expression of all dsRNAs (Fig. 2).

We then examined the silencing efficiency of these in vitro-synthesized dsRNAs. We mixed all five dsRNAs and applied this mixture to *M. micrantha* leaves of uniform size and shape under normal growth conditions. More specifically, we evenly applied each leaf with 60 μg of this dsRNA mixture; DEPC-treated leaves served as negative control. We performed this procedure using three biological replicates and collected samples daily for qRT-PCR analysis to detect the levels of expression of target genes. As shown in Fig. 3A, we found that the expression of all targeted genes was reduced, with a decrease being observed on day 3 after treatment, whereas that of nontargeted genes (negative) remained unchanged. In addition, we treated another six groups of leaves of different size with dsRNAs and water, and monitored their morphological changes. Consistent with the qRT-PCR results, we noticed that 14 d after treatment with dsRNAs the leaves started to turn yellow and wilted, whereas the leaves in the control group looked healthy (Fig. 3B), indicating that dsRNAs specifically silenced the endogenous target genes.

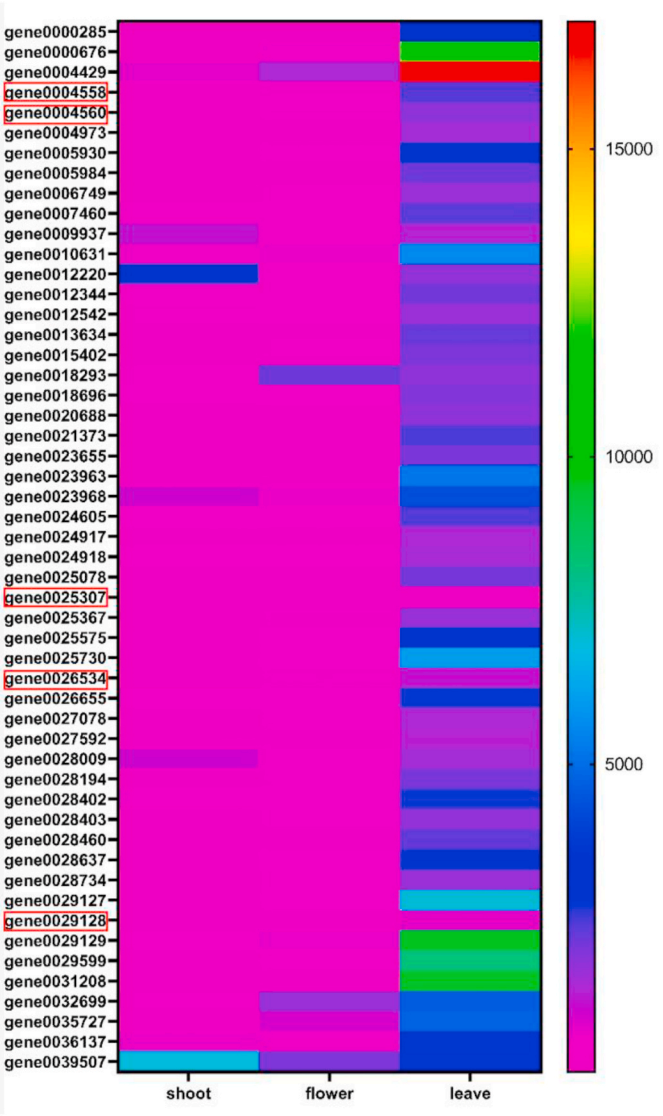

Fig. 1. Transcriptome analyses of *M. micrantha* stems, leaves, and flowers (excerpt). Genes that are involved in primary metabolisms were included in this analysis. Gene0004558, gene0004560, gene0026534, gene0025307, gene0029128 were selected as the target genes in the experiment (circled in red). The scale bar represents RPKMs (per million mapped reads).

3.3. RNAi nanomicrospheres achieved better silencing effect than dsRNAs

Nanospheres have been reported to serve as vectors to promote the entry of RNAi molecules into cells and protect them from degradation, thus improving their efficiency [20,22,23]. RNA nanospheres mainly consist of a single-stranded circular DNA with a positive strand, an antisense strand, and an unpaired short sequence that can be transcribed in vitro to form an RNAi molecule with multiple hairpin structures. Subsequently, millions of RNAi copies bind tightly and eventually form a nanosphere. Considering the thick waxiness surface of *M. micrantha* leaves, we wondered whether RNPs would silence the endogenous target genes in *M. micrantha* more efficiently than dsRNAs.

To synthesize RNPs, we first needed to cyclize ssDNA, which would serve as a template for rolling PCR. The cyclized ssDNA should run slower in gel electrophoresis than the uncyclized ssDNA (92 nt). As shown in Fig. 4A, we obtained the cyclized ssDNA for RNP synthesis and recovered it by gel extraction; however, we noticed that the cyclization efficiency was low.

As the molecular weights of RNPs cover an extensive and

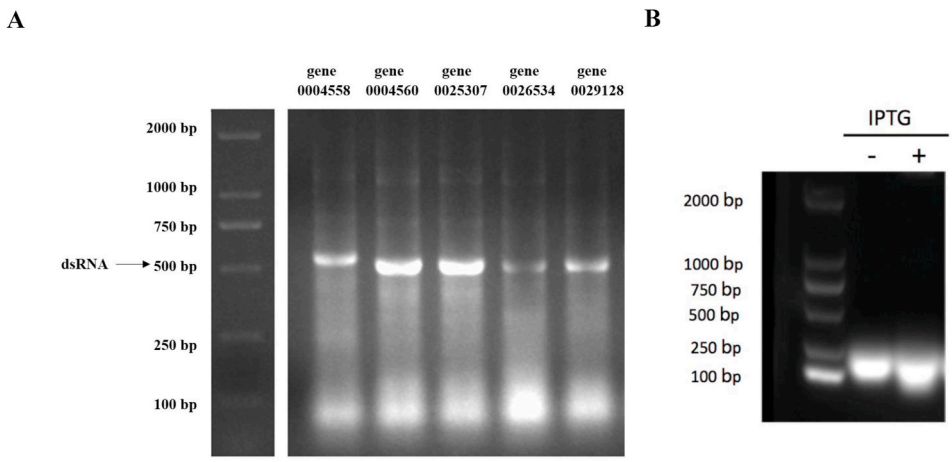

Fig. 2. Gel electrophoresis image of dsRNAs extracted from *E. coli*. A) Bands at the expected sizes (around 500 bp) corresponding to the transcription units were observed with varied intensity; B) The IPTG-induced and non-induced empty vector (*E. coli* without dsRNA plasmid) served as the negative control.

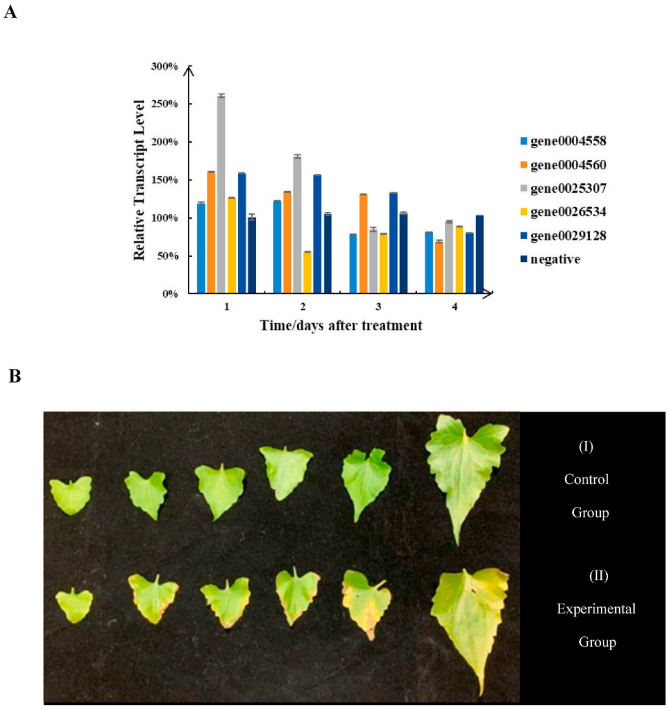

Fig. 3. The effects of exterior dsRNA application on target gene expressions and leaf morphology in *M. micrantha*. A) Suppression of target gene expression by exterior dsRNA as determined by quantitative RT-PCR. Following dsRNA application, the expression levels of the target genes decreased slowly, while the expression level of the negative control gene remained unchanged, indicating that the dsRNA molecules successfully down-regulated the expression of the target genes. B) The phenotypic result of leaf samples with different sizes after a fortnight of dsRNA treatment. (I) Control Group: six leaf samples remained healthy; meanwhile, Experiment Group (II) showed the appearance of wilting and breakage areas on leaf tip and leaf margin, which indicated that the treatment of dsRNA may affect the normal development of *M. micrantha* and damaged the morphological phenotypic of its leaves by constantly down-regulating the expression of target genes.

comprehensive range, different sizes of smear bands would appear in the RNP samples, and some RNP samples would remain in the gel well during electrophoresis. To determine whether RNPs were successfully synthesized, we first checked the synthesized RNPs using gel electrophoresis. As expected, we observed smear bands and remaining samples in the gel wells (Fig. 4B), suggesting the successful synthesis of RNPs. To observe and further confirm their structures, we recovered the synthesized RNPs from the gel, dripped them onto a silicon wafer for spray-gold treatment, and observed them under a scanning electron microscope (SEM). We noticed that RNPs formed petal-like structures on the surface, but the nanoscale polymer was not visible under SEM because only nanospheres with diameters ranging from 1.5 to 8.5 μm were obtained (Fig. 4C).

Given the successful synthesis of RNPs with the expected size, we aimed to test whether these RNPs were functional. To avoid individual variations among leaves, which might affect the accuracy of the results, we applied RNPs to leaves of different size and shape continuously for 10 d. Following treatment, we examined the level of expression of target genes by qRT-PCR, and recorded the changes in the morphological phenotypes of leaves daily. Similar to the treatment with dsRNAs, we used water-treated leaves as negative control. Our qRT-PCR results (Fig. 5A; revealed that on day 2 posttreatment, the relative transcript levels of target genes were all significantly decreased, except for that of gene0025307; no change was detected for the nontarget genes (negative). We subsequently observed that on day 5 posttreatment, the transcript levels of the three target genes began to increase, probably due to feedback regulation within the plants. As expected based on the function of target genes, all leaves treated with RNPs turned yellow and wilted after continuous treatment for 10 d, whereas those treated with water remained green and healthy (Fig. 5B). These results indicated that RNPs could partially permeate into the leaves of *M. micrantha*, triggering RNAi during transcription and leading to the downregulated expression of target genes, which resulted in wilting and yellow-turning leaves. In addition, by comparing the phenotypes of leaves treated with either dsRNAs or RNPs, we observed that RNPs were more efficient and associated with a longer duration of gene silencing than dsRNAs. This could be because the structure of RNPs might facilitate the entry of RNPs into plant cells and prevent the degradation of RNAs.

3.4. *shRNA-induced gene silencing was the most efficient among all three tested RNAi molecules*

We observed that although RNPs displayed better silencing efficiency than dsRNAs, the cost of RNP synthesis was slightly higher. Therefore, we investigated a third type of RNAi molecule, shRNA, formed by a single-stranded RNA containing siRNA that is reverse complementary to target genes. We induced the expression of shRNA by IPTG and then subjected them to 1% agarose gel electrophoresis. As shown in Fig. 6, following IPTG induction an additional bright band with a size of approximately 51 bp was detected below the 5S rRNA, whereas no band was detected in the sample lacking IPTG, suggesting the

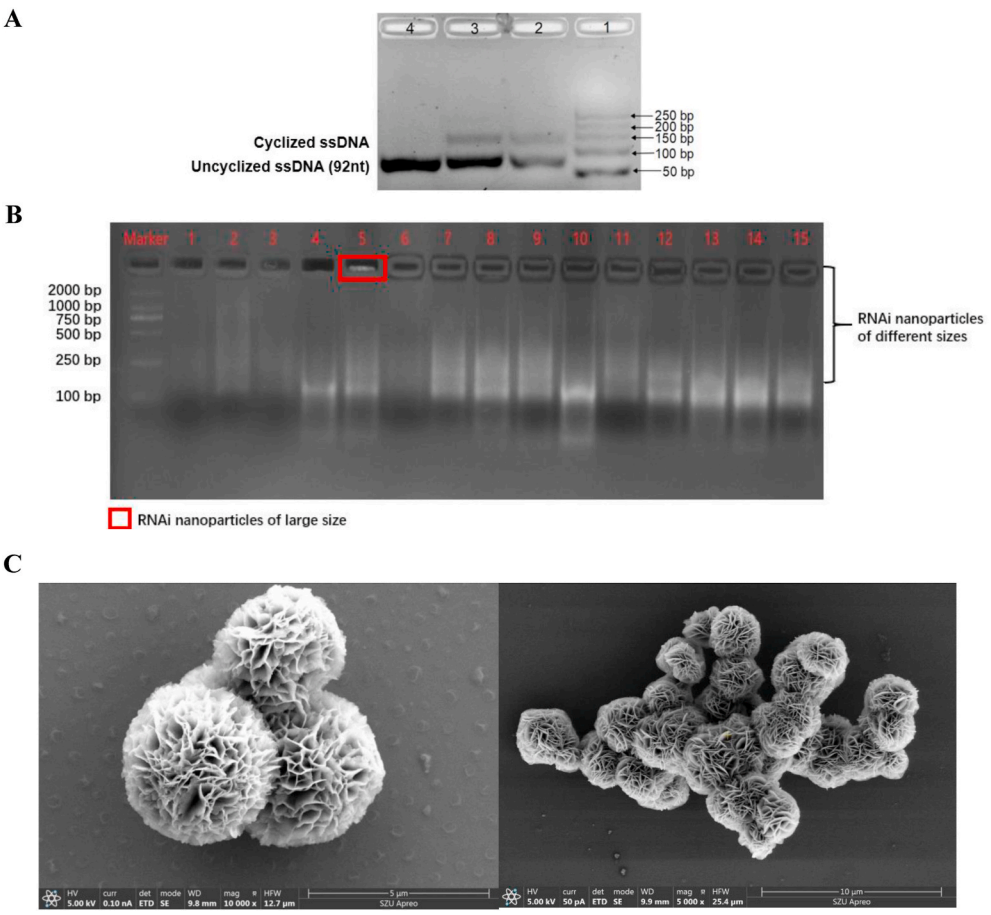

Fig. 4. Stage and final results of RNP synthesis. A) The gel electrophoresis image of cyclic DNA. The cyclized ssDNA theoretically run slower than the uncyclized one. The weak band around 150 bp represented the successfully cyclized ssDNA, which was right above the uncyclized ssDNA (92 nt). B) The gel electrophoresis image of RNP. The smear band and remaining samples (circled in red) in the gel well represented RNPs in varied sizes, indicating that the RNPs were successfully synthesized. C) The SEM plot of RNPs. A petal-like structure was observed on the surface of RNPs with diameters ranging from 1.5 μm to 8.5 μm.

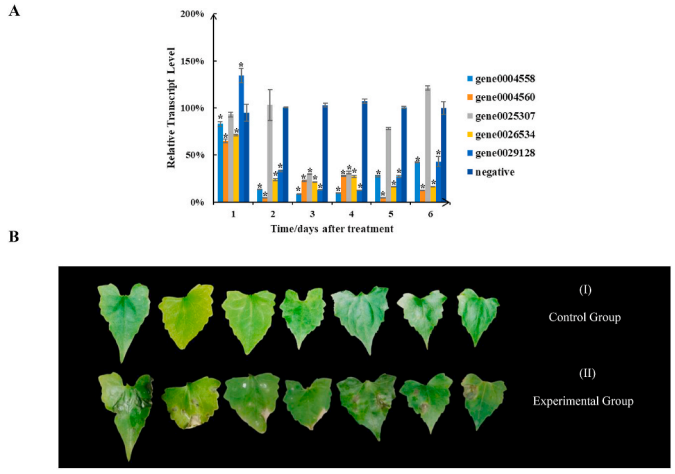

Fig. 5. The effects of RNP application on target gene expressions and leaf morphology in *M. micrantha*. A) Suppression of target gene expression by exterior RNP as determined by quantitative RT-PCR. Following RNP application, the expression levels of the target genes decreased significantly, then started to rise on day 4/5 posttreatment. Asterisk (*) indicates that a statistically significant difference between the exerimental group and the control group ($p < 0.05$, student's *t*-test). B) The morphological phenotypic results of leaf samples with different sizes on day 10 after RNP treatment. (I) Control Group: seven leaf samples remained healthy; meanwhile, Experiment Group (II) showed the appearance of yellowing and necrotic areas on the surface of the leaf samples with wilting and broken leave tips, which indicated that the treatment of RNP could significantly inhibit the normal development of *M. micrantha* by constantly down-regulating the expression of target genes.

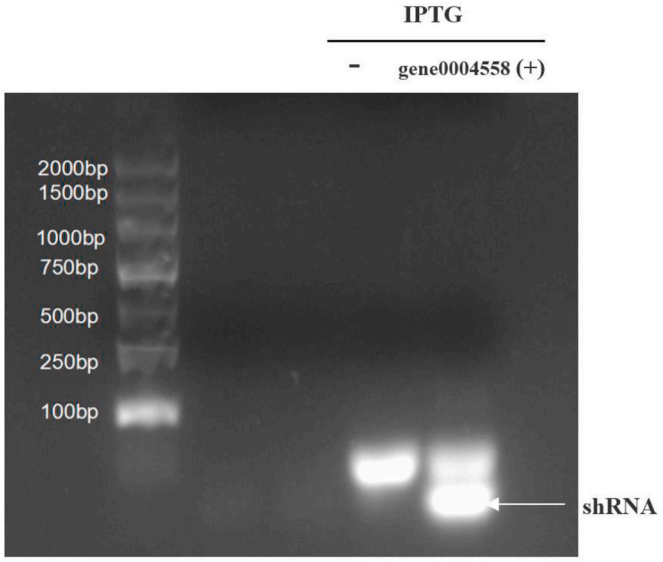

Fig. 6. Gel electrophoresis image of shRNAs extracted from *E. coli*. The band at expected size corresponding to shRNAs was detected and marked with an arrowhead. The same *E. coli* strain without IPTG induction served as the negative control.

successful expression of shRNA.

To quickly evaluate the silencing efficiency of shRNAs, we first mixed the 5 shRNAs in equal proportions and applied the mixture to *M. micrantha* leaves for 7 consecutive days. Water-treated leaves were

used as negative control. We collected samples daily, and performed qRT-PCR to detect the relative expression levels of target genes. Our results revealed (Fig. 7A) that the expression levels of all target genes were significantly decreased on day 2 posttreatment with shRNAs compared with those treated with water, whereas no significant change was observed for nontarget genes (negative). On day 4 posttreatment, we noticed that the levels of expression of target genes were increased in shRNA-treated leaves, whereas those of nontarget genes remained unchanged. These findings were similar to the results of RNP-treated samples and might probably be due to some unknown feedback regulation in plants. We also recorded the phenotypical changes in leaves treated with either the shRNA mixture or water for 7 d (Fig. 7B). We found that leaves treated with shRNAs gradually turned yellow and shriveled over the 7 d treatment period, whereas those of the control continued to grow. Our analyses of the wilting efficiency and gene expression suggested that similar to dsRNAs and RNPs, shRNAs specifically and effectively silenced the endogenous target genes. Moreover, a comparison among dsRNA-, RNP-, and shRNA-mediated gene silencing indicated that shRNA-induced silencing was more efficient than that induced by dsRNAs and RNPs. In addition, to further validate the species specificity of Micrancide, we mixed the shRNA molecules and sprayed them on *Arabidopsis thaliana* and tobacco leaves, respectively. As shown in Appendix Fig. 2, both Arabidopsis and tobacco leaves remained green after 10-day treatment, suggesting that the Micrancide is *M. micrantha*-specific.

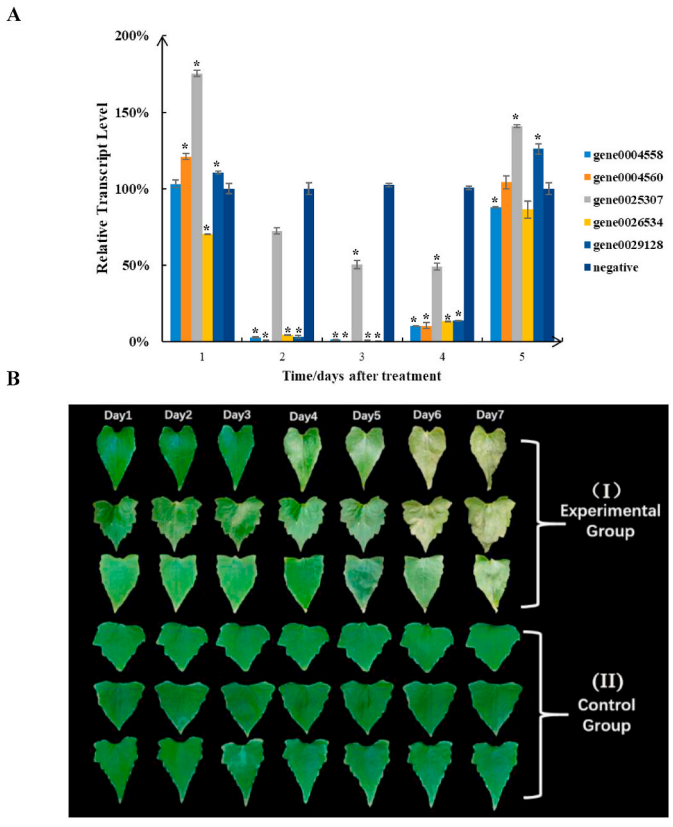

Fig. 7. The effects of shRNA application on target gene expressions and leaf morphology in *M. micrantha*. A) Suppression of target gene expression by exterior shRNA as determined by quantitative RT-PCR. After the treatment of shRNA, the expressions of all target genes were significantly decreased except for gene0025307. The expression started to recover on day 4 posttreatment. Asterisk (*) indicates that a statistically significant difference between the exerimental group and the control group ($p < 0.05$, student's *t*-test). B) The phenotypic changes of leaf samples with different sizes after a seven-day shRNA treatment. (I) Experiment Group: the leaf samples gradually etiolated and died during the period of shRNA treatment; meanwhile, the Control Group (II) remained green and happy, which indicated that the treatment of shRNA could inhibit the normal development of *M. micrantha* by efficiently down-regulating the expression of target genes.

4. Discussion

4.1. shRNA is the best spray-induced gene silencing carrier for application to M. micrantha

As a novel and efficient nontransformative strategy, the SIGS technique has been widely applied in agricultural and mammalian research. However, few studies have compared the silencing efficiency of different RNAi molecules.

Here, we designed three types of RNAi molecules, dsRNAs, RNPs, and shRNAs, the silencing mechanism of which is shown in Fig. 8. We targeted *M. micrantha* genes encoding the chlorophyll a/b-binding proteins, and examined the silencing efficiency by spraying the corresponding RNAi molecules on the leaves of *M. micrantha*. Our results suggested that shRNA was the best silencing carrier among the three RNAi molecules. Studies have shown that dsRNAs are absorbed into plant cells via stomata, microinvasive, or vesicular transport in the form of RISC, and then move systemically inside the plants. Nevertheless, the gain results indicated that the introduction of dsRNAs, which are easily recognized by the host plant as viruses, triggers the most significant innate immune response [24]. Rao et al. found that siRNAs were 10-fold less efficient than shRNAs in forming RISCs with AGO and other proteins in cells [25]. As shRNAs were found to be assimilated to endogenous miRNA in vivo, they were continuously synthesized by host cells, leading to a significant increase in shRNA silencing efficiency [26].

In contrast, by tracking the degradation of fluorescence-labeled siRNAs in vivo, we found that less than 1% dsRNAs remained in cells 48 h posttreatment, suggesting the high degradation of exogenous siRNAs. Therefore, in comparison with siRNAs, shRNAs possess the advantages of long half-life, self-replication, and high inhibition efficiency, enabling them to continuously and efficiently silence target genes. Taken together, dsRNAs were less efficient than RNPs and shRNAs. However, RNPs produced by in vitro transcription have the advantage of high copy number but require the participation of various enzymes, such as T7 RNA polymerase, thus raising the cost of RNPs relative to that of shRNAs expressed by the *E. coli* strain HT115 (DE3). In addition, because of their large size and high negative charges, RNPs need to be combined with some cytotoxic cationic reagents, such as PEI to permeate cells,

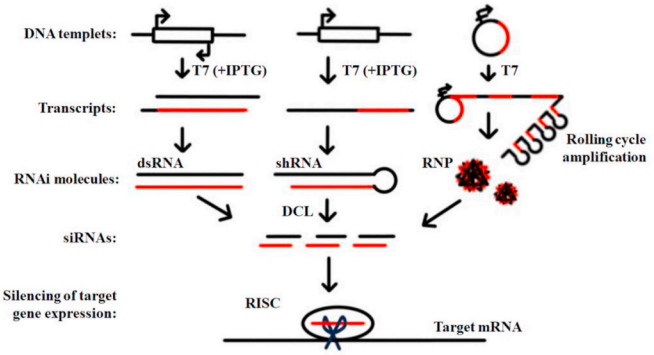

Fig. 8. Generation and action model of dsRNA, shRNA and RNP. Three types of RNAi molecules were generated in this study. dsRNAs were generated from bi-directional transcription. RNPs were generated from rolling PCR with a single-stranded circular DNA template, which consisted of a positive-strand, an antisense strand, and an unpaired short sequence; shRNAs were transcribed from a single-stranded DNA consisting of a positive-strand, an antisense strand, and an unpaired short loop sequence. Following external application, these RNAi molecules were processed into siRNAs, which were then loaded into AGO1 to mediate the silencing of their target genes.

limiting their use in agriculture and clinical settings. Furthermore, we found that the reaction system, including the dosage of enzyme and reaction time, could not be standardized, resulting in various sizes of gained RNPs. Taken together, our experimental evidence indicated that shRNAs are likely the optimal carriers associated with high efficiency and ecofriendliness; this should also be applied to other plant species.

4.2. Chlorophyll a/b binding proteins are essential for the growth and development of M. micrantha

M. micrantha is an invasive plant that is harmful to the ecosystem. Therefore, there is an urgent need to develop effective biocontrol methods to prevent the rapid growth and spread of *M. micrantha*. The genome of *M. micrantha* has already been sequenced and assembled, but functional studies of genes essential for its growth and development are still lacking. This is the first study to investigate the biological functions of *M. micrantha* genes.

In our study, we selected the genes encoding chlorophyll a/b-binding proteins as targets for the following reasons. First, the expression levels of chlorophyll a/b-binding proteins were much higher than those of other proteins in the *M. micrantha* transcriptome, especially in leaves and stems. Second, the genes encoding chlorophyll a/b-binding proteins are conserved among plant species, and their functions have been well studied. Two light-harvesting complexes (LHCs), LHCI and LHCII, are present in plants and are involved in light energy capture, energy transfer, and stress response. Chlorophyll a/b-binding proteins are membrane proteins in plant photosystems that belong to LHCII. By binding to 12 types of chlorophyll molecules, chlorophyll a/b-binding proteins are essential pigment binding proteins in PSII that are central to photosynthesis, which is essential during plant growth and development. Therefore, regulation of the expression of chlorophyll a/b-binding proteins could control the growth and eventual lead to the death of plants.

According to our results, all three types of synthesized RNAi molecules successfully knocked down the expression of chlorophyll a/b-binding proteins. The leaves turned yellow and eventually wilted, further confirming that chlorophyll a/b-binding proteins play vital roles in leaf development and the entire growth and development process of plants. Five genes from the chlorophyll a/b-binding protein gene family were selected as target genes, namely gene0004558, gene0004560, gene00026534, gene00029128, and gene00025307. We observed significant phenotypic changes of browning and wilting of leaves after the experimental period of application, as well as a trend of lower relative expression of targeted genes in the treatment groups, indicating that the wilting phenotypes were correlated with the silencing of target genes. Although it might be relatively tricky for RNAi bioherbicides to cause the mortality of *M. micrantha* as quickly as chemical herbicide (18% 2,4-D) [27], there remains potential for increasing the speed-to-effect via the utilization of coformulants (e.g., detergent) that might improve the shRNA-uptake by plant cells and its silencing efficiency [28–30].

4.3. Exogenous RNAi molecules might trigger the defense mechanism in plants

We assumed that the expression of endogenous genes would be kept downregulated as long as the RNAi molecules were functional in plant cells. Our qRT-PCR results showed that the expression of target genes was significantly increased on day 1 after treatment with the shRNA mixture; however, their expression level began to decrease on day 2. Similar results were also found in the leaves treated with dsRNAs and RNPs. We speculated that this might be related to endogenous defense mechanisms in plants. Due to the lack of research on the development of SIGS-based RNAi herbicides, few presumptions have been proposed on the rebound of the expression of target genes in plants. Plants can actively degrade exogenous RNAi molecules or impede the formation of the RISC assembly. Importantly, the antiviral defense mechanisms in plants have been widely studied. The antiviral defense mechanism, in which RNAi is essential for plant antiviral resistance [31], refers to the specific recognition and degradation of RNA sequences by plant cells against foreign invaders, such as viruses. Three types of proteins are mainly involved in the mechanism, including Dicer-like enzyme, RNA-dependent RNA polymerase, and AGO enzyme [32].

Moreover, the proteins involved in the antiviral mechanism widely exist in diverse plant species [33,34], suggesting that the mechanism is conserved in plants. Hence, we speculated that shRNAs triggered the defense mechanism in *M. micrantha*, leading to decreased gene silencing efficiency and an increase in the levels of expression of target genes. However, as the increasing number of RNAi molecules was absorbed into host cells through continuous application, the expression of target genes was ultimately suppressed.

5. Conclusion

To date, ecologically sustainable control methods are urgently required for ecological stability under the fierce attack of the invasive alien plant *M. micrantha*. Using high-throughput sequencing of *M. micrantha* transcriptome and bioinformatic analyses of sequence specificity, the highly expressed genes encoding chlorophyll a/b-binding proteins, namely gene0004558, gene0004560, gene00025307, gene00026534, and gene00029128, were selected as targets for RNAi molecules. Observation of morphological defects in *M. micrantha* leaves and reduced levels of expression of target genes indicated that all three types of RNAi molecules successfully and precisely silenced the endogenous target genes. Moreover, shRNAs were found to be the optimal carrier of the RNAi molecule and could be used as an RNAi-based herbicide, possessing the advantages of high silencing efficiency, feeblish immune response, and low cost compared with RNPs and dsRNAs. Several points should be considered in future studies. First, we need to estimate the potential for increasing the silencing efficiency of the bioherbicide against the invasive plant *M. micrantha* via its use in a mixture with pesticide adjuvants to enhance the infiltrability of shRNAs to the host plant. Second, it is necessary to determine the duration of exogenously spraying shRNAs on the leaves and stems of *M. micrantha*. Third, a further demonstration of the most suitable dosage and period of application of the bioherbicide is needed. Finally, further exploration via field experiments would be needed to verify the overall feasibility of using the shRNA-based spray herbicide to curb the reckless invasion of alien species.

CRediT authorship contribution statement

Jiantao Mai: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Lingling Liao:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision, Project administration. **Rongsong Ling:** Methodology, Software, Formal analysis, Data curation, Visualization. **Xiaolong Guo:** Validation, Investigation, Formal analysis. **Jingying Lin:** Investigation. **Beixin Mo:** Conceptualization, Funding acquisition. **Weizhao Chen:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Yu Yu:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors have no known competing interests to declare.

Acknowledgments

This research was financially supported by grants from the Shenzhen Science and Technology Program, China (Grant No.

JCYJ20190808115005598), National Natural Science Foundation of China (Grant No. 31801078), Guangdong Innovation Research Team Fun (Grant No. 2014ZT05S078), Natural Science Foundation of SZU (Grant. No. 2019080), and the Undergraduate Academic Competition Project of Shenzhen University (Grant No. 803-0000290846).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.11.005.

References

[1] Shao H, Peng S, Wei X, Zhang D, Zhang C. Potential allelochemicals from an invasive weed Mikania micrantha H.B.K. J Chem Ecol 2005;31:1657–68. https://doi.org/10.1007/s10886-005-5805-0.
[2] Zhang LY, Ye WH, Cao HL, Feng HL. Mikania micrantha H. B. K. in China – an overview. Weed Res 2004;44:42–9. https://doi.org/10.1111/j.1365-3180.2003.00371.x.
[3] Liu B, Yan J, Li W, et al. Mikania micrantha genome provides insights into the molecular mechanism of rapid growth. Nat Commun 2020;11:340. https://doi.org/10.1038/s41467-019-13926-4.
[4] Cock MJW. Potential biological control agents for Mikania micrantha HBK from the Neotropical region. Trop Pest Manag 1982;28:242–54. https://doi.org/10.1080/09670878209370717.
[5] Zhijie L, Jianghua H. Research progress on the control of *M. micrantha*. J Zhongkai Univ Agric Technol 2018;31:66–71. https://doi.org/10.3969/j.issn.1674-5663.2018.01.012.
[6] Dongguang H, Xianye Z, Qijie Z, Yongjun W, Liao Wen-bo. The chemical control of M. micrantha in the country park of Hong Kong. J South China Normal Univ (Soc Sci Ed) 2007;109–14:131. https://doi.org/10.3969/j.issn.1000-5463.2007.03.019.
[7] Qijie Z, Bosun W, Wenbo L, Yongjun W, Qiying L. Effectiveness of four herbicides on the harmful weeds *M. micrantha*. Ecol Sci 2001;20:32–6. https://doi.org/10.3969/j.issn.1008-8873.2001.01.006.
[8] Gaofeng X, Ying Y, Shicai S, et al. Evaluation of the controlling methods on inhibiting the secondary invasion of *M. micrantha* H.B.K. Ecol Environ Sci 2017;26:911–8. https://doi.org/10.16258/j.cnki.1674-5906.2017.06.001.
[9] Zhiyuan W, Nan M, Xianjiao T. Development and utilization prospects of invasive weed *M. micrantha*. Weed Sci 2013;31:8–11. https://doi.org/10.3969/j.issn.1003-935X.2013.03.002.
[10] Rosa C, Kuo YW, Wuriyanghan H, Falk BW. RNA interference mechanisms and applications in plant pathology. Annu Rev Phytopathol 2018;56:581–610. https://doi.org/10.1146/annurev-phyto-080417-050044.
[11] Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002;296:550–3. https://doi.org/10.1126/science.1068999.
[12] Sijen T, Fleenor J, Simmer F, et al. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 2001;107:465–76. https://doi.org/10.1016/s0092-8674(01)00576-1.
[13] Tabara H, Grishok A, Mello CC. RNAi in *C. elegans*: soaking in the genome sequence. Science 1998;282:430–1. https://doi.org/10.1126/science.282.5388.430.
[14] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–11. https://doi.org/10.1038/35888.
[15] Koch A, Kumar N, Weber L, Keller H, Imani J, Kogel KH. Host-induced gene silencing of cytochrome P450 lanosterol C14 α-demethylase-encoding genes confers strong resistance to Fusarium species. Proc Natl Acad Sci USA 2013;110:19324–9. https://doi.org/10.1073/pnas.1306373110.
[16] Nunes CC, Dean RA. Host-induced gene silencing: a tool for understanding fungal host interaction and for developing novel disease control strategies. Mol Plant Pathol 2012;13:519–29. https://doi.org/10.1111/j.1364-3703.2011.00766.x.
[17] Koch A, Biedenkopf D, Furch A, et al. An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. PLoS Pathog 2016;12:e1005901. https://doi.org/10.1371/journal.ppat.1005901.
[18] Wang M, Jin H. Spray-induced gene silencing: a powerful innovative strategy for crop protection. Trends Microbiol 2017;25:4–6. https://doi.org/10.1016/j.tim.2016.11.011.
[19] Voinnet O, Baulcombe DC. Systemic signalling in gene silencing. Nature 1997;389:553. https://doi.org/10.1038/39215.
[20] Lee JB, Hong J, Bonner DK, Poon Z, Hammond PT. Self-assembled RNA interference microsponges for efficient siRNA delivery. Nat Mater 2012;11:316–22. https://doi.org/10.1038/nmat3253.
[21] Xu DH, Huang C, Liu LY, Song TS. New progress of the highly efficient siRNA design. Hereditas (Beijing) 2006;28:1457–61. https://doi.org/10.1360/yc-006-1457.
[22] Hui C. Study on RNAi regulation and siRNA delivery based on aptamer. Masters thesis. Shanghai University; 2017. CNKI.CDMD.2.1018.701792.
[23] Jeon H, Han S, Kim H, Lee JB. Surface modification of RNA nanoparticles by ionic interaction for efficient cellular uptake. J Ind Eng Chem 2019;70:87–93. https://doi.org/10.1016/j.jiec.2018.10.013.
[24] Ishibashi K, Saruta M, Shimizu T, et al. Soybean antiviral immunity conferred by dsRNase targets the viral replication complex. Nat Commun 2019;10:4033. https://doi.org/10.1038/s41467-019-12052-5.
[25] Rao DD, Vorhies JS, Senzer N, Nemunaitis J. siRNA vs. shRNA: similarities and differences. Adv Drug Deliv Rev 2009;61:746–59. https://doi.org/10.1016/j.addr.2009.04.004.
[26] McAnuff MA, Rettig GR, Rice KG. Potency of siRNA versus shRNA mediated knockdown in vivo. J Pharm Sci 2007;96:2922–30. https://doi.org/10.1002/jps.20968.
[27] Xuping L, Jianfeng L, Ying H, Qizhao C. Safety and control effect of herbicide Mieweijing against Mikania micrantha. For Pest Dis 2009;28:15. https://doi.org/10.3969/j.issn.1671-0886.2009.01.011.
[28] Oftedal L, Myhren L, Jokela J, et al. The lipopeptide toxins anabaenolysin A and B target biological membranes in a cholesterol-dependent manner. Biochim Biophys Acta 2012;1818:3000–9. https://doi.org/10.1016/j.bbamem.2012.07.015.
[29] Mattei B, Lira RB, Perez KR, Riske KA. Membrane permeabilization induced by Triton X-100: the role of membrane phase state and edge tension. Chem Phys Lipids 2017;202:28–37. https://doi.org/10.1016/j.chemphyslip.2016.11.009.
[30] Shaohong F, Yingze N, Hongquan Y, Xianya W. Effects of surfactant Silwet-77 on the floral-dip transformation of *B. napus*. Mol Plant Breed 2004;2:661–6. https://doi.org/10.3969/j.issn.1672-416X.2004.05.010.
[31] Niehl A, Heinlein M. Perception of double-stranded RNA in plant antiviral immunity. Mol Plant Pathol 2019;20:1203–10. https://doi.org/10.1111/mpp.12798.
[32] Silhavy D, Burgyán J. Effects and side-effects of viral RNA silencing suppressors on short RNAs. Trends Plant Sci 2004;9:76–83. https://doi.org/10.1016/j.tplants.2003.12.010.
[33] Rodríguez-Leal D, Castillo-Cobián A, Rodríguez-Arévalo I, Vielle-Calzada JP. A primary sequence analysis of the argonaute protein family in plants. Front Plant Sci 2016;7:1347. https://doi.org/10.3389/fpls.2016.01347.
[34] Qin L, Mo N, Muhammad T, Liang Y. Genome-wide analysis of DCL, AGO, and RDR gene families in pepper (*Capsicum annuum* L.). Int J Mol Sci 2018;19. https://doi.org/10.3390/ijms19041038.