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ORIGINAL PAPER

Plant-mediated RNAi of a gap gene-enhanced tobacco tolerance against the *Myzus persicae*

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Abstract Plant-mediated RNAi has been developed as a powerful weapon in the fight against agricultural insect pests. The gap gene *hunchback* (*hb*) is of crucial importance in insect axial patterning and knockdown of *hb* is deforming and lethal to the next generation. The peach potato aphid, *Myzus persicae* (Sulzer), has many host plants and can be found throughout the world. To investigate the effect of plant-mediated RNAi on control of this insect, the *hb* gene in *M. persicae* was cloned, plant RNAi vector was constructed, and transgenic tobacco expressing *Mphb* dsRNA was developed. Transgenic tobacco had a different integration pattern of the transgene. Bioassays were performed by applying neonate aphids to homozygous transgenic plants in the T2 generation. Results revealed that continuous feeding of transgenic diet reduced *Mphb* mRNA level in the fed aphids and inhibited insect reproduction, indicating successful knockdown of the target gene in *M. persicae* by plant-mediated RNAi.

Keywords RNAi · *hunchback* · *Myzus persicae* · Transgenic tobacco

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Introduction

RNA interference (RNAi), first discovered in *Caenorhabditis elegans*, is machinery for sequence-specific post-transcriptional gene silencing in most eukaryotic organisms (Fire et al. 1998). Double-stranded RNA (dsRNA)-mediated RNAi has been developed as one of the most promising tools to study gene function, particularly in organisms for which stable transgenesis is not available (Hannon 2002; Huvenne and Smagghe 2010). Dicer RNaseIII-type enzymes digest cytoplasmic dsRNAs into small interfering RNAs (siRNA) duplexes composed of 21–23 nucleotides (nt). These siRNA duplexes are incorporated into a multiprotein RNA-inducing silencing complex (RISC) where the antisense strand guide RISC to its homologous target mRNA for endonucleolytic cleavage (Dykxhoorn et al. 2003; Meister and Tuschl 2004). RNAi technology has also become a powerful weapon in biological control of agricultural insect pests (Gordon and Waterhouse 2007; Price and Gatehouse 2008). So far, a variety of feasible methods have been established to deliver exogenous dsRNA to organisms, for example microinjection, feeding, and soaking (Baum et al. 2007; Chen et al. 2010; Liu et al. 2010; Rosa et al. 2012; Tian et al. 2009; Zhang et al. 2010; Zhao et al. 2011; Zhu et al. 2011).

In addition, gene knockdown by expressing dsRNA in plants has also been exploited to control insect pests (Pitino et al. 2011). In 2007, prodigious progress was achieved in constructing transgenic plants expressing

insect dsRNA for insect pest management. Transgenic corn plants expressing western corn rootworm (WCR) *Diabrotica virgifera virgifera* vacuolar ATPase (V-ATPase) subunit dsRNAs showed a significant reduction in WCR feeding damage in a growth chamber assay (Baum et al. 2007). When cotton bollworm larvae (*Helicoverpa armigera*) were fed transgenic plants expressing double-stranded RNA (dsRNA) specific to CYP6AE14, a cytochrome P450 gene (CYP6AE14) from cotton bollworm, levels of its transcript in the midgut decreased and larval growth was retarded (Mao et al. 2007). The hexose transporter gene NlHT1, the carboxypeptidase gene Nlcar, and the trypsin-like serine protease gene Nltry are highly expressed in the *Nilaparvata lugens* midgut. When *N. lugens* nymphs were fed on rice plants expressing dsRNAs of the three targeted genes, RNA interference was triggered but lethal phenotypic effects after dsRNA feeding were not observed (Zha et al. 2011).

Myzus persicae (Sulzer), known as the green peach aphid, is an agricultural pest worldwide. Green peach aphid feeds on hundreds of host plants in more than 40 plant families (Annis et al. 1981). This sap-sucking insect can attain very high densities on young plant tissue, causing water stress, wilting, and reduced growth rate of the plant. Prolonged aphid infestation can cause appreciable reduction in yield of root crops and foliage crops. But, the major damage caused by green peach aphid is by transmission of a variety of plant viruses. Indeed, this aphid is believed by many to be the most important vector of plant viruses, for example potato virus Y and potato leaf roll virus, to members of the nightshade/potato family Solanaceae, and various mosaic viruses to many other food crops throughout the world. Nymphs and adults are equally capable of virus transmission (Namba and Sylvester 1981).

The gap gene *hb*, which codes for a zinc-finger-containing transcription factor, is a key regulatory gene in the anteroposterior patterning in a number of insects (Jürgens et al. 1984; Lehmann and Nüsslein-Volhard 1987; Liu and Kaufman 2004; Patel et al. 2001; Schröder 2003; Tautz et al. 1987). *hb* mRNA can be synthesized maternally and zygotically. The maternal RNA is distributed homogeneously in the embryo and is under the control of the posterior maternal factor *nanos* (*nos*). Zygotic expression of *hb* is regulated by the anterior maternal gene *bicoid* (*bcd*) (Wolff et al. 1995). In *Drosophila*, loss-of-function alleles for *hb* lead to defects in the anterior region, for example deletions of gnathal and thoracic segments (Finkelstein and Perrimon 1990; Tautz et al. 1987). The single depletion of maternal and zygotic *hb* by parental RNAi in both *Tribolium* and *Nasonia* leads to serious defects in the head and thorax. Knockdown of both *hb* and *otd*, another gap gene, results in failure to form the head, thorax, and anterior abdomen (Lynch et al. 2006; Schröder 2003). In the milkweed bug *Oncopeltus*, *hb* (*Of'hb*) RNAi depletion results in transformations of gnathal and thoracic regions into an abdominal identity, and impaired posterior elongation and segmentation (Liu and Kaufman 2004). All these reports suggest that knockdown of the *hb* gene in insects is deforming and lethal to the next generation.

The potential of *hb* as an RNAi target has been reported by Mao and Zeng (2012). In the study, feeding of *hb* dsRNA was lethal to the pea aphid, *Acyrthosiphon pisum*. Here, we first report plant-mediated RNAi of the *hb* gene in *M. persicae*. A 1,325-bp fragment of *Mphb* cDNA was cloned for *M. persicae*. A vector for in-planta expression of *Mphb* dsRNA was constructed and transformed into tobacco. DNA blot showed different integration of the transgene. When *M. persicae* nymphs were fed on *Mphb* dsRNA-expressing tobacco, *Mphb* mRNA level was reduced and aphid fecundity was impaired. These results suggested that the *hb* gene can be used as a plant-mediated RNAi target for biocontrol of sap-sucking insects.

Materials and methods

Insect and plant

Tobacco plants (*Nicotiana tobacum* cv. Samsun NN) used for insect rearing and transgenic transformation were grown at 28 °C under a 14 h light and 10 h dark cycle. *M. persicae* aphids obtained from peach trees in Beijing were reared on tobacco plants at 24–25 °C under a 14:10 h light:dark regimen in a greenhouse.

Gene cloning and annotation

Total RNA was extracted from pooled adults by use of Tranzol reagents (Transgene, Beijing, China). DNA contamination was removed by digesting RNA solution with DNase (Ambion, Austin, TX, USA). cDNA

Table 1 Primers used in the experiments

Target genes	Forward	Reverse	Use	Product size (bp)
Mphb	AAAAANCACAARTGCAAACANTG	CCCATGTGSATMGWGTASAKGA	cDNA cloning	1,325
Mphb	TGTCGACCAGCCTCAAGCAGCATC (underline indicates *Sal* I site)	CGGATCCCGACGAGGAGTTGTTATT (underline indicates *Bam*H I site)	Vector construction	427
Mphb	TGCGACTACAAGTGCGTGAG	TGCTGCTTGAGGCTGTGC	qRT-PCR	125
18S rRNA	TCAACACGGGAAACCTCACCA	CACCACCCACCGAATCAAGAA	qRT-PCR	80

was synthesized by use of TransScript First-Strand cDNA Synthesis SuperMix (Transgene, Beijing, China) with anchored Oligo(dT)$_{18}$ primer.

Degenerate primers (Table 1) were designed according to *hb* sequence of other insect species to obtain a partial fragment (1,325 bp) of the *Mphb* cDNA. The PCR was performed at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 57.6 °C for 30 s, and 72 °C for 80 s, finishing with an extension step at 72 °C for 10 min. PCR products were purified by use of the TIANgel Midi Purification Kit (Tiangen, Beijing, China) and then sequenced.

The *Mphb* cDNA sequence obtained was run blast against GenBank. Amino acid encoded by *Mphb* cDNA was aligned with Hb proteins of other insect species to analyze conserved motifs.

Vector construction and plant transformation

Construction of plant RNAi vectors pCAMBIA2300-*Mphb* was based on the pCAMBIA2300 plasmid vector (CAMBIA, Canberra, Australia) and pUCCRNAi vector. Specific primers (Table 1) were designed to amplify a 427-bp target sequence of the *Mphb*. The PCR products were recovered and inserted at inverted repeats into the *Sal* I/*Bam*H and *Xho* I/*Bgl* II sites of the pUCCRNAi vector to obtain a hairpin RNAi construct, which was then inserted into the *Pst* I site of the binary vector pCAMBIA2300 to form a plant RNAi vector pCAMBIA2300-*Mphb*. This final vector and an empty pCAMBIA2300 vector were introduced into *Agrobacterium tumefaciens* strain EHA105. The plant transformation was performed according to the methods described by Horsch et al. (1985). The well-rooted plantlets were transplanted to soil and grown under greenhouse conditions to obtain homozygous transgenic plants in the T2 generation.

DNA detection

Genomic DNA was isolated from tobacco leaves by use of a DNA extraction kit (Tiangen, Beijing, China). Primers were designed to amplify a 440-bp fragment of the CaMV 35S promoter.

For Southern hybridization, DNAs (20 µg) were digested with *Eco*R I, subjected to electrophoresis on a 1.0 % agarose gel, and transferred to Hybond-N nylon membranes. An *Mphb* fragment labeled by the Dig-High Prime method was used as probe. Hybridization and washing processes were carried out according to the instruction manual of the Dig High Primer DNA Labeling and Detection Starter Kit II (Roche, Germany).

Aphid bioassays

T2 homozygous plants (lines 13, 25, and 32) with *Mphb* dsRNA expression were challenged by neonate aphids and tested for effects on aphid survival, biomass, and fecundity. Empty vector-transformed plants were used as controls. For all experiments, plants were grown at 24 ± 1 °C with 70 % relative humidity under a 16-h light and 8-h dark cycle. At 4–5 leaf stage, 10 neonatal nymphs of *M. persicae* in instar 1 were deposited on a top leaf of each plant. Aphid mortality was recorded daily. At 14 days post inoculation (dpi), all aphids per plant were weighed and counted. In every experiment, eight plants were used per phenotype. All experiments were repeated three times.

Quantitative real-time PCR

Accumulation level of *Mphb* mRNA in *M. persicae* reared on transgenic tobacco was investigated by qRT-

PCR using an IQ-5 Real-Time System (Bio-Rad, California, USA). Total RNAs were isolated from feeding aphids at 2, 7, and 14 dpi and cDNA was synthesized according to conventional procedures. qRT-PCR was performed in a final volume of 25 µl containing cDNA produced from 2 µg total RNA, 11.25 µl SYBRH Green Real-time PCR Master Mix (TOYOBO, Japan), and 200 nM each of forward and reverse *Mphb* specific primers (Table 1). Standard curves were obtained by tenfold serial dilution and three technical replicates of each reaction were performed. *M. persicae* 18S rRNA (GenBank Accession Number: AF487712.1) was used as internal control and specific primers (Table 1) were designed for normalization. Means and standard errors for each time point were obtained from an average of three independent sample sets. Quantification of the relative changes in gene transcript level was performed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

Cloning and characterization of the *M. persicae hunchback*

A 1,325 bp fragment of *Mphb* (GenBank Accession Number: KC481323) was cloned by using degenerate primers designed to match conserved regions known to flank *hb* homeodomanis. *Mphb* cDNA had the highest identity of 90 % with *Acyrthosiphon pisum hb* (*Aphb*) mRNA sequence (GenBank Accession Number: NM_001162510.1). Similar to the orthologue *Aphb*, the 441 amino acids encoded by *Mphb* also contains four zinc fingers (MF 1–4), a C box, and a Basic box (data not shown). All these motifs are conserved among Hb proteins.

Construction and molecular analysis of transgenic RNAi plants

The dsRNA-expressing construct pCAMBIA2300-*Mphb* was developed for a 427-bp fragment of the *Mphb* cDNA under the control of CaMV 35S promoter and the nopaline synthase (nos) terminator cassette (Fig. 1). Tobacco leaf discs were infected by *A. tumefaciens* strain EHA105 harboring pCAMBIA2300-*Mphb* or empty pCAMBIA2300 vector. A total of 57 independent kanamycin-resistant transformants were regenerated (39 *Mphb* transformants and 18 empty vector transformants). Most had wild-type morphology and growth.

Genomic DNAs were extracted from transgenic plants. Positive transformants were determined by PCR analysis of the 35S promoter. Twenty-eight *Mphb* transformants and 11 empty vector transformants showed the presence of CaMV 35S promoter sequence, while wild type plants did not show specific amplification (results not shown). These T0 transformants were reproduced to obtain homozygous T2 transgenic lines.

Integration pattern of the *Mphb* dsRNA-expressing cassette in PCR-positive plants (13, 25, and 32) were investigated by Southern hybridization. The three transgenic transformants showed one integration copy, but no *Mphb* hybridization band was observed with genomic DNA from empty vector plant (Fig. 2). The unique hybridization patterns indicated that each investigated plant resulted from independent transformation events.

Aphid resistance of dsRNA-expressing tobacco

T2 *Mphb* dsRNA-expressing homozygous plants (lines 13, 25 and 32), together with empty vector plants, were challenged by neonate aphids and tested for effects on aphid survival, growth, and reproduction. Each tobacco plant was inoculated with 10

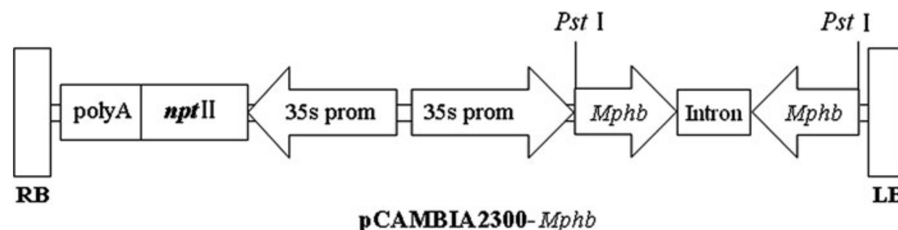

Fig. 1 Construction of the plant RNAi vector. Plant RNAi vector pCAMBIA2300-*Mphb* was constructed on the basis of the pCAMBIA2300 plasmid vector and pUCCRNAi vector. *LB* left border; polyA cauliflower mosaic virus terminator; *npt* II, kanamycin resistance gene; 35S prom, 35S cauliflower mosaic virus promoter; *Mphb*, *M. persicae hunchback gene*; *OCS*, polyA signal from octopine synthase; *RB* right border

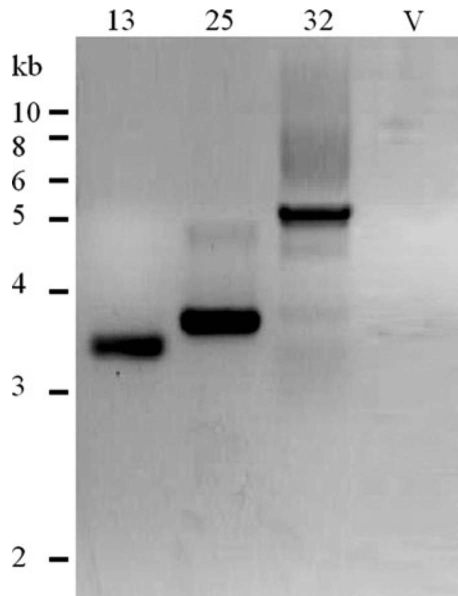

Fig. 2 Southern blot analysis of transgenic tobacco. Total DNA (20 μg) from T0 *Mphb* plants (*13*, *25*, and *32*) and empty vector plants (*V*) was digested with restriction enzyme *Eco*R I and subjected to 1.0 % agarose gel electrophoresis. DNA samples were transferred to a nylon membrane and hybridized with the Dig-labeled DNA probe. Three transformants showed different integration patterns

neonatal nymphs and aphid survival was investigated 7 days post inoculation (dpi). No statistical difference in survival was observed between *Mphb* dsRNA-expressing lines and control plants (empty vector plants) (results not shown). At 14 dpi, aphids on *Mphb* dsRNA-expressing plants had statistically smaller populations than those on control plants. The aphid population on each control plant increased from 10 insect per plant to an average of 107 per plant in a 14-day period. But aphid number on the *Mphb* dsRNA-expressing plant was a maximum of 93 at 14 dpi (Fig. 3a). Aphids on *Mphb* dsRNA-expressing plants also had lower insect biomass compared with those on empty vector plants (Fig. 3b). On this day, each *Mphb* dsRNA-expressing plant yielded a biomass of approximately 13 mg. But each empty vector plant produced an average of 15.5 mg.

Depletion of *Mph*b mRNA after ingestion of transgenic tobacco

At 2, 7, and 14 dpi, *Mphb* mRNA accumulation level after ingestion of transgenic tobacco was analyzed by

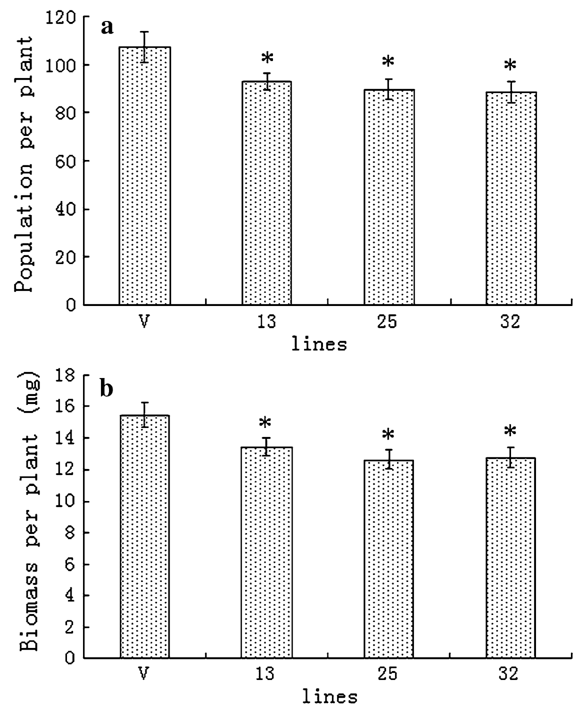

Fig. 3 Inhibition of the transgenic tobacco on population and biomass of *M. persicae*. **a** mean population produced on a given plant; **b** mean insect biomass produced on a given plant. Values are expressed as mean ± SE of eight replicates. *Asterisks* indicate significant differences between the treatment and the control determined by a *t* test (*, $p < 0.05$). Compared with empty vector plants (V), T2 homozygous *Mphb* plants (lines *13*, *25*, and *32*) showed suppression of fecundity and biomass of the *M. persicae* aphids

quantitative real-time PCR. In nymphs fed on *Mphb* plants, *Mphb* mRNA level had an obvious decrease and the *Mphb* transcripts abundance was reduced by approximately 12 % on day 2. The reduction level then increased with elongation of feeding period and reached 31.3 % on the seventh day and 32.4 % on the 14th day (Fig. 4).

Discussion

So far, dsRNA-mediated RNAi has been demonstrated in several insect orders, including Coleoptera (Tomoyasu et al. 2008), Diptera (Dzitoyeva et al. 2001), Hymenoptera (Lynch and Desplan 2006), Hemiptera (Chen et al. 2010; Liu et al. 2010; Mao and Zeng 2012; Zhu et al. 2011), Orthoptera (Meyering-Vos and Muller 2007), Lepidoptera (Terenius et al. 2011; Turner et al.

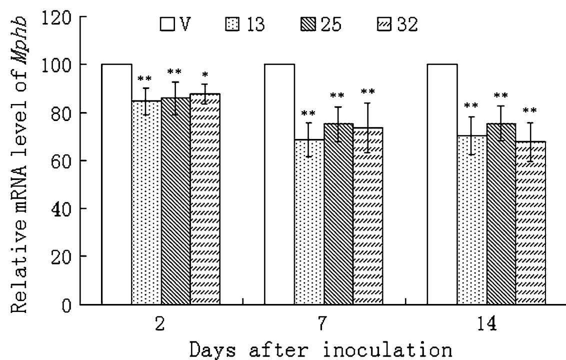

Fig. 4 *Mphb* mRNA depletion after feeding on dsRNA-expressing tobacco. *Mphb* mRNA level was analyzed by qRT-PCR 2, 7, and 14 days after ingestion of transgenic plants. 18S rRNA was used as internal control for normalization. Normalized *Mphb* expression was expressed as the proportion of that recorded for the vector control plant. Each kinetic point was expressed as mean ± SE of three replicates. *Asterisks* indicate significant differences in *Mphb* transcripts levels between the treatment (lines *13*, *25*, and *32*) and the control (*V*) determined by a *t* test (*$p < 0.05$; **$p < 0.01$)

2006) and Isoptera (Zhou et al. 2008). In these reports, dsRNA was usually delivered to insects by artificial feeding or injection. But these two methods are not applicable in farmland. Expressing dsRNA in a plant is another effective approach exploited to control insect pests. Transgenic plants expressing target gene dsRNA had significant resistance against Lepidoptera species, for example western corn rootworm (WCR) *D. virgifera virgifera* vacuolar (Baum et al. 2007) and cotton bollworm (*H. armigera*) (Mao et al. 2007). Furthermore, plant-mediated RNAi successfully depleted target gene expression in sap-sucking insect. When *N. lugens* nymphs were fed on rice plants expressing dsRNAs of the three targeted genes, RNA interference was triggered but lethal effect was not observed (Zha et al. 2011). Aphids (Hemiptera: Aphididae) are exclusive phloem feeders and are among the most economically important pest insects of temperate agriculture. In addition to the effect of feeding, aphids also transmit plant viruses. Now, development of transgenic crops expressing toxins derived from the bacterium *Bacillus thuringiensis* (Bt) has provided effective plant protection against some insect pests, but Bt toxins have little toxicity against sap-sucking insects (Chougule and Bonning 2012). Here, we report plant-mediated RNAi in *M. persicae*, a phloem-sucking agricultural pest throughout the world. Transgenic plants expressing of *Mphb* dsRNA not only suppressed target gene expression, but also impaired insect fecundity. The significance of this study is that it demonstrated the feasibility of plant-mediated RNAi in biocontrol of sap-sucking insect pests.

hunchback, which encodes a zinc-finger-containing transcription factor, is crucial for axial patterning in insects (Lehmann and Nüsslein-Volhard 1987; Liu and Kaufman 2004; Patel et al. 2001; Schröder 2003; Tautz et al. 1987). *Acyrthosiphon pisum* Hb protein contains six zinc finger domains and two other conserved motifs, C box and Basic box. In this study, C box and Basic box was present in *Mphb* protein, but we identified only four zinc fingers, not six. It remains possible that *Mphb* contains two other zinc fingers, because we did not obtain a full length of *Mphb* cDNA. In addition, sequencing of genomic DNA revealed that the *Mphb* middle fragment obtained contains no introns (data not shown). This suggested that, similar to its homologs in other insect species, the whole *Mphb* locus contains no introns. Similarly, *M. persicae* should have only one copy of *hb*.

Aside from the crucial functions in the early embryonic anteroposterior patterning, *hb* is important in sequential cell fate specification within the *Drosophila* central nervous system (CNS) (Novotny et al. 2002). But up to now, there are no further reports about how the *hb* functions in CNS development of other insects. Although defective and lethal phenotypes were all absent in our RNAi experiments, we inferred that the reduction of insect population resulted from impairment of the *hb* functions in the early embryonic anteroposterior patterning, not the role in specifying the CNS development. Further investigation is required to confirm this. In this study, both population and biomass of aphids reared on *Mphb* RNAi plants were suppressed. We inferred that the reduction of biomass resulted from the inhibited population, because average individual weight of aphids fed on *Mphb* RNAi plants and control plants was no different (data not shown).

Parental RNAi of *hb* is deforming and lethal to the next generation (Lehmann and Nüsslein-Volhard 1987; Liu and Kaufman 2004; Patel et al. 2001). In *Oncopeltus fasciatus*, maternal depletion of *hb* transcripts resulted in different classes of defect in most progeny (Liu and Kaufman 2004). Continuous feeding of *Acyrthosiphon pisum hunchback* (*Aphb*) dsRNA mixed in an artificial diet led to a maximum 50 % reduction of *Aphb* transcripts and more than doubling of insect lethality, indicating that *hb* was a good RNAi

target for management of insect pests. In this study, ingestion of dsRNA-expressing tobacco only resulted in a 17.6 % reduction of fecundity and 18.4 % suppression of biomass. In addition, defective phenotypes frequently appeared after *hb* depletion in other insect species but were absent in this feeding bioassay. So, what is the reason? It is less likely that other paralogues in *M. persicae* compensate for suppression of *Mphb*, because such insects as *A. pisum* usually have one *hb* only (Huang et al. 2010). One explanation is that *Mphb* dsRNA was not expressed at a high level in transgenic plants and uptake amount of siRNA was not sufficient to trigger defective or lethal effect and to cause high population suppression. Another possible reason is that ingested siRNA is not sufficient to penetrate into target parental and embryonic cells of the *M. persicae* aphids, because RNAi efficiency may vary for different target genes, different insect species, and different organs. Further work is needed to verify this speculation.

No guarantee of sufficient uptake of dsRNA or siRNA by insects is, maybe, a drawback of plant-mediated RNAi. Neither dsRNA injection nor artificial feeding has this problem, because large amounts of dsRNA synthesized at high concentration can be delivered to insects by these two methods. Another problem we must mention is that it is relatively easier to realize plant-mediated RNAi in chewing mouthparts insects than in stylet mouthparts species. This is because the former insects, for example Lepidoptera, take up all of the plant tissue with chewing mouthparts. Thus, dsRNA expressed in cells can be easily delivered to the insect digestive tract, whereas stylet mouthparts insects, for example aphids, are quite different because they suck sap from plant phloem. Theoretically, plant-mediated RNAi can be transformed into an applicable method for bio-control of stylet mouthparts insects only if three requirements are all met:

1. dsRNA of the target sequence is synthesized at a high level;
2. enough dsRNA is processed into siRNA; and
3. sufficient dsRNA or siRNA is ingested by insects and penetrates the nucleus.

Lack of any of these conditions will lead to failure of RNAi. Transgenic plants expressing dsRNA specific to *MjTis11*, a transcription factor in a root knot nematode, did not result in a lethal phenotype (Fairbairn et al. 2007). In another study, RNA interference was triggered but lethal phenotypic effects after dsRNA feeding were not observed when *N. lugens* nymphs were fed on rice plants expressing dsRNAs of three target genes (Zha et al. 2011). These plant-mediated RNAi cases indicated that further work is urgently needed to increase dsRNA level in transgenic plants, to promote dsRNA or siRNA uptake by sap-sucking insects, and to achieve expected phenotypes after target gene depletion.

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