Insect science

Insect Science (2024) 0, 1–10, DOI 10.1111/1744-7917.13415

ORIGINAL ARTICLE

miRNA-mediated insect-resistant transgenic rice poses no risk to a non-target parasitoid, *Cotesia chilonis*, via direct feeding or through its target host

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Abstract MicroRNAs (miRNAs) have started to play an important role in pest control, and novel miRNA-based transgenic insect-resistant plants are now emerging. However, an environmental risk assessment of these novel transgenic plants expressing insect miRNAs must be undertaken before promoting their application. Here, transgenic *miR-14* rice, which has high resistance to the rice stem borer *Chilo suppressalis*, was used as an example for evaluation in this study. Taking the tier 1 risk assessment method in *Bacillus thuringiensis* (Bt) crops as a reference, the effects of the direct exposure of a non-target parasitoid, *Cotesia chilonis*, to a high concentration of miRNA were evaluated. The results showed that direct feeding with *miR-14* at high concentration had no significant effects on the biological parameters of *Co. chilonis*, whereas when *miR-14* was injected into *Ch. suppressalis*-parasitized larvae, the development duration of *Co. chilonis* was significantly affected. In combination with the real conditions of the rice paddy field, it could be inferred that transgenic *miR-14* rice has no significant negative effects on the important non-target parasitoid, *Co. chilonis*. These results will provide a foundation for the establishment of a new safety evaluation system for novel RNAi-based transgenic plants.

Key words *Chilo suppressalis*; *Cotesia chilonis*; *miR-14*; non-target effects; rice

Introduction

MicroRNAs (miRNAs) are important small non-coding RNAs widely found in plants and animals, and their main roles are to regulate the expression of endogenous genes and to defend the genome from invasive nucleic acids (Carthew & Sontheimer, 2009). As a result of intensive research on non-coding RNAs, miRNAs have started to play an important role in pest control (Razna & Cagan, 2019; Zhang *et al.*, 2021; Chi *et al.*, 2023). It has been demonstrated that feeding the cotton bollworm, *Helicoverpa armigera*, with artificial miRNA that targets the *ecdysone receptor* (*ecr*) gene results in significantly higher mortality and developmental defects (Yogindran & Rajam, 2016). Injection with *miR-311-3p* leads to the reduced egg deposition and progeny viability of *Bactrocera dorsalis* (Zhang *et al.*, 2023). Meanwhile, when insect miRNAs were transferred into plants, novel miRNA-based transgenic insect-resistant plants emerged (Faisal *et al.*, 2021; Mann *et al.*, 2023). Two transgenic tobacco lines carrying *miR-24* or *miR164b*, respectively, resulted in larval mortality or adult deformity in *H. armigera* (Agrawal *et al.*, 2015; Saini *et al.*, 2018). It has also been shown that the expression of cotton bollworm miRNA precursors (pre-miRNAs) in tobacco could

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result in a significant increase in cotton bollworm mortality (Bally *et al.*, 2020). The larval growth, pupation duration, pupal weight and eclosion rate of *Chilo suppressalis*, a destructive insect pest of rice, were significantly inhibited by feeding with transgenic rice expressing its endogenous miRNA (*Csu-novel-miR53*) (Liu *et al.*, 2022). Similarly, the exogenous expression of *miR-14*, *miR-15* or *Csu-novel-260* in rice all led to high resistance to *Ch. suppressalis* (Jiang *et al.*, 2017; He *et al.*, 2018; Zheng *et al.*, 2021).

Now that the success of transgenic *Bacillus thuringiensis* (Bt) insect-resistant plants has been tempered by the sporadic emergence of resistance in the pests targeted, the emergence of the RNAi-based approach could provide an alternative to Bt-toxin technology (Agrawal *et al.*, 2015). However, the environmental risk assessment of these novel transgenic plants expressing insect miRNAs must not be neglected before promoting their application. Until now, few studies have focused on safety evaluations. When *Apis mellifera* adults ingested pollen from the new *Ch. suppressalis* resistance rice plants carrying *Csu-novel-260*, their survival rate was not affected significantly compared with the controls. Although the 3′ untranslated region of the *disembodied* gene, which was the target of *Csu-novel-260* in *Ch. suppressalis*, shared 58.06% nucleotide sequence similarity between *Ch. suppressalis* and *A. mellifera*, no potential target site of *Csu-novel-260* was detected in *A. mellifera*. This indicated that the new miRNA-treated rice has no risk to the non-target insect *A. mellifera* (Chen *et al.*, 2021). Recently, another study showed that neither *Csu-novel-260* nor *Csu-miR-14*, added into the miRNA toxicity diet, had negative effects on the non-target arthropod *Folsomia candida* (Zhou *et al.*, 2023). However, the pupation of *Cnaphalocrocis medinalis*, another important rice pest, was inhibited by the transgenic *Csu-novel-260* rice plants (Wen *et al.*, 2021). This indicates a broader insecticidal spectrum for this new transgenic rice, but on the other hand the non-target effects of miRNAs must be taken into consideration.

Thus, the transgenic *miR-14* rice, which has high resistance to the rice stem borer *Ch. suppressalis*, was used as the evaluation event in this study, and *Cotesia chilonis*, the dominant parasitoid wasp of the rice stem borer, was used as the non-target organism. First, a high concentration of *miR-14*, which was 100 times higher than the level found in the transgenic rice, was directly fed to adults of *Co. chilonis*. As the eggs and larvae of the wasp develop in the larval body of *Ch. suppressalis*, *miR-14* was also injected into parasitized stem borer larvae. Using these two approaches, the effects of *miR-14* on the non-target parasitoid were assessed across the complete life cycle, from egg to adult. These results will provide a foundation for the establishment of a new safety evaluation system for novel RNAi-based transgenic plants.

Materials and methods

Insects and rice plant

The rice stem borer *Ch. suppressalis* and its parasitoid *Co. chilonis* were all collected from the experimental farm of Jinhua Academy of Agricultural Sciences, Zhejiang Province, China. The colony of *Ch. suppressalis* was reared on an artificial diet in the laboratory (Han *et al.*, 2012). The rearing conditions were 25 ± 1 °C, with a 16 h light : 8 h dark (16L : 8D) photoperiod and with a relative humidity of 75%–85%.

The transgenic *miR-14* rice seeds were developed by Prof. Fei Li from Institute of Insect Science, Zhejiang University. This transgenic rice line has been reported to show high resistance to the rice stem borer in the laboratory (He *et al.*, 2018). The non-transgenic parental control rice line used was Zhong Hua 11 (ZH11). The accelerating germination of rice seeds were conducted in a climate chamber under conditions of 28 ± 1 °C, with a 16L : 8D photoperiod and a relative humidity of 40%–60%. The rice seedings were then planted in a glasshouse held under the same conditions. All rice plants were cultured with nutrient solution, as reported by Yoshida *et al.* (1976).

Detection of miRNA content in transgenic miR-14 rice

Rice leaf, sheath and pollen at the flowering stage of transgenic *miR-14* rice plants, as well as harvested seed, were sampled to extract total RNA with the TRIzol method. miRNA was reverse-transcribed by the stem-loop method (miRNA 1st Strand cDNA Synthesis Kit; Nanjing Vazyme Biotech Co. Ltd, Nanjing, Jiangsu, China). The reaction system included 1 μg of total RNA, 2 μL of 5 × gDNA Wiper Mix and RNase-free water was added to make up the volume to 10 μL, which was then held for 2 min at 42 °C to remove genomic DNA. A previous reaction mixture at the last step was added into 1 μL of stem-loop primer (Table S1), 2 μL of 10 × reverse transcription mix and 2 μL of HiScript II Enzyme Mix. The reaction conditions were 25 °C for 5 min, 50 °C for 15 min and 85 °C for 5 min.

Absolute quantitative analysis was used to detect the miRNA content in rice tissues. A standard curve was

constructed with a plasmid clone of the target sequence from the polymerase chain reaction (PCR) product with the primers qmiR-14/URP (Table S1). The PCR product was cloned into pCE2-TA/Blunt-Zero vector (5 min TA/Blunt-Zero Cloning Kit; Nanjing Vazyme Biotech Co. Ltd) and then sent to Zhejiang Sunya Biotech Co. Ltd (Hangzhou, Zhejiang, China) for sequencing. Standard plasmid diluted in a gradient to 7 different concentrations was taken as the template for quantitative PCR (qPCR). The reagents used for qPCR were ChamQTM SYBR® qPCR Master Mix (Without ROX) (Nanjing Vazyme Biotech Co. Ltd). The qPCR reaction system contained 3 μL of cDNA, 1 μL of specifically designed *miR-14* primer, 1 μL of miRNA universal primer (UP), 12.5 μL of 2 × SYBR and 7.5 μL double distilled water (ddH_2O). Three replicates were performed for each concentration.

Direct feeding of miR-14 agomir of Ch. suppressalis to Co. chilonis adults

Newly emerged females of *Co. chilonis* were transferred individually into feeding tubes (2.5 cm in diameter, 9 cm in height). The wasps were fed with miR-14 agomir of *Ch. suppressalis* (Shanghai GenePharma Co. Ltd, Shanghai, China) dissolved in 10% sucrose water to a final miRNA concentration of 0.1 μg/μL. A non-insect (*Caenorhadits elegans*) miRNA mimic (*cel-mir-239b*), which has not been found in insects, was used as a negative control, and each treatment contained 50 replicates. The survival rate of wasps was recorded after feeding for 12 and 24 h. At the same time, after the survival rate observations 9 wasps were collected and randomly divided into 3 groups to detect the miRNA content by qPCR.

To determine whether the parasitic ability of the wasps was affected by *miR-14*, female wasps fed for 24 h were used to parasitize the 4th instar larvae of *Ch. suppressalis* individually (Teng *et al.*, 2016b). The parasitized stem borer was reared on an artificial diet, as described before. Then the development time and the emergence rate of the wasp offspring were recorded, as well as offspring number per parasitized larva. Ten replicates for each treatment were conducted, and the non-insect miRNA mimic was used as the negative control.

Injection of the miR-14 agomir into parasitized Ch. suppressalis larvae

Before the *miR-14* injection, *Ch. suppressalis* larvae at the 4th instar were exposed to a 1-d-old mated female wasp. Once a single oviposition was observed, the parasitization was confirmed and the parasitoid wasp was removed and the parasitized stem borer larvae were reared individually on an artificial diet. On the 2nd day after parasitism, *miR-14* agomir was injected into the stem borer abdomen every 3 d with an auto-nanoliter injector (Drummond Nanoject II™; Drummond Scientific Company, Broomall, PA, USA) (He *et al.*, 2018) until the parasitoid wasp offspring began to emerge from the stem borer. The quantity of *miR-14* agomir injected was 300 μg (2 μg/μL × 150 nL) each time. A non-insect miRNA mimic was used as the control, and there were 10 replicates for each treatment. The number of emerged wasps was recorded, as well as the development time from egg to adult duration. Nine newly emerged parasitoid wasp larvae were randomly divided into 3 groups and sampled for qPCR to detect the miRNA content.

The probable target gene of miR-14 detected in Co. chilonis wasps

The *miR-14* and its precursor sequences of *Co. chilonis* wasps were obtained by local blast from the genome and transcriptome data (Ye *et al.*, 2022). The RNAhybrid method was used to predict the target of *miR-14* in *Co. chilonis* (Krueger & Rehmsmeier, 2006). If the binding free energy of one target predicted by both methods was less than −18 kJ/mol, it was considered a possible target site for *miR-14*. Then the predicted target sites were selected for qPCR validation. The qPCR method used here was similar to that used in previous miRNA content detection experiments, except that reverse transcription cDNA was obtained by the One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransScript®; TransGen Biotech Co., Ltd, Beijing, China). The comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method was used to calculate the relative transcript levels of related genes (Bustin *et al.*, 2009).

Statistical analysis

Comparisons of the survival rates of the wasps were performed by chi-square test, whereas comparisons of other biological parameters, *miR-14* content and gene transcript levels were performed by Student's *t*-test. A one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used in the comparison of *miR-14* content in hemolymph of *Ch. suppressalis* at different times. For all comparisons, $\alpha = 0.05$. All statistical analyses were conducted with SPSS 19.0 (IBM, Armonk, NY, USA).

Table 1 *miR-14* contents in different plant tissues of transgenic *miR-14* rice.

Plant tissue	*miR-14* rice (μg/g total RNA)	ZH11 (control) (μg/g total RNA)
Leaf	0.79 ± 0.12	$(9.64 \pm 1.67) \times 10^{-6}$
Sheath	0.18 ± 0.04	$(5.59 \pm 1.26) \times 10^{-6}$
Pollen	0.06 ± 0.01	$(7.23 \pm 1.89) \times 10^{-6}$
Seed	0.11 ± 0.02	$(3.75 \pm 0.74) \times 10^{-6}$

Mean $\pm$ standard error, $n = 8$.

Results

miR-14 content in different tissues of transgenic miR-14 rice

The standard curve of the qPCR for *miR-14* was calculated as $y = -0.3457x + 11.965$ ($r^2 = 0.998$), where y is the logarithmic value of the plasmid copy number and x is the Ct value. This standard curve could also be used for the subsequent quantification of *miR-14* in *Ch. suppressalis* and *Co. chilonis*. Based on this, the *miR-14* content in different tissues of transgenic rice was calculated to range from 0.06 to 0.79 μg/g total RNA, whereas the *miR-14* content in the non-transgenic parental control ZH11 was extremely low and virtually undetectable (Table 1).

Direct feeding of Co. chilonis with miR-14 agomir of Ch. suppressalis

According to the miR-14 contents measured above, a high concentration of miR-14 (0.1 μg/μL) was used in the direct feeding of *Co. chilonis*. After feeding for 24 h, the *miR-14* content measured in the wasp body increased compared with that measured after the first 12 h of feeding (Fig. 1A). Given that the longevity of a female adult *Co. chilonis* was about 2−3 d (Teng *et al.*, 2016a), to avoid the effects of natural mortality, only the 24 h survival rate was recorded. This showed that no significant difference was detected between the group fed with *miR-14* and the control group after 12 or 24 h feeding time (12 h, $\chi^2 = 0.80$, $P = 0.371$; 24 h, $\chi^2 = 3.15$, $P = 0.076$; Fig. 1B). Moreover, when the wasps fed with *miR-14* were used to parasitize *Ch. suppressalis*, the developmental duration (egg-larval duration, from parasitizing to cocooning: $t = 1.22$, $P = 0.237$; pupal duration from cocooning to emergence: $t = 0.57$, $P = 0.578$; Fig. 1C, D), emergence rate ($t = 0.92$, $P = 0.371$; Fig. 1E) and fecundity ($t = 0.70$, $P = 0.490$; Fig. 1F) of their offspring did not significantly differ compared with the control group.

Table 2 *miR-14* content in hemolymph of *Chilo suppressalis* and its larval parasitoid *Cotesia chilonis* after *miR-14* was injected into parasitized *Ch. suppressalis* larvae.

Sample	*miR-14* content (μg/g total RNA)
Ch. suppressalis larvae not injected with *miR-14*	0.10 ± 0.05^{B}
Ch. suppressalis larvae injected with *miR-14* agomir, after 2 h	0.88 ± 0.13^{A}
Ch. suppressalis larvae injected with *miR-14* agomir, after 72 h	0.23 ± 0.13^{B}
Parasitoids from *Ch. suppressalis* larvae injected with *miR-14* agomir	5.67 ± 0.66
Parasitoids from *Ch. suppressalis* larvae injected with non-insect miRNA mimic	5.40 ± 0.36

Mean $\pm$ standard error, $n = 5$. One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used in the comparison of *miR-14* content in hemolymph of *Ch. suppressalis* at different times, and the same letter indicated no significance ($P > 0.05$). A Student's *t*-test was used in the comparison of *miR-14* content in the parasitoids from *Ch. suppressalis*.

Indirect effect of miR-14 agomir on the parastioid Co. chilonis via its host Ch. suppressalis

The *miR-14* concentration detected in hemolymph of *Ch. suppressalis* larva was significantly increased after injection with *miR-14* agomir (Table 2), and it remained at a relatively higher level after 72 h, despite some degradation. As for the parasitoids emerging from parasitized *Ch. suppressalis* larvae, a slight increase in *miR-14* content was detected in the *miR-4* injection group, compared with the control group.

When the parasitized stem borer larvae were injected with *miR-14*, the development duration from parasitizing to cocooning (approximately equal to the egg plus larval developmental time) of wasps in its hemolymph was significantly reduced ($t = 2.40$, $P = 0.031$; Fig. 2A), whereas the pupal duration increased significantly after the wasp larvae emerged from the stem borer ($t = 2.37$ d, $P = 0.036$; Fig. 2B). However, there were no significant

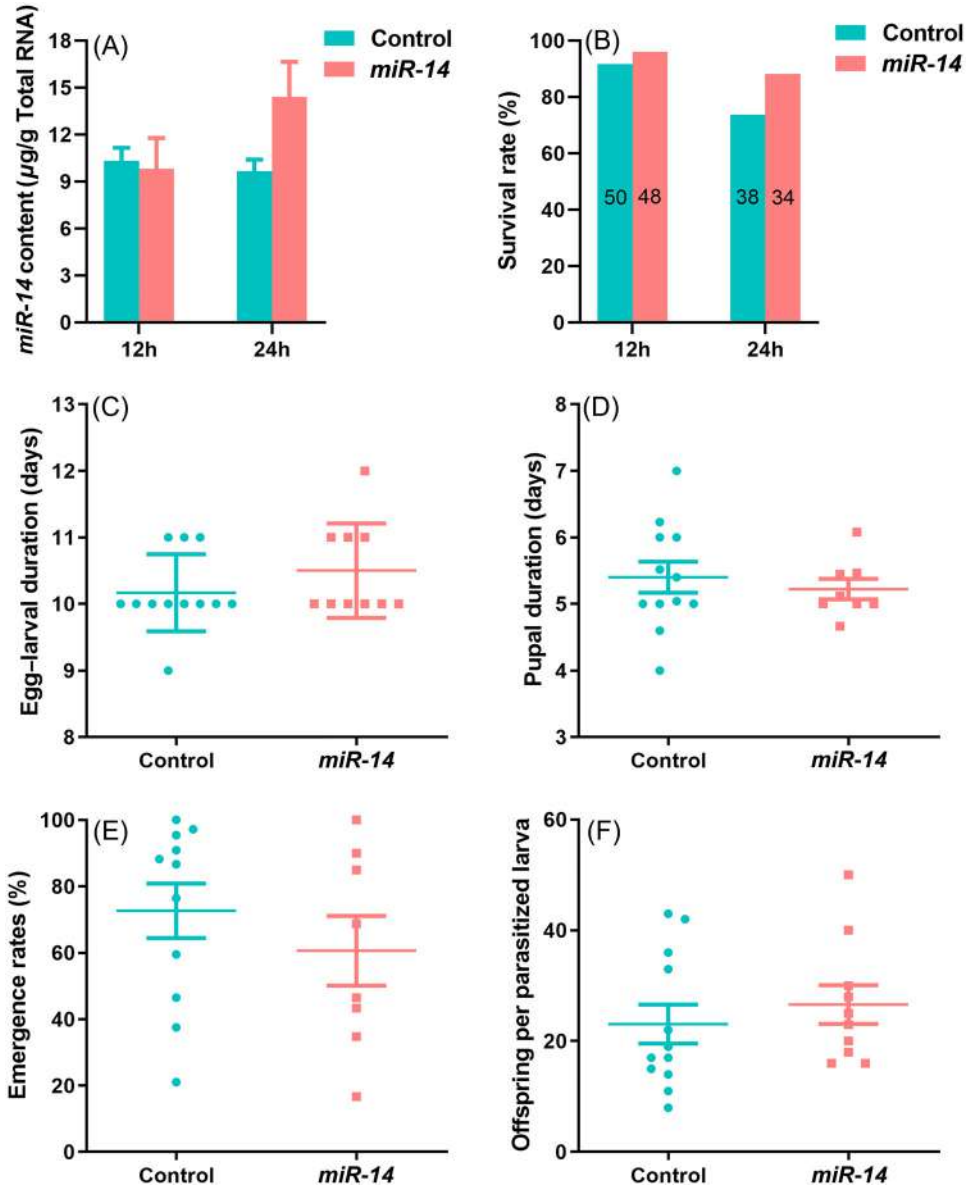

Fig. 1 The effects of direct feeding with *miR-14* on *Cotesia chilonis* adult survival and biological parameters of the offspring. (A) *miR-14* content in *Co. chilonis* after being fed with *miR-14*; error bars indicate the standard error, $n = 3$. A Student's *t*-test was used in the comparison between the group fed with *miR-14* and the control group; the absence of asterisks indicates that there was no significant difference in the comparison (and below). (B) The survival rate of *Co. chilonis* fed with *miR-14*; the number in the column is the sample size; a chi-square test was used for the comparison of survival rates. (C) Egg–larval duration from parasitizing to cocooning of the wasp offspring after being fed with *miR-14*. (D) Pupal duration from cocooning to emergence of the wasp offspring after being fed with *miR-14*. (E) Emergence rates of the wasp offspring after being fed with *miR-14*. (F) Wasp offspring number after being fed with *miR-14*.

differences in the emergence rate ($t = 0.03$, $P = 0.979$; Fig. 2C) and offspring number ($t = 0.37$, $P = 0.715$; Fig. 2D) of the wasps that emerged from the stem borers injected with either *miR-14* or the non-insect miRNA mimic.

The probable target gene of miR-14 detected in Co. chilonis wasps

Based on the genome and transcriptome data, the *miR-14* mature sequence and its precursor sequence was

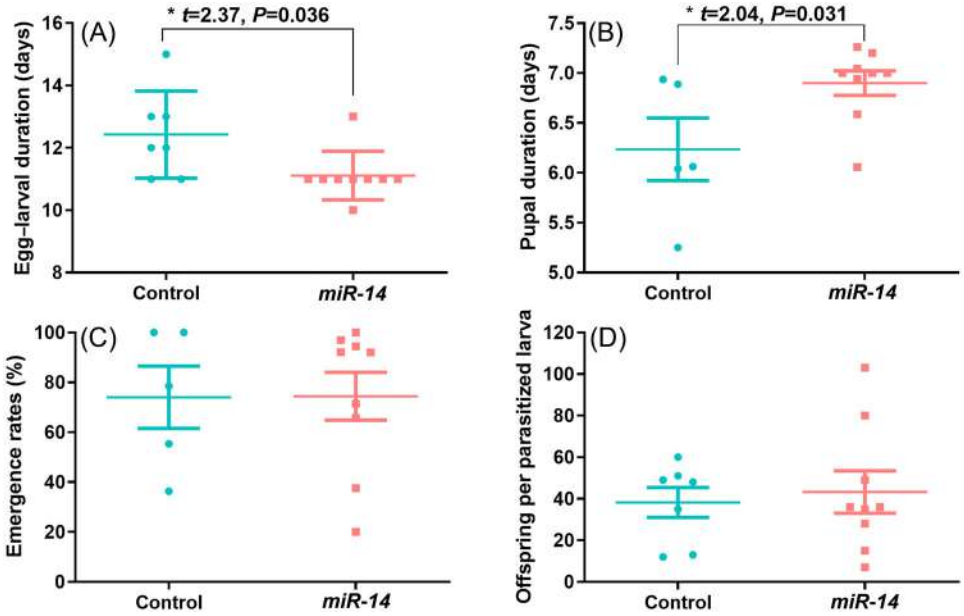

Fig. 2 Indirect effect of *miR-14* agomir on the biological parameters of the parasitoid *Cotesia chilonis* via its host *Chilo suppressalis*. (A) The duration from parasitizing to cocooning for wasps emerging from *Ch. suppressalis* larva injected with *miR-14*. A Student's *t*-test was used in the comparison between the group fed with *miR-14* and the control group; asterisks denote significant differences among comparisons (*$P < 0.05$); no asterisks indicates that no significant differences were found among the comparisons (and below). (B) Pupal duration of wasps from *Ch. suppressalis* larva injected with *miR-14*. (C) Emergence rates of wasps from *Ch. suppressalis* larva injected with *miR-14*. (D) Wasp offspring per parasitized larva.

predicted for *Co. chilonis* (Fig. 3A). The sequences of the 3′ arms of mature *miR-14* in *Co. chilonis* and *Ch. suppressalis* were found to be entirely consistent. In a previous study it was reported that *miR-14* in *Ch. suppressalis* could interact with 2 genes: *ecdysone receptor* (*ecr*) and *spook* (*spo*) (He *et al.*, 2018). Thus, the minimum free energy of the binding between the 3′ untranslated region (UTR) of these 2 genes and *miR-14* in *Co. chilonis* was predicted by the RNAhybrid method (Fig. 3B, C). This showed that the minimum free energy of the binding was no more than −18 kJ/mol, which indicted that both *spo* and *ecr* might be the target of *miR-14* in *Co. chilonis*. Although the qPCR experiments showed that the mRNA expression levels of these 2 genes were not significantly reduced for wasps fed with *miR-14* directly (Fig. 3D, E), the expression level of the *spo* gene in wasps that emerged from *Ch. suppressalis* larvae injected with *miR-14* significantly differed from that in the control group (Fig. 3F, G).

Discussion

In this study, to clarify the non-target effects of transgenic *miR-14* rice, the non-target organism *Co. chilonis* was exposed to high concentrations of the miRNA. We drew upon lessons learned from the tier 1 risk assessment of Bt crop safety, in which high concentrations of Bt protein were fed directly to non-target organisms (Romeis *et al.*, 2008). So far, there is still no risk evaluation protocol for miRNAs, and limited studies have focused on the safety assessment of miRNA-mediated transgenic plants. Notably, there have been some cases of non-target effects from similar non-coding RNA: small interfering RNA (siRNA). Most of these studies also used high concentrations of double-strand RNA (dsRNA, which could be cleaved into siRNAs) to feed the non-target organism, and the non-target effects have always been attributed to sequence matches between the siRNA and the homologous gene in the non-target organism (Baum *et al.*, 2007; Dang *et al.*, 2022; Chen *et al.*, 2023). These results could provide some insight into the non-target assessment of miRNAs.

According to the quantification data, the *miR-14* content in transgenic rice was 0.06−0.79 μg/g total RNA. Whereas in this study, both the feeding concentration (0.1 μg/μL) and the injection concentration (2 μg/μL) of the miRNA were more than 100 times higher than the highest *miR-14* content (0.79 μg/g total RNA) found in the leaves of transgenic rice. Moreover, the efficiency for

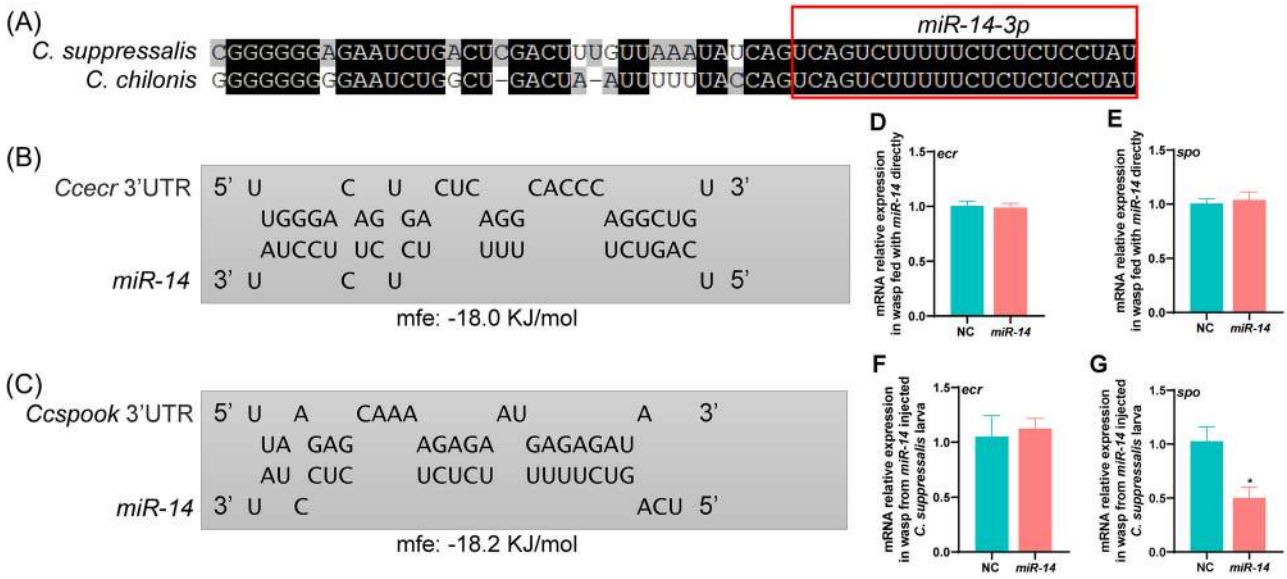

Fig. 3 Target prediction for *miR-14* in *Cotesia chilonis*. (A) Alignments of the precursor sequence of *miR-14* in *Chilo suppressalis* and *Co. chilonis*; the sequence framed in red is the 3′ arm of the mature sequence of *miR-14*. (B, C) Sequence alignment of the 3′ untranslated regions (UTRs) of the *ecdysone receptor* (*ecr*) and *spook* genes and *miR-14* in *Co. chilonis*; mfe indicates the minimum free energy of the binding between the miRNA and the 3′ UTRs predicted by RNAhybrid. (D, E) The mRNA relative expression of *ecr* and *spook* genes for wasps fed with *miR-14* directly. (F, G) The mRNA relative expression of *ecr* and *spook* genes for wasps from *Ch. suppressalis* larva injected with *miR-14*. A Student's *t*-test was used in the comparison between the *miR-14* and the NC groups. Asterisks denote significant differences among the comparisons (*$P < 0.05$); no asterisks indicates that no significant difference was found among the comparisons.

the total RNA extracted from plant tissue was lower than 1%. In this case, the feeding or injection concentration of *miR-14* was higher than the concentration found in transgenic plants by a large order of magnitude. We detected increased *miR-14* content in adult wasps after being fed with *miR-14* and in newly emerged larvae from *Ch. suppressalis* larvae injected with *miR-14* agomir, which indicated that the wasp *Co. chilonis* could acquire exogenous miRNA through direct feeding and through the hemolymph of its host. However, *miR-14* could also be detected in the control group (fed with non-insect miRNA mimic), the main reason was that the *miR-14* sequence in *Co. chilonis* was similar to that in *Ch. suppressalis*. The sequence from the wasp itself could also be amplified in the qPCR experiment, meaning that increases in *miR-14* obtained through the hemolymph or through direct feeding could not be easily discerned. A previous study has shown that the *miR-14* sequences in mature stem borers and rice planthoppers are almost same (He *et al.*, 2018). Based on the mechanism of action of miRNAs, the non-target risk assessment for this conserved miRNA should be a priority.

In the practical situation of a rice paddy field planted with transgenic *miR-14* rice, *Co. chilonis* could acquire the exogenous *miR-14* by feeding directly on the rice pollen. Thus, the wasp was fed with a high concentration of *miR-14* directly in the first evaluation of the transgenic rice. Compared with the control group, direct feeding with *miR-14* had no significant effects on the biological parameters of *Co. chilonis*, including the survival rate, ability to parasitize and developmental duration. These results were similar to previous results demonstrating that the survival rate of *A. mellifera* adults was not affected by another *Ch. suppressalis* miRNA, *Csu-novel-260* (Chen *et al.*, 2021). Also, an artificial diet including the miRNAs *Csu-novel-260* or *Csu-miR-14* both pose no significant risks to the life-table parameters of the non-target arthropod *F. candida* (Zhou *et al.*, 2023). He *et al.* (2018) fed the brown planthopper with transgenic *miR-14* rice directly, and they also did not find any significant differences in biological parameters such as survival rate and egg production for the brown planthopper when compared with the non-transgenic rice. Moreover, it has been reported that most of the insect *miR-14* is targeted to genes related to the ecdysone synthesis regulation pathway, and that *Ch. suppressalis miR-14* targets 2 ecdysone pathway genes, *ecr* and *spo* (Varghese & Cohen, 2007; Varghese *et al.*, 2010; He

et al., 2019). The expression levels of the potential target genes of *miR-14* in *Co. chilonis* detected in the *miR-14* feeding group did not significantly differ to those in the control group. Thus, it could be inferred that direct feeding with *miR-14* posed no safety risk to *Co. chilonis*.

Cotesia chilonis is an egg parasitoid, which means that female wasps lay eggs into the hemolymph of *Ch. suppressalis*. By injecting *miR-14* into the parasitized larva of *Ch. suppressalis*, the wasps could be exposed to a high *miR-14* concentration from the embryonic to larval stages. In this scenario, it was found that the *Co. chilonis* developed faster in the host larvae injected with *miR-14*. Whereas after the wasp larva emerged from the hosts, their development time was significantly retarded. Combined with the fact that the transcript levels of the *spo* gene of *Co. chilonis* were significantly reduced in the *miR-14*-treated group, it was hypothesized that *miR-14* is also likely to target genes related to the ecdysone pathway in *Co. chilonis*. This might explain why the development time of the wasp was affected by *Ch. suppressalis miR-14*. However, its specific target sites still need to be verified in further experiments. In addition, the diminished quality of the parasitized host larva could also be attributed to the development effects of *Co. chilonis*. When the *miR-14* was introduced into the hemolymph of host larvae, their development was also significantly affected (He *et al.*, 2018). In the first level of the evaluation of the impact of Bt crops on natural enemies, differences associated with reduced prey quality were often observed in the biological parameters of the natural enemy (Chen *et al.*, 2009; Dang *et al.*, 2017).

In summary, no significant differences in the biological parameters of *Co. chilonis* were found from direct feeding with *miR-14* in this study. The reason for this result might be attributed to the fact that the quantity of *miR-14* obtained by non-target organisms through feeding was very limited and was not sufficient to affect their biological parameters. Therefore, in the actual rice paddy field, the safety risk of this new transgenic rice on non-target organisms, which can only obtain exogenous *miR-14* by direct feeding on rice plants, was relatively low. It was suggested that the transgenic *miR-14* rice would pose no significant negative effects on the non-target organism *Co. chilonis*. However, only one species of non-target organism was evaluated in this study, and more detailed evaluations of different types of non-target organisms are needed in the future. Also, higher tier evaluations in the greenhouse and in semi-field environments must be carried out.

Acknowledgments

This research was funded by Science and Technology Innovation 2030 – "Agricultural Biological Breeding" Major Project (2023ZD04062) and the Experimental Technology Research Program of Zhejiang University (SYB202126).

Disclosure

The authors declare that they have no conflicts of interest associated with this work.

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Manuscript received March 5, 2024
Final version received June 5, 2024
Accepted June 17, 2024

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 The primers used in the qRT-PCR for rice and *Cotesia chilonis*.