Improvement of host-induced gene silencing efficiency via polycistronic-tRNA-amiR expression for multiple target genes and characterization of RNAi mechanism in *Mythimna separata*

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Introduction

Small RNAs (sRNAs) are a family of regulatory non-coding RNAs (ncRNAs) including small interfering RNAs (siRNAs) and micro-RNAs (miRNAs). Both types of small RNAs are involved in RNA interference (RNAi), a natural gene regulation mechanism found in a diversity of eukaryotes (Carthew and Sontheimer, 2009). Generally, RNAi effects are elicited by sequence-specific double-stranded RNA (dsRNA). Dicer (Dcr) or Dicer-like proteins (DCLs), a family of RNaseIII endonucleases, cleaves the dsRNA to generate double-stranded siRNAs. One strand of the double-stranded siRNA is incorporated into the RNA-induced silencing complex (RISC), the main component of which is the RNA binding protein Argonaute (AGO). The RISC-incorporated guide RNA recognizes the target mRNA via sequence complementarity, leading to mRNA degradation or translational inhibition (Preali and Sontheimer, 2005). In some eukaryotes-like Caenorhabditis elegans, the SID1 (systemic interference deficiency 1) protein is responsible for movement of dsRNA(+)siRNAs between cells (Winston et al., 2002), while RdRp (RNA dependent RNA polymerase) amplifies the silencing signal by its RNA synthesizing activity (Peragine et al., 2004).

Many crops suffer yield losses from a variety of pathogens including viruses, nematodes, fungi, oomycetes and insect pests. RNAi was recently demonstrated as a useful tool for control of a variety of pathogens, as knockdown of pathogen genes that are essential for growth/development or infection processes would cause deleterious effects on pathogens (Rosa et al., 2018). The rationale for development of pathogen-resistant transgenic plants by RNAi technology is the cross-species interference of pathogen target genes by host expressed RNAi inducers, and therefore, it is called host-induced gene silencing (HIGS). HIGS is considered to be the next generation pathogen control strategy as the sequence-specific nature of RNAi allows it to target a selected mRNA of a defined pest or pathogen species, as long as the interfering sequences were carefully designed to possess high specificity.

Agricultural pests cause significant yield losses in major crops. For example, the desert locust disaster that has ravaged East Africa and many countries in South Asia this year has caused serious damage to agricultural production in the affected areas (Devi, 2020). Recently, RNAi has been developed as sequence-specific insecticides for insects from different taxonomic orders. Direct application of dsRNAs in fields for pest control was...
successfully demonstrated by spraying dsRNA on plant leaves or soaking of plant roots in dsRNA solutions (Li et al., 2015; Falli, 2014; San and Scott, 2016). Stable transgenic plants expressing target-specific hairpin dsRNA (hpRNA) were first reported to confer insect resistance in 2007 (Baum et al., 2007). Thereafter, this inspired researchers to develop various RNAi protocols towards a broad-spectra of insect species (Kim et al., 2015). Commercial application of transgene based HIGS for insect control, such as the transgenic rootworm-resistant corn MON87741, was also reported (Levine et al., 2015; US-EPA, 2017).

*Spodoptera frugiperda*, also known as the armyworm, is a major corn pest especially in the African continent (Stokstad, 2017). In China, *Mythimna separata* (Lepidoptera, Noctuidae), also known as oriental armyworm, is very similar to *S. frugiperda* and is also devastating to maize production. Transgenic maize expressing Bt toxin protein Cry1Ab has been reported, but field-evolved resistance to Cry1Ab was observed in *M. separata* populations (Chang et al., 2007). Previously, we and others successfully carried out RNAi research on *M. separata* (Ganbaatar et al., 2017; Wang et al., 2018; Zhai et al., 2017). Successful RNAi effects were also reported in *S. frugiperda* (Ghosh et al., 2016). In earlier efforts, we examined RNAi effects in *M. separata* via feeding of *in vitro* synthesized dsRNAs, bacterially expressed dsRNAs, and plant virus expressed RNAi effectors, and we identified two ideal target genes, *MsChi1* and *MsChi2*, both of which encode chitinase proteins (Bao et al., 2016; Cao et al., 2017; Ganbaatar et al., 2017). In this study, we first evaluated the HIGS effects on recipient *M. separata* by transgenic expression of hpRNAs for targeting *MsChi1* and *MsChi2* genes and then developed an artificial microRNA (amiR) based PTA (polycistronic-tRNA-amiR) system to improve the silencing efficiency by simultaneously targeting three insect genes. We used small RNA sequencing technology to examine the siRNA categories which were triggered by both HIGS and delivery of *in vitro* dsRNAs/riRNAs. The RNAi mechanism in *M. separata* was also examined by biochemical characterization of RNAi machinery proteins such as MsAGO2, MsDcr2 and MsSID1.

**Results**

**Feeding on hpMsChi1-Ox and hpMsChi2-Ox rice retarded *M. separata* larvae growth**

In our previous study, *M. separata* chitinase encoding genes *MsChi1* and *MsChi2* were selected as ideal RNAi target genes. Here, we developed transgenic rice stably expressing hpRNA for *MsChi1* and *MsChi2*, as more robust genetic transformation system was available in rice than in maize, and *M. separata* readily fed on rice the same as maize, especially under experimental conditions. PCR analysis with the primers for both the 35S promoter and target genes confirmed 13 lines for hpMsChi1-Ox rice and 8 lines for hpMsChi2-Ox rice (Figure S1). Southern blot analysis showed that both hpMsChi1-Ox and hpMsChi2-Ox expression cassettes had been integrated into the rice genomes with one to two copies, respectively, in different transgenic lines (Figure S2). The relative mRNA abundances of targets *MsChi1* and *MsChi2* were decreased by 54% and 60% in gut tissue of the larvae fed on hpMsChi1-Ox and hpMsChi2-Ox rice compared with the larvae on wild-type (WT) rice, respectively (Figure 1a). Phenotypic effects of *MsChi1* and *MsChi2* knockdown were evaluated by measurement of various larvae fitness parameters. Larvae mortalities increased at 3rd DAF (day after feeding), and were 43% and 41% higher upon MsChi1 and MsChi2 silencing than that of the larvae on WT rice at 7th DAF, respectively, although a mortality of 32% was also observed on WT rice at this time point (Figure 1b). A moderate but significant decrease by 20% in body weight was observed in the larvae fed on hpMsChi2-Ox rice compared with the larvae on WT rice, while feeding on hpMsChi1-Ox rice did not reduce body weight (Figure 1c). Body lengths were significantly decreased by 27% and 41% after feeding on hpMsChi1-Ox and hpMsChi2-Ox rice compared with WT rice (Figure 1d,e).

In order to confirm the dose-dependent effects of transgene-derived siRNAs on recipient insects, we selected three hpMsChi2-Ox transgenic lines for further analysis. MsChi2 siRNA level was gradually increased in hpMsChi2-Ox Line2, Line8 and Line13 (Figure 2a). Both RT-qPCR and RT-PCR experiments demonstrated that the degree of MsChi2 inhibition correlated with siRNA levels of different transgenic lines, among which the feeding of hpMsChi2-Ox Line13 caused the highest target mRNA decrease by 44% (Figure 2b,c). Consistent with this, the larvae fed on hpMsChi2-Ox Line13 showed the most retarded growth phenotype with regards to body weight and body length (Figure 2d,e). Growth inhibition was less severe in the larvae after feeding on transgenic rice Line2 and Line8 compared with Line13, demonstrating the correlation of inducer siRNA level and silencing efficiency.

**Signatures of the target-specific siRNAs upon HIGS in *M. separata***

To date, there are few reports which used sequencing technology to reveal siRNA features in insect species. Therefore, we carried out high-throughput sequencing of small RNAs from hpMsChi2-Ox rice (Os_Chi2), WT rice (Os_CK), gut tissue of the larvae fed on hpMsChi2-Ox rice (Ms_Chi2) and control larvae fed on WT rice (Ms_CK). A total of 23-39 million sRNA reads of 18-35nt were obtained for each library. The much higher ratio of target *MsChi2*-specific siRNAs in Os_Chi2 (23.5%) compared with that in Os_CK (0.03%) indicated the efficient processing and production of siRNAs from the MsChi2 hpRNA expressed in the transgenic rice. The data also indicated that no *MsChi2*-specific siRNAs were endogenously produced in *M. separata* (0% in Ms_CK), and therefore, MsChi2-specific siRNAs in Ms_Chi2 (0.87%) should be either directly acquired from Os_Chi2 rice or induced by orally acquired RNAi effectors from Os_Chi2 (Table 1). Because of the negligible representation of MsChi2 sRNAs in control Os_CK and Ms_CK, these two samples were not further analysed.

Among sRNAs, 20-24nt siRNAs are considered to play major roles in RNAi processes in eukaryotes including insects (Zamore et al., 2000); therefore, these siRNAs were retrieved for further analysis. 27-46% of 18-35nt siRNAs fell within 20-24nt siRNAs for each library. The percentage of target *MsChi2*-specific siRNAs in total 20-24nt siRNAs were 37.95% and 2.21% for Os_Chi2 and Ms_Chi2, respectively. The ratios of target *MsChi2*-specific 20-24nt siRNAs were 1.61- and 2.54-fold compared with those of 18-35nt siRNAs in Os_Chi2 and Ms_Chi2, respectively, suggesting that 20-24nt siRNAs might be functional siRNAs rather than non-specific RNA degradation products. Furthermore, above data showed that target *MsChi2*-specific 20-24nt siRNAs were highly represented in Ms_Chi2 (2.54-fold) compared with Os_Chi2 (1.61-fold), although the reason was unknown as the plant was very different from the insect (Table 1).

The full-length ORF of MsChi2 is 1902nt, and the 78-662nt region (585nt, designated as the trigger region hereafter) was
used for construction of hpRNA expression vector for hpMsChi2-Ox rice. In Ms_Chi2, almost all of the MsChi2-specific 20-24nt siRNAs were located in the trigger region, showing that feeding of hpMsChi2-Ox rice did not induce the production of secondary siRNAs spreading outside of the trigger region in the target MsChi2 transcript. Both sense and antisense siRNAs were detected in the trigger region, while distinct sense bias (84.12%) was observed in contrary to the antisense siRNA (15.88%) (Table 1). The size distribution of MsChi2-specific siRNAs in Ms_Chi2 showed strong 22nt representation (51.63%), showing that 22nt siRNAs might be the major siRNA class responsible for RNAi effects in M. separata (Figure 3a, Table S1). The 22nt siRNA predominance was also observed in the Os_Chi2 sample (47.58%), demonstrating that ectopic expressed MsChi2 hpRNA was mainly processed into 22nt siRNA by rice RNAi machinery (Figure 3b, Table S1). Hotspot regions were observed for both sense and antisense siRNAs, and they seemed to overlap as regard to different sizes of siRNAs in Ms_Chi2 (Figure 3c). Moreover, the MsChi2-specific siRNA patterns were similar between Ms_Chi2 and Os_Chi2, demonstrating that the siRNAs provided by Os_Chi2 might influence the siRNA signatures in the recipient Ms_Chi2 sample, or they shared the same RNAi dicing mechanism (Figure 3c,d). The latter seemed to be unlikely as MsActin-specific siRNAs did not show such 22nt siRNA predominance in later experiments.

Ingestion of MsActin dsRNA and siRNAs showed RNAi effects in recipient insects

We attempted to detect-specific siRNAs for MsChi2 via northern blot experiments in M. separata, but were not successful (data not shown). The possible reason is that the MsChi2 gene is expressed at a very low level; therefore, siRNAs derived from MsChi2 mRNA are too low in amount to be detectable (Ganbaatar et al., 2017). On the other hand, in our previous work we showed that total siRNA levels for MsChi2 were so low that the siRNA bands were very weak in northern blots using 32P labelled probes (Bao et al., 2016). In order to readily detect the siRNAs and to compare siRNAs from HIGS and dsRNA/siRNA feeding, we silenced the highly expressed housekeeping gene MsActin. For comparison of siRNAs derived from feeding with either dsRNA or siRNAs, we prepared both dsRNA and siRNAs for MsActin and fed them to the larvae.

Two different types of labelled dsRNAs were used to assess the efficiency of dsRNA absorption by the feeding larvae. First, when
Figure 2  Comparison of silencing efficiencies between different hpMsChi2-Ox lines on target MsChi2 gene. (a) Detection of MsChi2 siRNAs (Upper panel) in different transgenic lines via northern blot experiment. Rice U6 RNA (Lower panel) was visualized as internal control. (b) RT-PCR experiments for evaluation of MsChi2 expression. Housekeeping gene MsActin was used as internal control. (c) RT-qPCR experiment for evaluation of MsChi2 expression. Housekeeping gene MsActin was used as internal control, and 2−ΔΔCT method was used for calculation of MsChi2 level. (d) Phenotypes of the larvae before and after 7 days’ feeding. Scale bar is shown. (e) Body weight of the larvae before and after 7 days’ feeding. (f) Body length of the larvae before and after 7 days’ feeding. T-test was performed between treatment and control Wt groups. Single asterisk, \( P < 0.05 \); double asterisks, \( P < 0.01 \), triple asterisks, \( P < 0.001 \). ns, non-significant. Wt, Wt rice. Lines 2, 8 and 13 indicate different hpMsChi2-Ox lines.
M. separata were of antisense polarity. These data demonstrated that mapped small RNAs were of sense polarity, while less than 5% mapped to the unigenes. Interestingly, more than 95% of the libraries. When the 18-35nt small RNAs were aligned with the 8.5 million of total 18-35nt clean reads were detected in these ds1060, si893 and control insects (CK). Approximately 6.1 million of total 18-35nt clean reads were sequenced for the larvae fed with ds100, ds893, was in vitro digested with RNaseIII to produce corresponding sRNAs, si893. When dsRNA and siRNAs were fed to the ds893, was observed in the gut tissue by confocal microscopy (Figure 4b). These results indicate that both dsRNA and siRNAs are efficiently taken up by the insects upon oral delivery. Three sets of dsRNAs corresponding to different regions of MsActin, ds100 (100-340nt), ds893 (893-1367nt) and ds1060 (1060-1290nt), were in vitro synthesized. One of these dsRNAs, ds893, was in vitro digested with RnaseII to produce corresponding sRNAs, si893. When dsRNA and siRNAs were fed to the larvae, the body weight of ingested larvae was significantly decreased by 28-32% compared with the control GFP dsRNA treated group, demonstrating their RNAi effectiveness on the target insects (Figure 4c,d). Characteristics of small RNAs from MsChi2 dsRNA/siRNA fed insects Small RNAs were sequenced for the larvae fed with ds100, ds893, ds1060, si893 and control insects (CK). Approximately 6.1–8.5 million of total 18-35nt clean reads were detected in these libraries. When the 18-35nt small RNAs were aligned with the whole UniGene database of M. separata, approximately 55-64% mapped to the unigenes. Interestingly, more than 95% of the mapped small RNAs were of sense polarity, while less than 5% were of antisense polarity. These data demonstrated that endogenous sRNAs were produced in M. separata and these sRNAs originated from unigene expressed mRNAs. Among these unigene matched 18-35nt sRNAs, 61-72% were 20-24nt siRNAs (Table 1). Among the total 20-24nt siRNAs, 27.04%, 18.99%, 55.69%, 15.20% and 0.044% of them were identified to be of MsActin origin in ds100, ds1060, ds893, si893 and CK samples, respectively (Table 2). The higher percentage of target-specific siRNAs in MsActin-silenced insects than CK demonstrates again the effectiveness of RNAi in this insect. The representation of MsActin sRNAs in MsActin-silenced insects (15.20-55.69%) was greater than that of MsChi2 sRNAs in MsChi2 silenced insects (2.21%, Table 1). This indicated that target sRNAs were efficiently produced for the housekeeping gene MsActin via feeding of in vitro synthesized dsRNA/siRNA, as we expected. Both sense and antisense siRNAs are detected among the 20-24nt MsActin sRNAs, the majority of them being sense sRNAs with one exception in ds893 where 74.50% are antisense siRNAs. In sharp contrast to MsChi2 siRNAs resulting from HIGS, varying proportions (13.47-76.76%) of MsActin siRNAs were observed from outside the trigger region (Table 2). Sense siRNAs were found from outside the trigger region, indicating that they were derived from both ingested dsRNAs/siRNAs and endogenous MsActin mRNA. By contrast, almost all of the antisense siRNAs arose from the trigger region, demonstrating their origin from the exogenous trigger RNAs and no amplification of secondary siRNAs. Although heterogeneous distribution of siRNAs was observed on the entire MsActin ORF sequences, several hotspot regions were detected for both sense and antisense siRNAs. While sense siRNAs showed similar patterns between different samples regardless of the trigger RNAs, they showed a higher representation at specific trigger regions for each sample, respectively. The results demonstrated that a portion of the trigger-specific sense siRNAs were derived from ingested RNAs and the silencing activity spread outside of the trigger region onto the endogenous mRNA. The above features of MsActin-specific siRNAs applied to the siRNAs originating from ingestion of both dsRNAs and siRNAs (Figure 5a).

SiRNA peaks were confirmed by northern hybridization experiments in each sample via eight DIG-labelled probes which were complementary to the hotspot siRNA peaks (Figure 5b). MsActin-specific siRNAs generally displayed no size specificity among 20-24nt, while slight prevalence in 20nt siRNA was observed (Figures S3 and S4). It is different from the HIGS-derived MsChi2 siRNAs where 22nt predominance was observed. We used quantitative real-time PCR to evaluate the abundance of target MsActin mRNA levels by amplifying different regions of the mRNA via different sets of PCR primers. The results demonstrated that PCR product for the hotspot region showed reduced abundance while that for siRNA desolate region displayed no change (Figure 5c). The results demonstrated that siRNA peaks might be derived from target mRNAs via more frequent cleavage events in hotspot regions than the other regions. Silencing of multiple insect target genes via PTA technology We propose that efficient pest control should benefit from silencing of several target genes because of synergistic effects. Artificial miRNA (amiR) is a custom-tailored RNAi technology, which is based on miRNA biogenesis mechanisms and is suitable for expression of short RNAi effectors. Here, we developed a silencing system named PTA (Polycistronic-tRNA-amiR) which is based on miRNA biogenesis mechanisms and is suitable for expression of short RNAi effectors. Here, we developed a silencing system named PTA (Polycistronic-tRNA-amiR) which expressed multiple amiRs from the same expression cassette (Figure 5S). First, we determined the efficiency of PTA for silencing of two target genes GFP (Green fluorescent protein)

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**Table 1** Statistics of MsChi2 sRNA reads and percentages

| sRNA types | Os_CK | Os_Chi2 | Ms_CK | Ms_Chi2 |
|------------|-------|---------|-------|---------|
| 18-35nt total | 39 274 175 | 29 505 467 | 23 591 270 | 22 627 481 |
| 18-35nt | 13 189 | 6 934 686 | 17 (0%) | 196 995 |
| MsChi2 (ratio in 18-35nt total) | (0.03%) | (23.50%) | (0.87%) | |
| 20-24nt total | 15 744 872 | 13 429 704 | 7 753 773 | 6 305 721 |
| 20-24nt (ratio in 18-35nt total) | (40.09%) | (45.52%) | (32.87%) | (27.87%) |
| MsChi2 (ratio in 20-24nt total) | (0.06%), 2 fold† | 5 096 685 | |

† The fold value of 20-24nt MsChi2-specific sRNAs percentage compared to that of 18-35nt sRNAs was also shown. nd, not detectable.
and GUS (β-glucuronidase) in the N. benthamiana transient expression system. When two amiRs were designed for each target gene in the same PTA, it showed greater silencing effects on both target genes than when single amiR was used (Figure S6).

Based on the above result, we made a PTA construct targeting three M. separata genes, including previously defined MsChi2, MsActin and a gut expressed nuclease coding gene MsREase. In our previous results, target region-specific siRNA peaks were observed for both MsChi2 and MsActin. Although we are not sure about the importance of these hotspot siRNAs, we speculated that delivery of siRNA for this hotspot region might improve the silencing efficiency. Based on this, we selected the hotspot regions 377-411nt for MsChi2 and 991-1027nt for MsActin for amiR design. REase (RNAi efficiency-related nuclease) is a gut-specific nuclease responsible for dsRNA degradation in the intestine of the lepidopteran insect Ostrinia furnacalis. REase contains a PIN (PilT N-terminus) domain with nuclease activity, MsREase, which might be attributable to the combined effects of amiR triggered silencing and/or knockdown of MsREase. Phenotypically, the insects that were fed with PTA-Ox maize showed retarded growth (Figure 6c). Interestingly, the insects fed on PTA-Ox maize showed visible phenotypic abnormalities which were rarely seen in our previous publications for MsChi2 silencing via different approaches (Bao et al., 2016; Cao et al., 2017; Ganbaatar et al., 2017) and feeding with hpMsChi2-Ox rice here (Figure 6d). The developmental defects such as melanism were observed in various organs, and the insects showed growth failures in different moulting stages (Figure 6e). MsChi2 expression was also silenced in all these stages/organisms showing abnormality, demonstrating the systemic RNAi response in M. separata (Figure 6f,g). We also purified MsChi2 protein and verified its chitinase activity by chitin hydrolysis assays, demonstrating that MsChi2 was a bona fide chitinase (Figure S9). Insect chitinase genes are important in cuticle formation, moulting and remodelling in insects (Arakane and Muthukrishnan, 2010), and silencing of chitinase was demonstrated to retard insect growth in P. operculella, T. castaneum, Helicoverpa armigera and P. xylostella (Mamta and Rajam, 2016; Mohammaed et al., 2017; Noh et al., 2018; Zhu et al., 2019). In consideration of the above results, we speculate that the developmental abnormalities might be attributable to strong systemic silencing of the MsChi2 gene.
We selected three independent lines of PTA-Ox maize for further assay. Northern hybridization was performed to detect the accumulation of MsChi2-amiR level, which was the highest in PTA-Ox Line8, followed by Line2 and Line5, whereas no signal was detected in Wt maize (Figure S10a). Silencing efficiency was evaluated by measuring target MsChi2 mRNA level (Figure S10b, c), phenotypic changes (Figure S10d), body weight (Figure S10e) and body length (Figure S10f). Feeding of PTA-Ox Line8 caused the highest knockdown effects, whereas that of Line2 and Line5 only showed the moderate effects. These results confirmed the correlation of silencing efficiency and amiR expression level.

Analysis of core RNAi machinery components in *M. separata*

Transcriptome sequencing was carried out on *M. separata* previously (Liu et al., 2016), and ten gene families associated with RNAi mechanisms were identified here (Table S2). RT-PCR was performed to confirm the expression of the representative genes from each family in gut tissue of *M. separata* (Figure 7a). Interestingly, we could not retrieve any unigenes homologous to RdRp in *M. separata* in our transcriptome data and even from draft genome sequences of *Spodoptera frugiperda*, which is a closely related species of *M. separata* (Huan et al., 2019). These results were consistent with our small RNA sequencing data in which no secondary siRNA production was observed. We verified the expression of MsAGO2, MsDcr2, MsPIWI and MsSID1, subclasses of which were confirmed by evolutionary analysis (Figure S11), with predicted sizes of mRNAs using northern hybridization experiment (Figure 7b). For analysis of biochemical activities, GST-tagged recombinant MsAGO2 (PAZ domain), MsDcr2 (RNaseIII domain) and MsSID1 (N-terminal domain) proteins were expressed in *E. coli* and were purified using...
glutathione agarose columns (Figure S12). MsAGO2 bound to and retarded 32p labelled ssRNA mobility in EMSA assays via concentration-dependent manner, while this activity was abolished by non-labelled competitor (Figure 7c). MsDcr2 cleaved the dsRNA into a mixture of siRNAs with the sizes of 20-24nt, while control GST did not show this effect (Figure 7d). MsSID1

Table 2 Statistics of MsActin sRNA reads and percentages

| siRNA types                  | ds100     | ds1060    | ds893     | si893     | CK        |
|------------------------------|-----------|-----------|-----------|-----------|-----------|
| 18-35nt total                | 6 717 681 | 8 503 424 | 7 824 932 | 7 038 244 | 6 118 075 |
| Unigene mapped sRNA (ratio in 18-35nt total) | 4 286 343 (63.81%) | 5 155 561 (60.63%) | 4 662 552 (59.59%) | 3 900 626 (55.42%) | 3 592 639 (58.72%) |
| Unigene mapped sRNA\* (ratio in mapped siRNA) | 4 149 554 (96.81%) | 5 030 104 (97.57%) | 4 451 100 (95.46%) | 3 834 260 (98.30%) | 3 420 060 (95.20%) |
| Unigene mapped 20-24nt siRNA (ratio in mapped siRNA) | 2 640 319 (61.60%) | 3 423 475 (66.40%) | 2 894 340 (62.08%) | 2 795 783 (71.68%) | 2 582 199 (71.87%) |
| 20-24 MsActin (ratio in 20-24nt total) | 714 121 (27.04%) | 650 256 (18.99%) | 1 612 436 (55.69%) | 425 272 (15.20%) | 1 134 (0.044%) |
| 20-24\* (ratio in 20-24 MsActin ) | 643 032 (90.05%) | 594 125 (91.37%) | 411 138 (25.50%) | 309 174 (72.70%) | 1129 (100%) |
| Trigger region (ratio in 20-24 MsActin ) | 276 068 (38.66%) | 151 092 (23.24%) | 1395 209 (86.53%) | 209 131 (49.18%) | nd |
| Trigger-outside (ratio in 20-24 MsActin ) | 438 053 (61.34%) | 499 164 (76.76%) | 217 227 (13.47%) | 216 141 (50.82%) | nd |

18-35nt total, read numbers of 18-35nt sRNAs from each sample; * represents sense sRNAs and * represents antisense sRNAs; 20-24nt MsActin, 20-24nt sRNA reads matched to the MsActin ORF sequence; 20-24\*, 20-24 MsActin-specific sRNA reads with sense polarity; 20-24\-*, 20-24 MsActin-specific sRNA reads with antisense polarity; Trigger, MsActin-specific sRNA reads from the trigger region; trigger-outside, MsActin-specific sRNA reads derived from the region outside trigger sequence. nd, not detectable.

Figure 5 Analysis of MsActin-derived siRNAs and MsActin gene expression. (a) Distribution of 20-24nt siRNAs on the MsActin ORF sequence in MsActin-silenced insects. Vertical axis shows the number of siRNA reads. The black lateral line represents the full-length ORF of MsActin, and the regions for dsRNA or siRNA synthesis were indicated by different colours. Sense and antisense siRNAs are aligned above and below the grey line, respectively. siRNA peaks from different samples are shown by different colours as indicated, respectively. The position of the MsActin-specific probe for northern blot in (b) was indicated by short bordered lines and RT-qPCR primers in (c) are shown as lateral arrowheads. (b) Detection of MsActin-specific siRNAs by hotspot-specific probes. 5.8S rRNA band from total RNA for small RNA recovery is shown as the internal control. Probes 1-5 were antisense to MsActin mRNA for detection of sense siRNAs, while probes 6-8 were sense to MsActin mRNA for detection of antisense siRNAs. (c) Knockdown of the MsActin gene after feeding of different dsRNA/siRNAs for 3 days was evaluated by RT-qPCR. Three different MsActin-specific primers were used, and MsTubulin mRNA level was used as the internal control. Statistical differences between silenced group and the control dsGFP fed group were evaluated. Triple asterisks, \( P < 0.001 \)
showed clear binding activity to $^{32}$P labelled dsRNA in a dose-dependent manner, while this activity was abolished by a non-labelled competitor (Figure 7e). Taken together, these data verified the biochemical activity of RNAi machinery proteins MsAGO2, MsDcr2 and MsSID1, suggesting that these may be responsible for systemic RNAi activity in *M. separata*.

**Discussion**

Host-induced gene silencing (HIGS) has emerged as a new strategy for pest control, where the robust RNAi response in target insect species was the requisite. Although RNAi was used for functional genetic research in insects of many orders, RNAi efficiency was reported to vary among species (Terenius et al., 2011; Zhang et al., 2017). Although successful RNAi effects have been reported in insects of many families of Lepidoptera such as Sphingidae, Noctuidae, Plutellidae, Tortricidae and Papilionidae (Choi and Vander, 2019; Christiaens et al., 2018; Hameed et al., 2018; Poreddy et al., 2017; Wang et al., 2012; Wang et al., 2015), RNAi efficiency was reported to be relatively low in Lepidoptera, especially compared with the high RNAi sensitivity of Coleoptera and Diptera (Zhang et al., 2017). Here we showed the effectiveness of transgene triggered RNAi effects on the Noctuidae insect *M. separata*, with HIGS effects comparable to those for other lepidopteran insects such as *Manduca sexta* and *Helicoverpa armigera* (Mao et al., 2007; Poreddy et al., 2017).

Several recent publications have attributed the RNAi recalcitrance of Lepidoptera to the high nuclease activity in their...
intestinal cells (Shukla et al., 2016). To date, dsRNase activity was found in the intestines of many insects such as Bombyx mori, Locusta migratoria and Acyrthosiphon pisum, and the decrease in dsRNase activity was reported to increase RNAi efficiency in Ostrinia furnacalis (Lepidoptera) and Leptinotarsa decemlineata (Coleoptera) (Guan et al., 2018; Wang et al., 2016). Therefore, simultaneous silencing of both dsRNase encoding genes and the gene of lethal effects would increase pest control effects via HIGS. To this aim, we need an appropriate expression cassette for targeting several genes via short RNAi inducers which are packaged into the same construct and are efficiently processed upon expression. Artificial microRNA (amiR) technology is considered as the second generation of RNAi strategies because of its uniqueness, effectiveness and accuracy. As regard to pathogen control, amiR technology was first used for plant antiviral research (Niu et al., 2006) and recently has also been used for HIGS on insects including Helicoverpa armigera and Chilo suppressalis (Agrawal et al., 2015; Jang et al., 2017). In aphids, stably expressed amiR was reported to show higher HIGS effects than hpRNA (Guo et al., 2014). Recently, an insect miRNA based amiR, Plin-amiR, was developed for Helicoverpa armigera target gene silencing and it appeared to have improved RNAi efficiency (Bally et al., 2020). However, in all the above reports they used single amiR for expression. Here, we developed an expression cassette named PTA for three amiRs, targeting the dsRNase encoding gene MsRsEase and the essential growth-related genes MsActin and MsChi2. The amiR sequences are embedded between tRNA spacers to be cleaved at their borders to produce separate amiRs. The larvae fed with PTA-Ox maize showed stronger knockdown effects on target genes and more pronounced phenotypic abnormalities than MsChi2 silencing via different approaches (Bao et al., 2016; Cao et al., 2017; Ganbaatar et al., 2017) as well as hpMsChi2-Ox rice triggered HIGS. Furthermore, MsChi2 knockdown was observed to spread throughout the M. separata body and to persist in later growth stages, demonstrating the existence of systemic RNAi response in M. separata. The strong RNAi effects of PTA might be attributable to either better silencing efficiency of amiR than hpRNA, or improvement of RNAi effects by silencing of MsRsEase. Mechanistically, we showed that M. separata encodes a functional SID1 protein with dsRNA binding activity, which might also contribute to the systemic response. Several amiRs in same construct for multiple target silencing were only reported for Arabidopsis thaliana gene silencing (Zhang et al., 2018), while our study is the first to be used for silencing insect genes and trans-kingdom RNAi.

Although siRNAs are the major players in RNAi, there are few reports regarding siRNA characteristics and their implications in insect species. Northern blot experiments were carried out to detect siRNAs in insect species including the lepidopteran species Helicoverpa armigera (Mao et al., 2011), Spodoptera litura (Rajagopal et al., 2002) and Bombyx mori (Ulhirova et al., 2003) upon dsRNA or VIGS (virus-induced gene silencing) mediated RNAi. However, structural nuances of the siRNAs involved in insect RNAi processes are largely unknown. In this regard, high-throughput small RNA sequencing has advantages and has been broadly employed in discovery of endogenous small RNAs like miRNAs (Barquist and Vogel, 2015). However, there are few reports using sequencing technology to reveal siRNA features in insect species. Therefore, we sequenced small RNAs triggered by both HIGS and in vitro dsRNA/siRNA feeding. Upon HIGS, 22nt siRNA predominance with sense bias within only the trigger region was observed, while dsRNA/siRNA feeding produced siRNAs with sense strands spreading outside the trigger region. It was reported that hpRNA derived siRNAs in rice were mainly 21nt, 22nt and 24nt (Wang et al., 2013), while the proportion seem to vary under different situations (Wang et al., 2008). We showed that ectopic expressed MsChi2 hpRNA was mainly processed into 22nt siRNAs by rice RNAi machinery. RNaseII digestion of dsRNA was reported to produce 21nt siRNAs (Wuriyanghan et al., 2011). Therefore, it appeared that the features of orally acquired RNAi inducers determined the characteristics of the target siRNAs in recipient M. separata larvae. With regard to the siRNA sizes in insects, virus-derived siRNAs (vsiRNAs) in insects of different orders were heavily investigated. In Diptera such as Aedes aegypti and Drosophila melanogaster, vsiRNAs are predominantly 21nt derived from Dicer2 processing (Léger et al., 2013; Mueller et al., 2010). VsIRNAs were shown to be either 21nt or 22nt in Hemiptera such as Laodelphax striatellus and Homalodisca vitripennis (Li et al., 2013; Nandety et al., 2013). In Lepidoptera such as Bombyx mori, Helicoverpa armigera and the Spodoptera frugiperda Sf19 cell line, the prevailing vsiRNAs are 20nt siRNA from Dicer2 cleavage (Jayachandran et al., 2012; Mehrabadi et al., 2015; Zografidis et al., 2015). Compared with these, there are several reports of the investigation of siRNAs derived from insect endogenous genes after RNAi effects, most of which utilized dsRNAs from mixture samples (Carradec et al., 2015; Guan et al., 2018; Iwashita et al., 2015; Li et al., 2018). In the Coleopteran insect Diabrotica virgifera, delivery of dsRNAs produced trigger region-specific 21nt siRNAs without obvious hotspots (Li et al., 2018). In two lepidopteran insects, Ostrinia furnacalis and Helicoverpa armigera, delivery of dsRNAs produced siRNAs in target region with obvious siRNA peaks on both sense and antisense strands (Guan et al., 2018). Here, we showed that secondary siRNAs might not be produced in M. separata, while sense siRNA spread beyond the target region both at upstream and downstream of the target region. Interestingly, we could not retrieve any unigenes homologous to RdRp in M. separata in our transcriptome data or from draft genome sequences of S. frugiperda (Huan et al., 2019). These results were consistent with our data in which no secondary siRNA production was observed. We found many homologs of core RNAi genes in M. separata, and we chose AGO2 and Dicer2 homologs in M. separata for further analysis as they were reported to be involved in the siRNA pathway instead of the miRNA pathway in insect species such as Bombyx mori (Koliopoulos and Swevers, 2013). Furthermore, injection of dsRNA triggers expression of AGO2 and Dicer2 in M. sexta and B. mori (Garbutt and Reynolds, 2012; Liu et al., 2013). We showed that MsDicer2 specifically cleaved the dsRNA and that MsAGO2 exhibited clear binding activity to ssRNA. Although RNAi machinery related genes were heavily investigated in insects of different orders by both binding transcriptome sequencing and unigene analysis (Bansal and Michel, 2013; Firmino et al., 2013; Tomoyasu et al., 2008; Upadhyay et al., 2013), biochemical analysis of these proteins was generally lacking. Therefore, we provide biochemical evidence of insect, especially of Lepidoptera, RNAi pathway enzyme activity for AGO2 and Dicer2, which mechanistically support evidence for RNAi activity. In Lepidoptera, overexpression of SID1 was reported to enhance RNAi efficiency (Kobayashi et al., 2012; Mon et al., 2012). Silencing of MsChi2 in distant organs outside gut tissue and active dsRNA binding of MsSID1 illustrated that MsSID1 might contribute to systemic RNAi response in M. separata. All
our data support the state of the art of RNAi mechanism in *M. separata*. In *Bombyx mori*, RIP sequencing confirmed binding of AGO2 with siRNAs (Nie et al., 2013), which was consistent with our data. Collectively, we explained the silencing mechanism of *M. separata* which was amenable to RNAi.

Sequencing and structural analysis of target gene-specific siRNAs in insects has more implications for RNAi application, besides aiding understanding of RNAi mechanisms. Although dsRNAs are widely used in insect RNAi, siRNAs were also reported to be effective RNAi inducers in several insect species including *Manduca sexta*, *Acythesphorus pisum*, *Bemisia tabaci* and *Bactericera cockerelli* (Levin et al., 2005; Mutti et al., 2006; Upadhyay et al., 2011; Wuriyanghan et al., 2011). However, in all of the above experiments, researchers used mixtures of siRNAs, that is, which were RnaseII digested products of dsRNA, the same as our present study by si893. To date, only one group used specifically designed siRNA to inject *Bombyx mori* eggs and detected a stronger silencing effects on a target gene than the long dsRNA (Yamaguchi et al., 2011). Compared with long hprRNA (at least 50nt), short amiRNA (21-24nt) overcomes the disadvantages of off-target effects as longer sequences are prone to have more homologous sequences in target species. However, selection of short target sequences for amiRNA is challenging as we do not know which region of a target gene is suitable for amiRNA design or whether or not it competes over other regions. Although Dicer processing of the dsRNA into siRNAs is the key step in RNAi pathway, it is elusive how dsRNA is recognized and cleaved. It was reported that Dicer has sequence cleavage preferences in different species (Hoehener et al., 2018). The in vivo rule of dsRNA processing in insects could be uncovered by small RNA sequencing as the siRNA peaks always appeared at the same region, which was evidenced in our results. Taken together, small RNA sequencing will help to define siRNA regions for amiRNA design, and the combination of this with the PTA strategy we developed here would be helpful for future HIGS study.

### Experimental procedures

#### Construction of binary RNAi hairpin vector and rice transformation

Interfering sequences for *MsChi1* (accession No. AY508698.1) and *MsChi2* (accession No. AB201283.1) were amplified from *Oryza sativa* L. spp. (japonica) transformation and selection were performed according to Wuriyanghan et al., 2009. Positive transformants were screened by Basta selection (20mg/mL BlpR), and transgenic seedlings were confirmed by genomic DNA PCR analysis on the target *MsChi1* or *MsChi2* gene and the 35S promoter. For Southern blots, five µg of the genomic DNA was digested with the restriction enzyme EcoRI and separated by 0.8% (w/v) agarose gel electrophoresis. The DNA was denatured and transferred to nylon membranes in 20 X SSC solution by capillary transfer and fixed with UV cross-linker. Digoxin (DIG) labelled probes for *MsChi1* and *MsChi2* were used for hybridization, and the hybridization signal was detected by chemiluminescence method according to Ganbaatar et al., 2017. The fresh leaves of transgenic rice and control wild-type rice were collected and used for feeding assays.

### Host-induced silencing of *Mythimna separata* genes

#### Insect feeding and bioassays

Laboratory-adapted *M. separata* eggs were provided by The Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS). Synchronous third-instar larvae were used in feeding bioassays. The number of surviving larvae was recorded at every other day. After seven days feeding, surviving larvae were collected and the body weight and body length were measured. For RT-qPCR experiments on target gene expression and siRNA sequencing, larvae were collected after three days' feeding and total RNAs were extracted from dissected gut tissues. RNA isolation, RT-PCR, RT-qPCR and northern blot for siRNA detection were performed as Ganbaatar et al., 2017. Ratio of mRNA abundance between *M. separata* gene silenced and control sample was calculated by 2^ΔΔCt method for RT-qPCR. Three biological replicates were performed, and statistical analysis was performed between treated and the control group.

#### dsRNA/siRNA synthesis, labelling, larvae feeding and bioassays

Interfering sequences for *MsActin* (accession No. GQ856238.1) were amplified using gene-specific primers containing the T7 promoter sequence 5'-TAATACGACTCACTATAGGGAGA-3' at the 5' end. Three fragments of 241 bp (100–340), 231 bp (1060–1290) and 475 bp (893–1367) were used as the templates to synthesize dsRNAs, namely ds100, ds1060 and ds893, by the 5 X MEGAscript® T7 kit (Caø et al., 2017). SiRNA was produced by treatment of ds893 with ShortCut® RnaseII and was named si893. 100 ng/µl dsRNA or 50 ng/µl siRNA was mixed with artificial diet for feeding experiments. ds893 was chosen for labelling of dsRNA and siRNA. In dsRNA synthesis reactions, UTP was partly replaced with DIG-UTP or Cy3-UTP to obtain labelled dsRNA. Cy3-labelled dsRNA was digested with ShortCut® RNaseII to obtain Cy3-labelled siRNA. The labelled dsRNA/siRNA was fed to the larvae. After feeding of DIG-labelled dsRNA for 4 h and 24 h, gut RNA was extracted, resolved on a 1% agarose gel, transferred to nylon membranes and DIG RNA signals on the membrane were visualized with BCIP/NBT alkaline phosphatase substrate solution. After feeding of Cy3-labelled dsRNA or siRNA for 4 h, gut tissue was sectioned, and Cy3 signals and the nuclei were visualized using a 550 nm (Cy3) and 360 nm (DAPI) for excitation and 570 nm (Cy3) and 460 nm (DAPI) for emission under a laser scanning confocal microscope (Carl Zeiss, LSM 710).

#### Small RNA sequencing, bioinformatic analysis and detection with northern blot hybridization

Three µg of total RNA for each sample was used for generation of small RNA libraries. Synthetic oligonucleotide adapters were ligated to the RNA using T4 RNA ligase. Adapter ligated RNA was reverse transcribed and PCR amplified. PCR products were resolved on 8% polyacrylamide gels, and DNA fragments of 140-160bp were recovered from the gel. The library was sequenced on the Illumina Hiseq 2500 platform and 50bp single-end reads were generated. Small RNAs were mapped to reference sequences by Bowtie2 (version 2.4.1) without mismatches to analyse the target-specific sRNAs. The sorted.bam files resulted from Bowtie2 and samtools (version 1.10) were used for analysis of small RNA size distribution. The small RNAs were visualized by the artemis software (version 18.1.0). Target-specific siRNAs were manually checked via local BLAST, and the numbers of siRNAs were comparatively analysed between treatment and control groups. Small RNA northern blot detection was
performed as in the literature using DIG-labelled RNA probes (Ganbaatar et al., 2017).

**Screening of RNAi machinery genes, evolutionary analysis, RT-PCR and northern blot analysis**

*M. separata* unigenes were obtained by transcriptome sequencing in our previous publication (Liu et al., 2016). *M. separata* RNAi machinery genes were identified from transcriptome data by using homologous gene sequences from other insect species as a query. The protein sequences were multiple compared using ClustalV2. MEGA-X was used to build the phylogenetic tree via the maximum likelihood method, and the bootstrap method was used to verify the quality of evolutionary tree with 1000 times of inspection. Regular PCR was performed using *M. separata* gut cDNA to detect the presence of the mRNAs for RNAi machinery genes. Northern hybridization experiments were carried out using *M. separata* gut RNA and 32P-UTP-labelled negative strand probes according to our previous publication (Diao et al., 2019).

**Synthesis of PTA expression cassette and transient knockdown analysis in *N. benthamiana***

We modified PTG (polycistronic-tRNA and CRISPR guide RNA) (Xie et al., 2015) to obtain PTA (polycistronic-tRNA-amIR) for silencing of multiple target genes. Gene-specific amiR was designed and synthesized according to literature (Diao et al., 2019) using *Arabidopsis thaliana* miR159b as the backbone. Multiple tRNAs and amiRs sequences were assembled by GoldenGate assembly, amplified with border-specific primers and cloned into the pMD19T vector. *KpnI*/*XbaI* digested PTA fragments were subcloned into the pBI121 vector to produce recombinant PTA vectors. For transient silencing assays, two amiRs for the *GUS* (β-glucuronidase) and GFP (Green fluorescent protein) genes were designed and assembled into one PTA construct. Recombinant vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into *N. benthamiana* leaves together with GFP expressing TMV-GFP or GUS expressing pBI121-GUS vectors. As a control, single amiR constructs for GFP or GUS were used in parallel experiments. Three days post-infection, target GFP or GUS mRNA expression was evaluated by RT-qPCR using *NbActin* (accession No. NM_102866.3) as an internal control. GFP fluorescence was also detected using a hand held UV lamp (365 nm).

**PTA construct for *M. separata* gene silencing, maize transformation and insect bioassays**

For multiple gene silencing, we chose *MsChi2*, *MsActin* and *MsRase* (Unigene No., Cluster-3827.32653) as the target genes, and the PTA vector was constructed as described above and transformed into *Agrobacterium tumefaciens* strain EHA101. Maize transformation was performed on immature embryos from the inbred line maize cultivar B104 according to the literature (Vi et al., 2019; Xiang et al., 2018). The complete plantlets with healthy roots were transplanted into soil pots and the transgenic seedlings were confirmed using regular PCR with genomic DNA as template. The fresh leaves of transgenic maize and control wild-type maize were collected and used for feeding assays.

**Protein expression, purification and biochemical analysis**

Protein expression and purification procedures followed the published methods (Wurzyanghan et al., 2009). Briefly, the PAZ domain of MsAGO2, the RNaseIl domain of MsDcr2, N-terminal domain of MsID1 and the whole ORF of MsChi2 were expressed as GST fusions in *E. coli* strain BL21 (DE3) by pGEX-4T-1 recombinant vectors via IPTG induction. Recombinant GST-tagged proteins were purified and confirmed by SDS-PAGE or protein gel blotting using a mouse anti-GST monoclonal antibody.

Biochemical analysis was performed according to Fifa et al., 2005. For RNA labelling, ds100 was treated with CIP to dephosphorylate the 5' ends, purified by phenol/chloroform extraction and end-labelled by [32P] ATP and T4 polynucleotide kinase to obtain 32P-labelled dsRNA. For ssRNA, 26nt short RNA 5'-GGGCAUCGCCGACCUGAAAGG-3' was end labelled the same way. Non-labelled RNA was used as competitor. Electrophoretic mobility shift assay (EMSA) was performed to investigate the RNA binding ability of MsAGO2 and MsID1 proteins by using 32P labelled ssRNA and dsRNA, respectively. The protein/RNA samples were resolved on native TBE polyacrylamide gels and visualized by autoradiography. dsRNA cleavage assays followed the procedures of Li et al., 2015 and Hodgson et al., 2019. Briefly, 32P labelled ds100 was mixed with purified MsDcr2, incubated at 37°C for 30 min, and cleavage products were analysed by 15% (v/v) urea PAGE and visualized by phosphorimaging. For chitinase activity assays, 3 mg/ml insect chitin was incubated with purified MsChi2 protein in 20 mM sodium phosphate buffer (pH = 6.0) and incubated at 30°C for different times. The product N-acetylglucosamine was reacted with P-Dimethylaminobenzaldehyde in boiling water for 7 min and centrifuged at 5000 rpm for 10 min, and the supernatant was detected in a microplate at 585 nm (Liu et al., 2017).

**Accession numbers**

*MsChi1*, AY508698.1; *MsChi2*, AB201283.1; *MsActin*, GQ856238.1; *NbActin* and NM_102866.3. Other *M. separata* sequences were obtained from transcriptome data (Liu et al., 2016).

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**Author contributions**

HW designed the experiments and wrote the manuscript; WB, AL and YZ performed most of the experiments and prepared the figures; YZ, QZ and ZZ analysed the sequencing data; PD and TY performed protein purification experiment; HD participated in insect culture experiment; XL participated in transgenic maize development.

**Conflicts of interests**

The authors declare no conflicts of interests.
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Figure S1. PCR detection of transgenic rice.

Figure S2. Southern blot detection of transgenic rice.

Figure S3. Analysis of MsActin derived 20-24nt siRNAs.

Figure S4. Size distribution of 20-24nt siRNAs on the MsActin ORF sequence in MsActin silenced insects.

Figure S5. The rationale for construction of the PTA. (poly-cistronic-tRNA-amIR) system and its mechanism for multi-target gene silencing.

Figure S6. Confirmation of PTA effects via transient expression in *N. benthamiana* plants.

Figure S7. Analysis of MsREase gene.

Figure S8. PCR detection of PTA-Ox transgenic maize.

Figure S9. Chitinase activity of MsChi2 protein.

Figure S10. Comparison of silencing efficiencies between different PTA-Ox lines on target MsChi2 gene.

Figure S11. Evolutionary analysis of four important *M. separata* genes involved in RNAi responses.

Figure S12. Detection of purified proteins.

Table S1. Size, abundance and polarity of MsChi2 derived siRNAs.

Table S2. Information of *M. separata* RNAi related genes identified in transcriptome data.

Table S3. Primers used in this study.