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Preparation of ShRNA-LDH nanoparticle and its application in biological control of *Mikania micrantha*

MO Yilin^{1,2}, CHEN Weizhao¹, HUANG Lijuan¹, WU Feiyan^{1,2}, XIAO Nian^{1,2},
YU Yu¹, and LIU Xuedong¹

1) College of Life Sciences and Oceanography, Guangdong Provincial Key Laboratory for Plant Epigenetics, Shenzhen University, Shenzhen 518071, Guangdong Province, P. R. China

2) Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley 94720, USA

Abstract: Ribonucleic acid interference (RNAi) is a highly efficient, safe, and environmentally friendly method in plant protection. Exogenous spraying of short hairpin RNAs (shRNAs) targeting light-harvesting chlorophyll a or chlorophyll b binding protein genes onto the leaves of *Mikania micrantha* (*M. micrantha*) H. B. K. can efficiently silence the expression of these genes. To improve the effectiveness of shRNA-mediated control of *M. micrantha*, layered double hydroxide (LDH) clay nanosheets with a diameter between 10 to 100 nm and good biocompatibility and environmental friendliness were prepared as shRNA carriers in this study. We found that the optimal binding mass ratio of LDH to shRNA was 1:10. ShRNA spraying assays showed that *M. micrantha* leaves with micro-wound were more sensitive to shRNA, and spraying LDH directly to *M. micrantha* had no effect on plant growth. To compare the effects of LDH-shRNA and shRNA alone on the control of *M. micrantha*, we sprayed LDH loaded with six different shRNA respectively or shRNA alone onto *M. micrantha* leaves, and a more severe necrotic phenotype was observed on the 6th day after LDH-shRNA-sprayed. Taken together, our study successfully developed an LDH-based shRNA carrying system to significantly improve the effectiveness of RNAi in the control of *M. micrantha*.

Key words: RNA interference; plant protection; *Mikania micrantha*; light harvesting chlorophyll a/b binding protein; clay nanosheets; short hairpin RNA

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ShRNA-LDH 纳米颗粒的制备及薇甘菊生物防治

莫亦琳^{1,2}, 陈伟钊¹, 黄丽娟¹, 吴飞燕^{1,2}, 肖念^{1,2}, 于宇¹, 刘学东¹

1) 深圳大学生命与海洋科学学院, 广东省植物表观遗传学重点实验室, 广东深圳 518071; 2) 加州大学伯克利分校分子与细胞生物学系, 美国伯克利 94720

摘 要: 核糖核酸干扰(ribonucleic acid interference, RNAi)技术在植物保护中具有高效、安全和环保的特点. 外源喷施短发夹RNA(short hairpin RNA, shRNA)可以有效沉默薇甘菊(*Mikania micrantha*)叶绿素a/叶绿素b结合蛋白相关基因. 为提高喷施shRNA对薇甘菊的防治效果, 制备直径为10~100 nm的层状双氢氧化物(layered double hydroxide, LDH)黏土纳米片为shRNA的载体, 该载体具有良好的生物相容性, 具环境友好. 研究发现, LDH结合shRNA的最佳质量比为1:10. shRNA喷施实验表明, 有轻微创伤的薇甘菊叶

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Corresponding author: Postdoctoral researcher LIU Xuedong. E-mail: xuedong007@126.com

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片对 shRNA 更敏感, 并且 LDH 直接喷施对薇甘菊的生长几乎没有任何影响. 为比较 LDH-shRNA 和单独的 shRNA 对薇甘菊的控制效果, 利用分别搭载了 6 个 shRNA 的 LDH 纳米颗粒或单独的 shRNA 喷施薇甘菊叶片, 在喷施 6 d 后观察到叶片喷施 LDH-shRNA 有更加明显的坏死表型. 研发的基于 LDH 的 shRNA 携带系统可显著提高 RNAi 在防控薇甘菊中的有效性.

关键词: 核糖核酸干扰; 植物保护; 薇甘菊; 叶绿素 a/b 结合蛋白; 黏土纳米片; 短发夹核糖核酸

Mikania micrantha (*M. micrantha*) H. B. K. is a perennial vine belonging to Asteraceae family, native to Central and South America, which is listed in the top 100 most harmful invasive species in the world by the International Union for Conservation of Nature (IUCN)^[1]. In China, it was first found at Hong Kong in 1884, then it was introduced to Shenzhen, Guangdong in 1984, and spread throughout the south China, causing great damage to forests, crops, and orchards in Shenzhen, Dongguan, Zhuhai, Huizhou, Zhongshan and other Pearl River Delta Areas^[2]. The growth rate of *M. micrantha* is extremely fast, and it possesses the ability to reproduce sexually and asexually. It can quickly colonize the invaded habitats, and hinder native species growth through competing for nutritional resources. Moreover, it can cover a large portion of the invaded habitats rapidly, and preventing other plants from performing photosynthesis and leading to their death^[3]. According to the investigation on invasion of *M. micrantha* in Neilingding Island, Shenzhen, its coverage area has reached 60%, and the annual loss to forest ecology and biodiversity has reached 4.5 to 10.1 million RMB^[4]. In addition, the invasion of *M. micrantha* led to the withering of mangroves in Shenzhen Bay, resulting in the loss of the mangrove habitat area and greatly weakening the carbon storage of mangroves^[5]. Therefore, there is an urgent need to prevent and control the rapid growth and spread of *M. micrantha*. The present methods of controlling *M. micrantha* include invasive plant monitoring, manual or mechanical eradication, chemical herbicide application, natural enemy adaptation, plant community reconstruction, and other comprehensive ecological strategies^[6]. Although these traditional methods can control the spread of *M. micrantha* to a certain degree, they still possess disadvantages such as high cost, low efficiency, and serious environmental hazards. Therefore, it is necessary to develop a low-cost, efficient, and environmentally friendly method to prevent and control the spread of *M. micrantha*.

Ribonucleic acid interference (RNAi), also called post-transcriptional gene silencing, is a highly conservative gene regulation mechanism in eukaryotes, which plays a vital role in the growth, development and responses to abiotic and biotic stresses of plants and animals^[7]. The current RNAi technology mainly uses double-stranded RNA (dsRNA) or short hairpin RNA (shRNA) to induce silencing of target genes. The mechanism of RNAi technology is to cleave foreign dsRNAs or shRNAs into 21 to 23 base pair (bp) small interfering RNA (siRNA) by Dicer-like protein. Then, the siRNAs are loaded into argonaute protein to generate an RNA-induced silencing complex (RISC), which binds to and cleaves the cognate mRNA, resulting in silencing of the target gene^[7]. At present, RNAi technology has been developed as a multi-functional, efficient, safe, and environmentally friendly plant protection approach, which has been successfully used for the protection of plants against plant viruses, bacteria, fungi, insects, and nematodes^[7]. However, the mechanism of dsRNA uptake by plant cells remains elusive, and the approaches of delivering dsRNA into plant cells, as well as increasing the stability of dsRNA, require further modification and improvement.

In recent years, layered double hydroxide (LDH), is also known as clay nanosheets, has been used as an efficient carrier for the delivery of anion or nucleic acid due to its outstanding physical properties, low cytotoxicity, and degradability^[8]. LDH nanosheets are composed of divalent metal hydroxides and trivalent metal hydroxides, with diameters ranging between 10 and 100 nm. Their general structural formula is $[M_{1-x}^{2+}M_x^{3+}(\text{OH})_2]^{x+} \cdot (A_{x/m}^{m-} \cdot n\text{H}_2\text{O})^{x-}$, where M^{2+} can be Mg^{2+} , M^{3+} can be Al^{3+} , and A^{m-} can be NO_3^- ^[9]. The existence of metal hydroxide layer enables LDH nanosheets to load negatively charged biomacromolecules, such as DNA, RNA, and protein. Studies have shown that LDH loaded with dsRNA or DNA can be directly sprayed to the surface of plant leaves, which protect plants from virus infection

for up to 20 d^[10-11]. In addition, LDH nanoparticles can also be used as nonviral vectors to effectively deliver dsRNA and other biomolecules into pollen cells, leading to endogenous gene silencing^[12].

In previous studies, we screened and identified a gene family encoding light harvesting chlorophyll a/b binding proteins through transcriptome sequencing, which play important roles in regulating the growth and development of *M. micrantha*. And spraying shRNA on its leaves could successfully silence the expression of these genes^[13]. In order to further improve the efficiency of exogenous shRNA in *M. micrantha* control, we used LDH to load shRNA. The LDH can protect shRNA from being degraded in the environment, and therefore to prolong the effective time of shRNA. Additionally, the LDH can also promote the absorption efficiency of plant cells to shRNA. Taken together, LDH-loaded shRNA has a more effective control of *M. micrantha*.

1 Materials and methods

1.1 Materials

M. micrantha seeds were collected in the field. The seedlings were grown in an artificial climate chamber maintained at 24 °C / 22 °C (day/night), 16 h/8 h (light/dark) photoperiod, with a 60% relative humidity. Seedlings from 6 to 8 weeks old were selected for shRNA treatment experiment.

Strains and plasmids: RNase III deficient *Escherichia coli* competent strains TH115 (DE3) were purchased from Aiji Biotechnology Company. The 6 pET28a shRNA plasmids were designed and constructed based on the sequences of the 5 light-harvesting chlorophyll a/b binding protein genes according to MAI et al^[13]. The gene number of 5 target genes, names and sequences of the accordingly designed shRNAs were listed in Table 1.

Finally, the DNA sequence of the designed shRNA was synthesized by Aiji Biosynthesis Company and constructed between the *Xho* I / *Xba* I sites of the pET28a.

1.2 In vitro shRNA induction

For *in vitro* shRNA induction, the clones carrying specific constructs were picked out in 10 mL LB liquid medium supplemented with kanamycin, the culture was grown at 37 °C, with shaking at 180 r/min overnight. Then, 1 mL of the cultured bacteria was added to 100 mL fresh LB liquid medium with the same antibiotic. The culture was incubated at 37 °C, with shaking at 180 r/min for 3 h to 4 h until $D(600) = 0.5$. Next, IPTG was added to a final concentration of 0.4 mmol/L, and the culture was incubated from another 3 h to 4 h to induce expression of the shRNA. Then, the bacteria were collected by centrifugation at 4 °C, 5×10^3 r/min for 10 min.

For shRNA extraction, the Trizol reagent was added at the ratio of 0.1 g/mL, samples were vortexed, and incubated for 5 min at room temperature. Then, 1/5 volume of trichloromethane was added, samples were vortexed, incubated for 3 min at room temperature, centrifuged at 4 °C, 12×10^3 r/min for 15 min. The supernatant was transferred to a new tube, an equal volume of precooled isopropanol was added. The mix was vortexed, and incubated at room temperature for 10 min, and then centrifuged at 4 °C, 12×10^3 r/min for 10 min to collect shRNA. The collected-shRNA was washed twice with 75% (volume fraction) ethanol, and left standing at room temperature for 3 to 5 min until the ethanol evaporated completely. Finally, an appropriate amount of DEPC water was added to dissolve the shRNA.

1.3 Preparation and characterization of LDH nanomaterials

$\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ and $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ were added to

Table 1 List of 6 shRNAs, their sequences and target genes

表1 六种 shRNA 及其序列和靶基因

target genes	shRNAs	shRNA sequence(5'→3')
Gene0004558	R1	UUGUAAGGGUUGUCUUGUUAAGACUUGCUUAACAAGACAACCCCUUACA
Gene0004558	R2	UUGGUUUGUAAGGGGUUGUCUUGACUUGCAAGACAACCCCUUACAAACCAA
Gene0025307	R3	UUUUUUUGCAUUCUCUGCCUGUGACUUGCACAGGCAGAGAAUGCAAAAAAA
Gene0026534	R4	UCUUA AUGUCUCCGGAUCAGAUACACUUGGAUCUGAUCCGGAGACAUUAAGA
Gene0028194	R5	UGUUA AUGGGAUCAGCCAAAUGGACUUGCCAUUUGGCUGAUCCCAUUAACA
Gene0029128	R6	UGCUAUCGGUUCGAUUGGAUCAGACUUGCUGAUCCAAUCCAACCGAUAGCA

methanol to final concentrations of 30 mmol and 10 mmol, respectively. Then, 2 mL of each solution was added slowly into 10 mL 16 mmol NaOH, the solution was stirred vigorously during the process. After stirring from 30 min to 40 min, the solution was centrifuged at 5×10^3 r/min for 10 min at room temperature. Discard the liquid, and using 40 mL methanol to resuspend the pellet. The suspension was transferred into the reaction kettle and reacted for 144 h at 100 °C. After the reaction is completed, the product was removed from the reaction kettle and cooled to room temperature. The product was centrifuged at 5×10^3 r/min for 10 min at room temperature to collect the pellet, and the pellet was then washed twice with deionized water, and dispersed with 40 mL deionized water. The aqueous suspension was shaken at room temperature at 180 r/min for 4 d to 6 d until the solution turns transparent. The final product obtained is an LDH suspension with a concentration of around 6 mg/mL to 7 mg/mL (50% yield).

1.4 Synthesis of shRNA loaded LDH (shRNA-LDH)

The shRNA (1 µg, Table 1) and LDH were mixed in a mass ratio ranging from 1:1 to 1:40, then the double distilled water (ddH₂O) was added up to 10 µL. The mix was then shaken at 350 r/min for 30 min at room temperature. The loading efficiency was detected by 10 g/L agarose gel electrophoresis.

1.5 Treatment of *M. micrantha* with shRNA and shRNA-LDH

Treatment of intact leaves with shRNA: the main vein in the middle of *M. micrantha* was regarded as a boundary to separate the leaves into the left and right half. 20 µg shRNA was sprayed on the right half of the leaves, and water was sprayed on the left half of the leaves as the negative control.

Treatment of micro-wounded leaves with shRNA: the leaves were separated as the above. A small amount of 400 mesh carborundum was applied all over the leave surfaces, followed by gently scrubbing the leaves with a cotton swab to generate scratches on the leaves. Then, 20 µg shRNA was sprayed on the right half of the leaves and the water were sprayed on the left half of the leaves as the negative control. Each experiment was performed with at least 6 leaves.

Treatment of intact leaves with LDH loaded shRNA: 100 µg of each shRNA was incubated with LDH

proportionally as described in section 1.4 respectively. Then, the LDH-shRNA solution was diluted to 10 mL with ddH₂O, and Silwet L-77 was added to the solution to a final concentration of 0.05% (v/v). Each LDH-shRNA solution was sprayed onto the leave surfaces of 6 seedlings as the experiment group. For the control, the same amount of LDH as in the LDH-shRNA samples was diluted to 10 mL with ddH₂O, and Silwet L-77, a surfactant that effectively moisturizes plant foliage, was added to a final concentration of 0.05% (volum fraction) before sprayed.

After shRNA treatment, the seedlings were continuously cultivated in an artificial climate chamber for symptom observation.

2 Results

2.1 *In vitro* induced transcription and LDH loading of shRNAs

Agarose gel electrophoresis results showed that the numbers of RNA bands appeared similar between bacteria with or without isopropyl β-D-thiogalactoside (IPTG) induction. This might because the size of 5S ribosomal RNA in *E. coli* is similar to the size of shRNA, therefore, causing the induced shRNA indistinguishable from the 5S ribosomal (Fig. 1(a)). However, the total content of RNAs from the bacteria with IPTG induction is significantly higher than that of the bacteria without IPTG induction. The higher RNA content indicates that shRNA was massively induced to transcribe.

The same amount of shRNA (1 µg) was incubated with different amount of LDH nanoparticles (from 1 µg to 40 µg) respectively, and the loading efficiency was detected by 10 g/L agarose gel electrophoresis. With the addition of LDH, more shRNAs were loaded onto LDH particle, less shRNAs enter the agarose gel, thus the bands of shRNA on the gel were weaker. Finally, the agarose gel electrophoresis result showed that 10 µg of LDH nanoparticles were sufficient to load 1 µg of shRNA. Therefore, the optimal mass ratio of shRNA to LDH is 1:10 (Fig. 1(b)).

2.2 Characterization of shRNA-LDH by transmission electron microscopy

Transmission electron microscopy (TEM) images showed that the prepared LDH nanoparticles were uniformly dispersed on the copper mesh, in a hexagonal

lamellar structure, with a diameter from 10 nm to 100 nm (Fig. 2(a)). Meanwhile, the LDH-shRNA particles stuck together, and a large amount of shRNAs were adhered to the sheet structure of LDH nanoparticles (Fig. 2(b)).

These observations suggest that the prepared LDH nanoparticles were well uniformly dispersed, and the positively charged LDH nanoparticles could successfully attract the negatively charged shRNA.

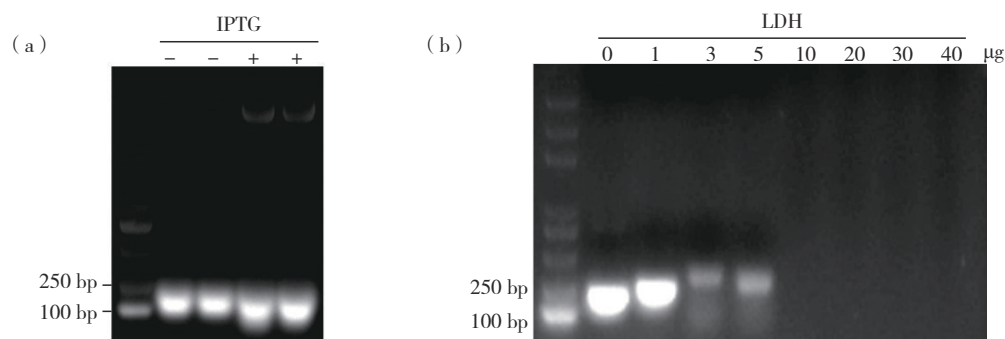


Fig. 1 (a) *In vitro* induction of shRNA transcription and (b) binding efficiencies of LDH to shRNA.

图1 (a) ShRNA体外诱导表达及(b)与LDH纳米颗粒结合效率

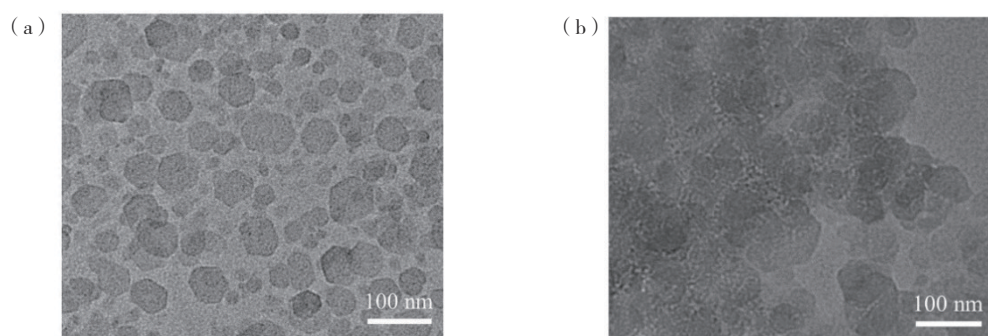


Fig. 2 Characterization of (a) LDH and (b) shRNA-LDH nanoparticles by TEM.

图2 (a) LDH和(b) shRNA-LDH纳米颗粒的透射电镜表征

2.3 Comparison of the effects of different treatment methods on the shRNA-based control of *M. micrantha*

In order to explore a more efficient method in shRNA-based control of *M. micrantha*, we sprayed shRNA with different approaches. The result showed that when sprayed above six different kinds of shRNAs directly on *M. micrantha* intact leaves, the symptoms on the leaf parts were all very mild, with no necrosis phenotype (Fig. 3(a)). However, if carborundum was used in advance to cause micro-wound on leaves, spraying the six kinds of shRNA could all result in different degrees of necrosis on the right half of the leaves, in which R2 caused the most severe necrosis symptom (Fig. 3(b)). The left half of the leaves (control) have no obvious phenotype, indicating that the micro-wound caused by scratches could not further develop into necrosis. The above results indicated that the micro-wound on the epidermal cells of *M. micrantha*

leaves can facilitate the entry of shRNA into the cells, and improve the efficiency of gene silencing.

2.4 Effects of shRNA-LDH on the control of *M. micrantha*

To identify the function of LDH-shRNA in the control of *M. micrantha*, the leaves were treated with LDH-shRNA, the LDH alone was used as a control. After spraying for 6 d, obvious necrosis symptoms can be seen on leaves sprayed with six different kinds of LDH-shRNA especially in LDH-R1 and LDH-R2 treatment leaves (Fig. 4(b)), and the necrosis symptoms on LDH-shRNA-sprayed leaves were much more serious than that in shRNA-sprayed leaves (Fig. 3(b) and Fig. 4(b)). Moreover, the leaves treated with LDH alone showed no significant changes (Fig. 4(a)), indicating that the severe necrosis symptoms were not caused by LDH. After 21 d, the treated leaves still showed severe necrosis symptoms. However, no systemic necrosis was observed in the upper

leaves (Fig. 4(c)). These results showed that LDH-shRNA leaves, but could not spread systematically. can generate strong necrosis symptoms on the treated

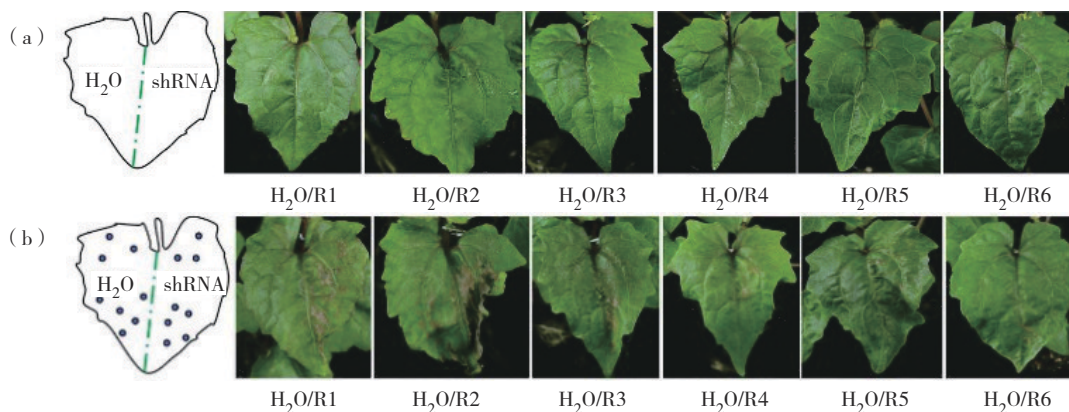


Fig. 3 Phenotypes of *M. micrantha* leaves at 6 days after direct spraying of shRNA with (a) intact leaves and (b) micro-wounded leaves.

图3 直接喷施 shRNA 6 d 后的 (a) 薇甘菊幼苗的完整叶片和 (b) 微伤叶片

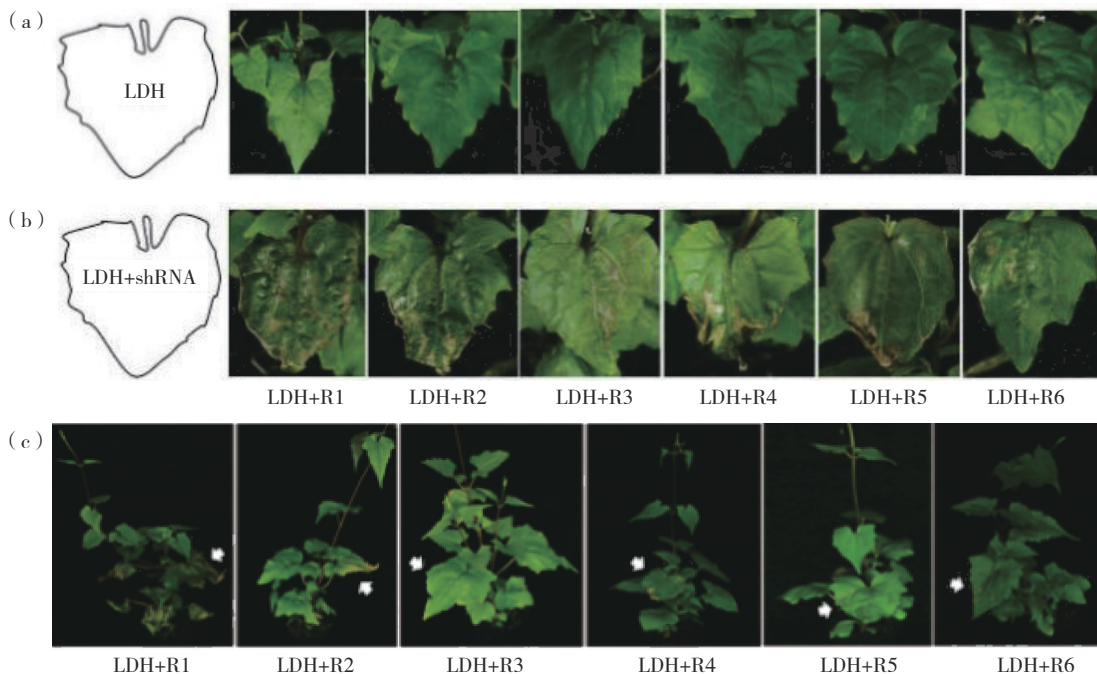


Fig. 4 Phenotypes of *M. micrantha* after spraying different LDH-shRNA. (a) Leaves of *M. micrantha* seedlings at 6 d after LDH treatment. (b) Leaves of *M. micrantha* seedlings at 6 d after LDH-shRNA treatment. (c) Leaves of *M. micrantha* seedlings at 21 d after LDH-shRNA treatment.

图4 薇甘菊喷施不同 LDH-shRNA 的表型 (a) 喷施 LDH 6 d 后; (b) 喷施 LDH-shRNA 6 d 后; (c) 喷施 LDH-shRNA 21 d 后

3 Discussion

M. micrantha is one of the worst invasive species, and has been called 'one mile a minute' weeds because of its vigorous, fast, and rampant growth characteristics, which were strongly related to its strong photosynthetic capability^[14]. The sequencing results of the *M. micrantha*

genome showed that it has a sedum acid metabolic pathway, which is not available in normal C3 plants^[1]. The sedum acid metabolic pathway can enable *M. micrantha* to absorb CO₂ during the night, and store in vacuoles in the form of organic acids, which can supplement the carbon fixation capacity in the day, and leading to a higher photosynthetic rate and achieve rapid growth^[1]. In addition, by regulating the biosynthesis of

chlorophyll a and chlorophyll b, the physiological responses and photosynthate distribution of *M. micrantha* can be regulated to adapt to different growth environments in a short time^[15]. Therefore, it is difficult to control *M. micrantha* effectively due to its rapid growth rate and excellent adaptability to the environment^[16].

The use of chemical herbicides and pesticides has caused great damage to the environment. Therefore, the rapid development of RNAi technology made it possible to become an effective substitute for traditional chemical herbicides and pesticides^[17]. Because genetically modified crops producing dsRNA to induce RNAi molecules mechanism are not yet accepted by the public, therefore, the rise and application potential of non-transgenic exogenous RNA spray attracted wide attention throughout the world^[18]. However, plant cells have very tough cellulose rich cell walls, with the thickness ranging from 0.1 μm to a few microns, which constitutes a natural physical barrier for the transfer of RNAi molecules inside the cells^[19]. Nanoparticles have the ability to penetrate the cell wall independently^[20]. Therefore, using nanoparticles to carry RNAi molecules can not only prevent RNAi molecules from being degraded by RNAase in the environment, but also help RNAi molecules pass through the plant cell walls and enter plant cells^[21]. Compared with spraying RNAi molecules alone, it can significantly improve the gene silencing effect^[22].

According to the previous transcriptome data of different organs and tissues of *M. micrantha*, our laboratory found that the chlorophyll a or chlorophyll b binding protein gene family was significantly accumulated in leaves of *M. micrantha*^[13]. By designing three kinds of RNAi molecules (dsRNA, RNAi nanosphere and shRNA), and using spray-induced gene silencing (SIGS) technology, we proved that RNAi technology has certain effects on *M. Micrantha* control^[13]. In this study, we found that compared with direct spraying of shRNA, LDH can improve the delivery efficiency of shRNA, maintain its stability, and prolong action time. For example, directly sprayed R2 had the strongest control effect, but when combined with LDH, LDH-R1 can also produce similar control effect with LDH-R2. These indicate that LDH could significantly improve the efficiency of RNAi technology on *M. micrantha* control.

However, due to the rapid growth rate of *M.*

micrantha, there is no obvious systemic gene silencing phenotype in the newly grown upper leaves. Future research will focus on silencing the genes related to growth and systemic movement using LDH-shRNA-based approaches, in order to explore effective ways to block the rapid growth of *M. micrantha* and achieve the goal of controlling *M. micrantha*.

Conclusion

LDH nanoparticles with a diameter of 10 nm to 100 nm were successfully produced. The shRNA can be successfully loaded onto the LDH nanoparticles, and the best loading efficiency can be achieved when the mass ratio of shRNA to LDH nanoparticles reached 1:10, in which shRNA could be completely loaded onto LDH nanoparticles. Compared with shRNA spraying, LDH-shRNA spraying has a better effect on *M. micrantha* control. We found that pre-micro-wounded leaves could facilitate the RNAi molecule delivering efficiency, and therefore improve the effect of *M. micrantha* control. Taken together, the developed LDH-based RNAi molecular loading approach in this study provides a highly efficient RNAi-based technology for controlling invasive weeds.

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作者简介: 莫亦琳(2002—), 深圳大学和加州大学伯克利分校联合培养本科生. 研究方向: shRNA在生物防治中的应用. E-mail: Lindsay_m@berkeley.edu

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