Suppression of Bedbug’s Reproduction by RNA Interference of Vitellogenin

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Abstract

Recent resurgence of the bedbug Cimex lectularius is a global problem on the public health. On account of the worldwide rise of insecticide-resistant bedbug populations, exploration of new approaches to the bedbug control and management is anticipated. In this context, gene silencing by RNA interference (RNAi) has been considered for its potential application to pest control and management, because RNAi enables specific suppression of target genes and thus flexible selection of target traits to be disrupted. In this study, in an attempt to develop a control strategy targeting reproduction of the bedbug, we investigated RNAi-mediated gene silencing of vitellogenin (Vg), a major yolk protein precursor essential for oogenesis. From the bedbug transcriptomes, we identified a typical Vg gene and a truncated Vg gene, which were designated as ClVg and ClVg-like, respectively. ClVg gene was highly expressed mainly in the fat body of adult females, which was more than 100 times higher than the expression level of ClVg-like gene, indicating that ClVg gene is the primary functional Vg gene in the bedbug. RNAi-mediated suppression of ClVg gene expression in adult females resulted in drastically reduced egg production, atrophied ovaries, and inflated abdomen due to hypertrophied fat bodies. These phenotypic consequences are expected not only to suppress the bedbug reproduction directly but also to deteriorate its feeding and survival indirectly via behavioral modifications. These results suggest the potential of ClVg gene as a promising target for RNAi-based population management of the bedbug.

Introduction

An important factor that makes insects serious pests is their high reproductive ability. Hence, understanding how insects produce plentiful offspring and how the reproductive capability can be suppressed have been among major subjects in researches on pest control and management [1–3]. In most insects, as in oviparous animals in general, females deliver a considerable amount of nutritious resources to their eggs, which is mainly mediated by a major yolk precursor protein called vitellogenin (Vg) [4–7]. Vg protein, a member of the large lipid transfer
protein superfamily [8], is mainly synthesized in the fat body and subjected to a variety of post-translational processing. Typically, Vg protein is proteolytically cleaved at particular sites, and the subunits are assembled together with lipids, carbohydrates and other nutrients, thereby forming a large oligomeric phosphoglycolipoprotein [6,7]. The complex is subsequently secreted into hemolymph and taken up by growing oocytes through endocytosis mediated by Vg receptors (VgR) [5].

The common bedbug *Cimex lectularius* (Hemiptera: Cimicidae) is a nuisance pest that feeds on blood of humans and other warm-blooded animals [9,10]. Although the bedbugs do not transmit fatal disease agents of humans, their bites annoy people by causing cutaneous manifestations, urticarial reactions and, occasionally, anaphylaxis [11]. The domestic infestation of this nuisance pest may result in adverse effects on mental health of residents, such as emotional distress, anxiety, insomnia and paranoia [12]. A single female bedbug can produce 200–500 eggs during its lifetime, which underlies its rapid population growth once infestation occurs [13]. The recent resurgence of the bedbug across the world, which might have been facilitated by increasing international travel and trade, is regarded as a global problem on public health [10,11]. Conventional eradication means are nowadays not effective, mainly because of the rise of insecticide-resistant populations of the bedbug [12]. Therefore, exploration of new approaches to the bedbug control and management is of urgent need.

In this context, gene silencing by RNA interference (RNAi) has recently attracted much attention not only in characterizing gene functions but also in controlling insect pests [14–17]. The high specificity of RNAi machinery against target nucleotide sequences conceptually enables eradication programs with minimal ecological side effects. It can also provide an alternative strategy for pest management without direct lethal actions, in which key genes responsible for pest status including fecundity, ability to utilize peculiar plant/animal hosts, pathogen transmitting capacity, resistance to chemical pesticides, etc., are targeted [14,18,19].

In diverse pest arthropods including cockroaches, fire ants and ticks, previous studies reported successful instances of RNAi targeting *Vg* gene [20,21] or *VgR* gene [22–24], which generally caused negative consequences in the ovarial development. Several studies reported that RNAi works in the bedbug, although those studies are not intended to population control of the nuisance pest [25,26]. In this study, in an attempt to develop a novel approach to reproduction control of the bedbug, we investigated *Vg* genes as a target of RNAi-mediated gene silencing.

### Materials and Methods

#### Insect

A laboratory strain of the bedbug JESC, which had been maintained at the Japan Environmental Sanitation Center for decades [27], was used in this study. Adults and nymphs were kept in plastic Petri dishes (9 cm in diameter and 2 cm in depth) with several pieces of pleated filter paper (2 cm x 3 cm) at 25°C under constant darkness. The insects were fed once a week on commercially-purchased rabbit blood (Kohjin Bio, Japan) warmed at 35–36°C using a membrane feeding system as described previously [27].

#### Identification of Bedbug Vg Genes

*Vg* genes of the bedbug were surveyed in the expression sequence tag (EST) database [28] and the RNA sequencing (RNAseq) database [29]. The short RNAseq reads (SRA accession number, SRP008480) were assembled using Trinity v2.0.2 [30]. Using BLASTX similarity searches against the Uniprot protein database (www.uniprot.org), we obtained partial fragments of the *Vg* genes. Then, full-length transcript sequences were obtained by PCR amplification and
DNA sequencing using the primers listed in Table 1 and the bedbug cDNA libraries constructed previously [28]. The Vg gene sequences determined in this study were deposited in the DNA Data Bank of Japan with the accession numbers LC115022 and LC115023.

### Molecular Phylogenetic and Evolutionary Analyses

Multiple alignment of Vg protein sequences was conducted using MUSCLE program [31]. Phylogenetic trees were constructed by maximum-likelihood and neighbor-joining methods using MEGA ver. 6.0 with 1,000 bootstrap replications [32]. Relative rate tests were performed using RRTree [33].

### Quantification of Vg Gene Expression

Reverse-transcription quantitative PCR (RT-qPCR) was conducted for evaluating expression levels of the Vg genes in different tissues of the bedbug essentially as described previously [28]. Adult females of 1–2 months after emergence were dissected in a phosphate buffered saline. Total RNA samples of the isolated tissues were purified and reverse-transcribed using RNAiso plus (TaKaRa), RNAeasy columns (QIAGEN) and Improm II Reverse Transcription System (Promega), and subjected to RT-qPCR using the primers listed in Table 1. A standard curve was drawn using the PCR fragment cloned into the pT7 blue plasmid (Novagen). Expression levels of the Vg genes were normalized by quantifying expression levels of elongation factor 1α.

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Table 1. Primers used in this study.

| Name         | Gene     | Sequence (5’ to 3’) | Usage^a |
|--------------|----------|---------------------|---------|
| ClVg_235_F   | ClVg     | GGA AAA CTC ACC GTC CAA CC | cloning |
| ClVg_370_R   | ClVg     | TTG TTG GAA AAG GGG AGT TG | cloning |
| ClVg_1660_F  | ClVg     | CCA TAC TCC AAA GCA TCA CC | cloning |
| ClVg_1686_R  | ClVg     | CGT TCG GCA TCA GAA GTG | cloning |
| ClVg_3341_R  | ClVg     | TTC GAC ATC AAC ATC GAC AC | cloning |
| ClVg_4021_F  | ClVg     | CGA TTC TCT TCC TTC GTG AG | cloning |
| ClVg_4106_R  | ClVg     | AAG TGG GAC ATT GGG TGA AG | cloning |
| ClVg_4501_R  | ClVg     | TCA AAC CTG ACA ACG ACA TCT AC | cloning |
| ClVg_4634_F  | ClVg     | TCC ACC CAC ATT ACA ACA CC | cloning/dsRNA |
| ClVg_4942_R  | ClVg     | GCT GTC TGT CCG TTG ACC TT | cloning/dsRNA |
| ClVg_5342_F  | ClVg     | ACA GCC GCA AAT CAC AAG AC | qPCR |
| ClVg_5474_R  | ClVg     | GGC ACT CGG GCA TCT TTC | qPCR |
| ClVgL_13_F   | ClVg-like | ATT TTT AAT CGT CCC GCC GC | cloning |
| ClVgL_83_R   | ClVg-like | AGG GAC GAA TGA TCC ATC GC | cloning |
| ClVgL_1380_F | ClVg-like | ACA ACG AGA CAG TGT CAT CCC | cloning |
| ClVgL_1431_R | ClVg-like | TGG TTT TCA AGC TCT CCA TG | cloning |
| ClVgL_2691_F | ClVg-like | TGC ATA CTC AAG CAG TCC GG | cloning |
| ClVgL_2778_R | ClVg-like | GCA ACG AAA CCT GGA AAG GC | cloning |
| ClVgL_4033_F | ClVg-like | ACC GAC AAG ATG AGC AGA GC | cloning/pPCR |
| ClVgL_4119_R | ClVg-like | TGC AAG ACG GAG TTT GAT CG | cloning/pPCR |
| ClEF1alpha_Fb| Ef1α     | TGG TAT CGA CAA ACG TAC CAT C | qPCR |
| ClEF1alpha_Rb| Ef1α     | GCT CGG CCT TGA GCT TGT C | qPCR |

^a Primers were designed for the following purposes: cloning, Vg gene sequence identification; dsRNA, gene fragment amplification for dsRNA synthesis; qPCR, gene expression quantification by RT-qPCR.

^b These primers were originated from Moriyama et al. [28].

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(EF1α) gene using the primers listed in Table 1. Each biologically independent sample was measured twice and averaged.

RNAi Treatment and Phenotype Inspection

Double-stranded RNA (dsRNA) was synthesized from the PCR product of the Vg gene using MEGAscript RNAi kit (Ambion), in which fragment amplification was conducted using the gene specific primers (see Table 1) attached to T7 promoter sequence. For control experiments, dsRNA of β-lactamase gene fragment from pT7 blue plasmid was synthesized. Adult insects within two weeks after emergence were collected and assigned to each treatment group. Three females and three males were placed in each plastic petri dish (6 cm in diameter and 1.5 cm in depth) with two pieces of pleated filter paper, where they were allowed to mate freely. After two days from the first blood meal, the females were injected with 20 ng or 200 ng of dsRNA dissolved in 0.5 μl of distilled water from the basement membrane of a hind leg using a fine glass capillary needle. These bedbugs were fed once a week, when the number of eggs and hatched nymphs were recorded. Four weeks after the injection, these females were subjected to histological analysis or RNA extraction. The ovaries of some females were dissected out, photographed, and measured under a stereoscopic microscope and digital camera system (S8Apo and EC3, Leica Microsystems). Total RNA was extracted from the whole body, and expression levels of the Vg gene were quantified as described above.

Statistics

Gene expression levels were compared by fitting to a generalized linear model (GLM) [34], where Gamma error structure with log link function was assumed. The following models were selected for each GLM analysis: binomial error structure with logit link function for survival and hatching rates; Gaussian error structure for oocyte size; and Poisson error structure with log link function for number of eggs. When sample overdispersion was observed, we adopted a generalized linear mixed model (GLMM) that considers individual variation as a random effect. If deviance reduction due to the treatment term was significant in chi-square test, we compared its effect between each treatment group by Tukey-type multiple comparisons. All statistical analyses were performed using R ver. 3.2 [35].

Results

Bedbug Vg and Vg-like Genes

From transcriptome databases of the bedbug [28,29], we obtained partial gene sequences exhibiting significant similarities to known Vg genes. By making use of the sequences, we cloned and identified full-length cDNA sequences of two putative Vg genes. Fig 1A shows the amino acid sequence (1,863 residues) deduced from the first Vg gene, which encoded all conserved structures typical of insect Vg proteins [6]. In addition to the well-conserved RXXR cleavage site flanked by polyserine motifs at the N-terminal region, this protein contained another RXXR cleavage site at the C-terminal region (Fig 1A), indicating possible multiple cleavages as known for Vg protein of the bean bug Riptortus pedestris [36]. The amino acid sequence inferred from the other Vg gene (1,449 residues) showed 36% sequence identity to the sequence of the former Vg protein. This sequence was somewhat diverged from those of conventional Vg proteins known from diverse insects, with a truncated lipid binding domain, so-called Vitellogenin_N, and without the polyserine motif (Fig 1B). In addition, there was an amino acid substitution in the well-conserved DGXR-GL/ICG motif at the C-terminal region [6]. Hereafter we refer to the former gene as ClVg, and the latter gene as ClVg-like.
In the NCBI non-redundant protein database, the ClVg protein was the most similar to Vg2 of Triatoma infestans (Hemiptera: Reduviidae) with 52% sequence identity, while the ClVg-like protein was the most similar to Vg of Apolygus lucorum (Hemiptera: Miridae) with 36% sequence identity. Molecular phylogenetic analyses were performed by including Vg protein sequences of hemipterans and other insects after removing the N-terminal region that was lacking in the ClVg-like protein (Fig 2). While both the ClVg protein and the ClVg-like protein were placed within the clade of the order Hemiptera, the ClVg-like protein was located outside the infraorder Cimicomorpha to which bedbugs, reduviid bugs, and mirid bugs belong. On the phylogeny, the ClVg-like branch was elongated (Fig 2), and relative rate tests showed accelerated molecular evolution in the lineage of ClVg-like gene (Table 2). Hence, although the ClVg-like protein was placed even outside of the belostomatid water bug Lethocerus deyrollei of the infraorder Nepomorpha, it seems doubtful that the ClVg-like gene diverged from the common ancestor of the Cimicomorpha and the Nepomorpha, considering the low resolution of the phylogeny and the accelerated molecular evolution in the lineage of the ClVg-like gene.

Tissue Specific Expression of Vg and Vg-like Genes

We investigated expression levels of the ClVg gene and the ClVg-like gene in dissected tissues of adult bedbugs. In adult females, the ClVg gene was highly expressed in the fat body, whereas it exhibited a low level of expression in the spermalege, a female-specific abdominal organ involved in traumatic insemination [37] (Fig 3A). Little expression of the ClVg gene was detected in adult males (Fig 3A). Expression patterns of the ClVg-like gene were similar to those of the ClVg gene, but the expression levels were $10^2$–$10^3$ times lower (Fig 3B). These results strongly suggest that the ClVg gene represents the primary functional Vg gene in the bedbug.

RNAi-Mediated Silencing of Vg Gene Expression

For RNAi-mediated silencing of the ClVg gene expression, dsRNA targeting the ClVg gene was injected into the hemocoel of adult females at the dose of 20 ng or 200 ng per insect. Expression levels of the ClVg gene were drastically suppressed even four weeks after the dsRNA injection (Fig 4). During the experimental period of four weeks, mortality rates were generally low (4–12%), and survival rates were statistically not different among the treatment groups (Fig 5A). On the other hand, the ClVg dsRNA injection resulted in remarkable phenotypic
consequences. The abdomen of adult females subjected to the ClVg RNAi was conspicuously swollen like fully-engorged bedbugs (Fig 6, “Dorsal” and “Lateral” columns). The swollen abdomen of the ClVg RNAi females was full of hypertrophied fat bodies, which was in contrast to much less developed fat bodies adhering to the inner wall of the thinner abdomen of the control females (Fig 6, “Inside” column). In the ClVg RNAi females, ovaries were atrophied with no mature oocytes, which were in contrast to well-developed ovaries containing mature oocytes in the control females (Figs 5B and 6, “Ovary” column). In the ClVg RNAi females, egg production was drastically and significantly suppressed in comparison with the control females (Fig 5C). Notably, in all eight replicate groups (three females each) injected with 200 ng...
dsRNA, and six of eight replicate groups injected with 20 ng dsRNA, females completely ceased egg production two weeks after the dsRNA injection. It is also notable that eggs produced by the ClVg RNAi females tended to suffer low hatching success (Fig 5D). Taken together, it was concluded that RNAi targeting the ClVg gene effectively inhibited the bedbug reproduction.

Discussion

Vg proteins are the major yolk precursor proteins whose structure and function are conserved among diverse insect species, although there are some diversity in number of cleavage sites, number of duplicated genes, and tissue-specific expression patterns [6,7]. In this study, we identified two Vg genes, ClVg and ClVg-like, in the bedbug (Fig 1). Some insects possess multiple Vg genes, whose sequences are usually highly similar to each other with retaining conserved functional domains and participating in vitellogenesis [38–41]. However, the two Vg genes of the bedbug are considerably different in their sequences. The ClVg gene exhibited canonical insect Vg protein structures including RXXR cleavage sites and conserved binding sites for a variety of nutrients (Fig 1A), and its transcription was highly up-regulated in the fat body of adult females (Fig 3A). RNAi-mediated silencing of the ClVg gene expression confirmed its primary contribution to oocyte maturation (Figs 5 and 6). These results indicate that the ClVg gene encodes the principal Vg protein involved in the conventional vitellogenic function in the bedbug. On the other hand, the ClVg-like gene lacked the N-terminal lipid-binding region (Fig 1B), although the ClVg gene and the ClVg-like gene are presumably derived from the common ancestral Vg gene (Fig 2). Since the ClVg-like gene still retains putative cleavage sites and some domains typical of insect Vg genes (Fig 1B), the possibility cannot be ruled out that the ClVg-like protein may be subjected to posttranslational processing and also incorporated into the oocytes as typical Vg proteins. However, expression levels of the ClVg-like gene in the fat body

Table 2. Relative rate tests for comparing the molecular evolutionary rate of ClVg protein sequence of the bedbug with those of ClVg-like protein sequence and other Vg protein sequences of hemipteran insects based on 1,235 unambiguously aligned amino acid sites.

| Lineage 1a | Lineage 2b | Outgroup | K1 | K2 | K1-K2 | K1/K2 | P-valuec |
|------------|------------|-----------|----|----|-------|-------|---------|
| Vg-like, Cimex lectularius | Vg, Cimex lectularius | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.643 | 0.360 | 0.283 | 1.79 | 1.0 x 10^-7 |
| Vg-like, Cimex lectularius | Vg, Apolygus lucorum | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.648 | 0.330 | 0.318 | 1.96 | 1.0 x 10^-7 |
| Vg-like, Cimex lectularius | Vg, Nesidiocoris tenuis | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.639 | 0.336 | 0.303 | 1.90 | 1.0 x 10^-7 |
| Vg-like, Cimex lectularius | Vg, Trigonotylus caelestialium | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.656 | 0.407 | 0.249 | 1.61 | 2.1 x 10^-7 |
| Vg-like, Cimex lectularius | Vg2, Triatoma infestans | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.647 | 0.354 | 0.293 | 1.83 | 1.0 x 10^-7 |
| Vg-like, Cimex lectularius | Vg, Lethocerus deyrollei | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.613 | 0.360 | 0.253 | 1.70 | 1.5 x 10^-7 |
| Vg-like, Cimex lectularius | Vg, Riptortus pedestris | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.666 | 0.499 | 0.167 | 3.33 | 2.8 x 10^-4 |
| Vg-like, Cimex lectularius | Vg, Geocoris pallidipennis | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.659 | 0.509 | 0.150 | 1.29 | 1.2 x 10^-3 |
| Vg-like, Cimex lectularius | Vg1, Plautia stali | Vg, Pediculus humanus; Vg, Nilaparvata lugens | 0.637 | 0.522 | 0.115 | 1.22 | 0.014 |

a Estimated mean distance between lineage 1 and the last common ancestor of lineages 1 and 2.
b Estimated mean distance between lineage 2 and the last common ancestor of lineages 1 and 2.
c P-value was generated using the program package RRTree [33].

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were incomparably lower than those of the \textit{ClVg} gene (Fig 3), refuting the possibility of substantial contribution of \textit{ClVg-like} protein to vitellogenesis. Considering its structural divergence (Fig 1) and accelerated molecular evolution (Table 2), it is conceivable, although speculative, that the \textit{ClVg-like} gene might have acquired a novel biological function distinct from the vitellogenic role of the \textit{ClVg} gene in the evolutionary course of the bedbug. In this context, previous studies suggested that Vg proteins may also be involved in hemolymph clotting [8,42], hormonal regulation [43], innate immune responses [44], and other biological roles. Further studies are needed to address what roles the \textit{ClVg-like} gene plays in the bedbug.

Although the main location of Vg protein synthesis is the fat body in diverse insects [6,7], there are several reports on Vg protein production outside the fat body. In a blood-sucking
reduviid bug *Rhodnius prolixus*, Vg protein is partially synthesized in the follicle cells, which is also incorporated into the oocytes together with the major Vg protein derived from the fat body [45]. In a blood-sucking tick *Haemaphysalis longicornis*, one Vg gene is exclusively expressed in the midgut, the other two Vg genes are mainly expressed in the fat body, and all the three genes are involved in oocyte maturation [21]. In the bedbug, we found that the ClVg gene is also expressed in a female-specific paragenital organ called the spermalege (Fig 3). The spermalege is a pouch-like mesodermal tissue attached to the abdominal inner wall of adult females of cimicid bugs [37], whose function is postulated as a counter adaptation against the traumatic insemination, a peculiar reproductive habit typical of cimicids [46,47]. Although the possibility that the ClVg protein synthesis in the spermalege may somehow contribute to vitellogenesis cannot be ruled out, a more likely possibility is that the ClVg protein may be recruited as a clotting element in this organ [8,42], considering that the spermalege is the site of frequent wounding by traumatic insemination.

We found that RNAi-mediated silencing of the ClVg gene expression effectively inhibits egg production in the bedbug (Figs 5 and 6). The significant inhibitory effects on egg laying became evident two weeks after the dsRNA injection (Fig 5C). The initial egg production is likely attributable to the Vg protein accumulated before the dsRNA injection. Note that we performed the dsRNA injection two days after blood meal, and the bedbugs normally start laying eggs three days after blood feeding [48]. It is also notable that the eggs produced by the ClVg RNAi females tended to suffer low hatching success (Fig 5D), which is also likely attributable to insufficient accumulation of the Vg protein during oogenesis. Furthermore, all females injected with 200 ng dsRNA and most females injected with 20 ng dsRNA completely stopped laying eggs two weeks after the dsRNA injection, in which no mature oocytes were found in the ovaries (Figs 5 and 6). On the basis of these results, we propose that the ClVg gene can be a promising target for RNAi-based control by inducing reproductive arrest of the bedbug.
We also found that abdominal inflation is another remarkable symptom of the ClVg RNAi in the bedbug. The external appearance of the insects looked like that just after full engorgement, but their abdomen was actually full of hypertrophied fat bodies instead of a blood-filled stomach (Fig 6). Such a phenotypic syndrome associated with Vg-RNAi was not observed in the cockroach Blattella germanica [20] and the tick Haemaphysalis longicornis [21], although
in the latter species the Malpighian tubules and the rectal sac were abnormally filled with white liquid [21]. The peculiar abdominal inflation may be relevant to feeding habit of the bedbug. Adult bedbugs ingest blood about two times of their own weight in a single meal by expanding intersegmental membranes of the abdomen, and the acquired nutritional resource is equivalent to production of 15–19 eggs [48,49]. The ClVg RNAi may inhibit not only the synthesis of Vg protein in the fat body but also the transportation of associated nutrients from the fat body to the oocytes. Conceivably, nutritional resources derived from blood meals are stagnated in the fat body, thereby resulting in its hypertrophy and consequent abdominal inflation.

In addition to the nutritional stagnation, the abdominal inflation due to the ClVg RNAi may influence the pest status of the bedbug by way of its behavioral modifications. First, because of the stuffed abdomen, the insects may have little room for further blood ingestion (see Fig 6), thereby presumably suppressing their blood-sucking activity. Second, considering the fact that fully-engorged female bedbugs frequently receive traumatic insemination because their swollen abdomen makes it difficult to take a guard posture against sexually aggressive males [37], it
seems likely that the ClVg RNAi females with the inflated abdomen may suffer continuous and repetitive harassment by conspecific males. It was reported that accumulated traumas reduce female longevity not only by damage of integument piercing but also by increasing risks of microbial infections [46, 47, 50, 51]. Experimental verification of these behavior-mediated effects of the ClVg RNAi deserves future studies.

The recent global resurgence of bedbug populations with resistance to broad-spectrum chemical insecticides has prompted exploration of new pest management strategies [12]. Our study demonstrated that an RNAi approach targeting the ClVg gene causes drastically suppressed egg production and remarkable abdominal inflation in adult females of the bedbug, which possibly reduces reproduction, feeding frequency and longevity of this notorious pest. These findings suggest the possibility that the ClVg gene is a promising candidate for RNAi-based population management of the bedbug, especially in an early phase of population growth on account of its non-acute effects on the insect reproduction. What needs to be solved for enabling practical applications is the development of technologies for efficient delivery of dsRNA into the bedbugs. Microinjection into individual insects is practically not feasible. The effectiveness of oral ingestion of dsRNA has been reported in several hemipteran insects including the blood-sucking reduviid bug Rhodnius prolixus [52–54]. For agricultural insect pests, application of transgenic plants that produce dsRNA of target insect genes has been attempted [55, 56]. For blood-sucking insect pests, however, using transgenic animals for supplying dsRNA-containing blood meal is unrealistic both practically and ethically. Therefore, future studies should be directed to the development of alternative dsRNA delivery methods like chemo-attractive feeding traps [57] and microbial vectors [58, 59], in parallel with survey of other target genes for more efficient control of bedbug populations.

**Author Contributions**

Conceived and designed the experiments: MM TH TF. Performed the experiments: MM TH MT. Analyzed the data: MM NN. Wrote the paper: MM TF.

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