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Dietary Risk Assessment of *Snf7* dsRNA for *Coccinella septempunctata*

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ABSTRACT

Recently, pest-resistant living modified (LM) crops developed using RNA interference (RNAi) technology have been imported into South Korea. However, the potential adverse effects of unintentionally released RNAi-based LM crops on non-target species have not yet been reported. *Coccinella septempunctata,* which feeds on aphids, is an important natural enemy insect which can be exposed to the double-stranded RNA (dsRNA) produced by RNAi-based LM plants. To assess the risk of ingestion of *Snf7* dsRNA by *C. septempunctata,* we first identified the species through morphological analysis of collected insects. A method for species identification at the gene level was developed using a specific *C. septempunctata* 12S rRNA. Furthermore, an experimental model was devised to assess the risk of *Snf7* dsRNA ingestion in *C. septempunctata. Snf7* dsRNA was mass-purified using an effective dsRNA synthesis method and its presence in *C. septempunctata* was confirmed after treatment with purified *Snf7* dsRNA. Finally, the survival rate, development time, and dry weight of *Snf7* dsRNA-treated *C. septempunctata* were compared with those of *GFP* and *vATPase* A dsRNA control treatments, and no risk was found. This study illustrates an effective *Snf7* dsRNA synthesis method, as well as a high-concentration domestic insect risk assessment method which uses dsRNA to assess the risk of unintentional released of LM organisms against non-target species.

Keywords: *Coccinella septempunctata,* Double-stranded RNA, Living modified organism, Risk assessment, *Snf7*

Introduction

Coccinella septempunctata, known as the seven-pointed ladybug, is a natural enemy for many pests. It is distributed across various habitats worldwide and is widely used for biological insect control in various crop systems. Both the larvae and adults feed on insects of Aphidoidea, Psylloidea, and Coccoidea superfamilies, which are found in the leaves and stems of various plants (Dolling, 1991; Kalushkov & Hodek, 2004; Yu *et al*., 2014; Zhang *et al*., 2011). *C. septempunctata* species are easy to breed and are ideal non-target organisms for studying potential toxicity, such as, *Bacillus thuringiensis* or RNA interference (RNAi)-based living modified organisms (Alvarez-Alfageme *et al*., 2012; Harwood *et al*., 2005; Harwood *et al*., 2007).

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Living modified (LM) organisms (RNAi gene silencing usi-double-stranded RNA (dsRNA)) is a powerful tool for targeted gene silencing in insects and offers a novel approach for insect control. Currently, RNAi technology is used as a biotechnological tool for the analysis of gene functions in various organisms and for pest control (Burand & Hunter, 2013; Fire *et al*., 1998; Katoch *et al*., 2013; Zhang *et al*., 2017). Insect toxicity assessment using environmental or dietary methods can be performed using sprays, bait, LM microorganisms, or LM plants (Fischer *et al*., 2016; Le, 2015; Liu *et al*., 2019; Pang & Mao, 1979; Zhang *et al*., 2017; Zhu *et al*., 2011; Zhu & Palli, 2019; Zotti *et al*., 2018). However, the success of the RNAi technique depends on the effectiveness of the in vivo action of the dsRNA in the insect species. RNAi efficacy may vary depending on the expression of the target gene, dsRNA degrading enzymes in the insect, concentration of dsRNA, and the method of delivery. According to previous reports, RNAi techniques have been used to treat western corn root-

worms (*Diabrotica virgifera virgifera*), red powder beetles (*Tribolium castaneum*), Colorado potato beetles (*Leptino-tarsa dece mlineata*), and two-spotted ladybugs (*Adalia bipunctata*) (Baum *et al*., 2007; Haller *et al*., 2019; Yoon *et al*., 2018). The western corn rootworm, which impacts corn yield, responds to a dsRNA diet. Several target genes of dsRNA in western corn rootworms have been evaluated. When the dsRNA encoded an essential functional protein, western corn rootworms larvae were reportedly killed at low concentrations (Baum *et al*., 2007). The RNAi target gene was found to be a homolog of yeast *Snf7*. LM crops which produce dsRNA targeting the *D. virgifera Snf7* showed insect resistance by killing western corn rootworms larvae.

The class E vacuolar sorting protein *Snf7* gene is present in various organisms as *Vps32, CeVps32.2, hSnf7,* and *At2g19830* (Kim *et al*., 2011; Peck *et al*., 2004; Tu *et al*., 1993; Winter & Hauser, 2006). Using the *Snf7* RNAi system, *Snf7* was confirmed to play a role in several cellular processes in mammals and nematodes (Kim *et al*., 2011; Lee *et al*., 2007; Ramaseshadri *et al*., 2013; Sweeney *et al*., 2006). Two methods have been applied to control pests using active *Snf7* dsRNA molecule. The first is the use of LM plant techniques to produce active *Snf7* dsRNA in plants, such as, in LM maize (MON87411). These LM maize plants produce *Snf7* dsRNA targeting the *Snf7* protein, leading to increased western corn rootworms larval mortality and reduced root damage (Bachman *et al*., 2020; Bolognesi *et al*., 2012). The second method is to treat crops externally with *Snf7* dsRNA, for example through trunk injections, food bait, microorganism delivery systems, or topical sprays (Hunter *et al*., 2012; Kunte *et al*., 2020; Li *et al*., 2015; Niu *et al*., 2018; Romeis & Widmer, 2020; San Miguel & Scott, 2016; Vogel *et al*., 2019; Zhang *et al*., 2010; Zhou *et al*., 2008). Although target pest can be removed using these methods, we must consider the potential risk of damage to other species. LM crops made with RNAi technology are imported with domestic import approval. These might be unintentionally released during transportation and might affect domestic species. Research on the effects of RNAi-based LMOs imported into South Korea on domestic species is insufficient. In particular, the risk assessments of *Snf7* dsRNA molecules in domestic natural enemies, such as, ladybugs, have rarely been conducted in South Korea.

In this study, we developed a risk assessment method for *C. septempunctata* using *Snf7* dsRNA molecules. To identify the species of ladybug before the *Snf7* dsRNA risk assessment, we compared the phenotypes of three species collected in South Korea (*Harmonia axyridis, C. septempunctata, and Cryptolaemus montruzieri*). The 12S *rRNA* gene-specific primer of *C. septempunctata* clearly differentiated *C. septempunctata* at the gene level. To determine whether assessing the risk to *C. septempunctata* using *Snf7* dsRNA treatment is feasible, treatment methods and checklists for each growth period were specified. A large amount of *Snf7* dsRNA was isolated for the risk assessment and its purity was confirmed. *C. septempunctata* larvae were treated with *Snf7* dsRNA for 1–5 days to confirm its presence in vivo, and the risk of *Snf7* dsRNA was determined by measuring the mortality, growth, and dry weight of *C. septempunctata.*

Materials and Methods

Insects

C. septempunctata, H. axyridis, and *C. montruzieri* were obtained from the Rural Development Administration (Jeonju, South Korea). Ladybug larvae and adults were bred in a growth chamber at 23 ± 0.5 °C, with a 16 h light and 8 h dark photoperiod and 50–70% relative humidity. The ladybugs fed on *Ephestia kuehniella* eggs. When the number of *C. septempunctata* required for the test was attained, first instar larvae were used for the risk assessment 1 day after hatching.

Identification of species

To analyze the morphological characteristics of the three ladybug species, we prepared three specimens, and observed and compared their phenotypes using a dissecting microscope (Olympus SZX16, Japan). For identification of *C. septempunctata,* we used two molecular marker genes, namely, *16S rRNA* and *12S rRNA*(Yao *et al*., 2011) (Table 1). The genomic DNA of *C. septempunctata, H. axyridis*, and *C. montruzieri* was extracted using an Animal Tissues Genomic DNA Extraction Kit (Tianlong, China). The PCR products of the *16S rRNA* and *12S rRNA* genes in *C. septempunctata* were analyzed using the National Center for Biotechnology Information Basic Local Alignment Search Tool.

Cloning, sequencing and dsRNA synthesis of *Snf7*, GFP, and vATPase A

Of the complete *Snf7* mRNA sequence (968 bp), only 240 bp were used to effectively produce insecticide effects. *Snf7* genes were cloned into the in vitro dsRNA-expressing L4440 vector. Based on the target gene sequence of the *Snf7* plasmid, *Snf7* dsRNA was synthesized using the T7 promoter and the RNA polymerase T7 MEGAscript kit (Ambion, Austin, USA) (Table 2). The synthesized *Snf7* dsRNA was quantified using a ND2000 spectrophotometer (Thermo Scientific, USA) and stored in an ultra-low temperature freezer at-80 °C before use. *GFP* and *vATPase* A dsRNAs were synthesized in the same manner and were used as negative and positive controls, respectively.

dsRNA expression analysis using RT-PCR

A total of 2 μg/μL *Snf7, GFP,* or *vATPase* A dsRNA was mixed into the artificial diet and administered to *C. septem-*

Table 1. List of specific oligonucleotide primers used to detect *C. septempunctata*

Primer Name	Sequence (5′–3′)	Product Size (bp)
16S-P1-F	CCGGTCTGAACTCAGATCACGT	682
16S-P1-R	CGCCTGTTTAACAAAAACAT	
16S-P2-F	TCTTCGCCTGTTTAACAAAAACATCTCTTTTT	529
16S-P2-R	GTTTTGTGGGGTGGCGCGAAGGGTATTGCCAA	
16S-P3-F	AAATTTGATTGGGGTGATAAAAA	164
16S-P3-R	TCGAGGTCGCAATCTTTTCT	
12S-P1-F	TACTATGTTACGACTTAT	525
12S-P1-R	AAACTAGGATTAGATACCC	
12S-P2-F	CGGGCGATGTGTACATATTTT	227
12S-P2-R	AGCAATTTTTATATCGTCGTTTTT	
12S-P3-F	CTTTCAAATCCAATTTCATTCTAAT	211
12S-P3-R	GTTCTGTAATTGATAATCCACGATTG	

Table 2. T7 promoter sequences used for *Snf7*, *GFP*, and *vATPase* A dsRNA synthesis

Primer Name	Sequence (5′–3′)	Product Size (bp)
DvSnf7-T7-F	TAATACGACTCACTATAGGGAGAATCCATGATATCGTGAACATC	240
DvSnf7-T7-R	TAATACGACTCACTATAGGGAGAGCAAAGAAAAATGCGTCGA	
GFP-T7-F	TAATACGACTCACTATAGGGAGAATGGTAGATCTGACTAGTAAA	240
GFP-T7-R	TAATACGACTCACTATAGGGAGAATCTGGGTATCTTGAAAAGCA	
*vATPase*A-T7-F	TAATACGACTCACTATAGGGAGAGCAGAACCAGGAAGTTACAC	700
*vATPase*A-T7-R	TAATACGACTCACTATAGGGAGATCGTAGAAGGAGGCGAGACG	

punctata larvae from each experimental group at the same time each day. After dsRNA treatment, three larvae per group were collected on day 1, 3, and 5 and immediately frozen in liquid nitrogen. Their RNAs were then transferred into RNA Stabilization Reagent (Ambion, Thermo Fisher Scientific) until RNA extraction. Reverse transcription (RT)-PCR primers were designed using the Primer 3 program (https://bioinfo.ut.ee/primer3-0.4.0) (Table 3) and synthesized by Macrogen Inc. (Seoul, Republic of Korea). RTPCR was performed by synthesizing cDNA from 500 ng/ μL RNA. Tubulin was used as the PCR control. mRNA expression levels of *Snf7, GFP,* and vATPase A in the *C. septempunctata* were detected by quantitative RT-PCR (Applied Biosystems, Waltham, MA, USA). The 25-μL reaction mix contained 19.5 μL of water, 0.5 μL of 2.5 mM dNTP mix, 0.5 μL of each forward and reverse primers (both 10 pmole), 2.5 μL of 10× Ex buffer, 0.5 μL of Taq polymerase, and 1 μL of template. Analyses were performed using a Proplex PCR system under the following cycle conditions: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 2 min; 1 cycle of final extension at 72 °C for 10 min. A 5 μL aliquot of each PCR product was analyzed using gel electrophoresis on a 2.5% (w/v) agarose gel at 135 V for 25 min and the images were captured using Chemi-Doc™ XRS+ (Bio-Rad, Hercules, CA, USA) (Lim *et al*., 2017). Three replicates were performed.

Risk assessment of *Snf7* dsRNA

To assess the risk of *Snf7* dsRNA against *C. septempunctata*, we treated the larvae with high concentrations of dsRNA (2 μg/μL). The concentration was selected with reference to prior experiments (Haller *et al*., 2019; Liang *et al*., 2019; Lu *et al*., 2020; Pan *et al*., 2020). One larva per one petri dish was treated with *Snf7, GFP,* or *vATPase* A dsRNA (2 μg/μL) mixed (1:1) in 50% sucrose. The experiment was carried out in three replicates of three larvae (one larva per petri dish). The control group was treated with only 0.5 M sucrose. *Snf7* dsRNA risk was evaluated based on the survival rate, development time, and dry weight of the larvae. All experiments were repeated three times. Analysis of variance was used for statistical analysis.

Results and Discussion

Identification of *C. septempunctata* using morphos-

Table 3. List of primers used to verify the expression level of *Snf7, GFP,* and *vATPase* A dsRNA expression

Primer Name	Sequence (5′–3′)	Product Size (bp)
DvSnf7-RT-F	GTATTTGTGCTAGCTCCTTCGA	120
DvSnf7-RT-R	TGCACTCCAAGCCCTCAAAA	
GFP-RT-F	AGAGGGTGAAGGTGATGCAA	83
GFP-RT-R	TTGGCCACGGAACAGGTAG	
VATPase A-RT-F	TATGTTGCAAGTGTGGCC	88
VATPase A-RT-R	AACTCTCTGTCCGGTGAG	
Tubulin-F	TTGGCCGACCAATGTACT	117
Tubulin-R	TCTTTCCATAGTCGACGGA	

pecies and molecular markers

LM crops which produce dsRNA targeting *D. virgifera Snf7* showed insect resistance by killing western corn rootworms larvae (Bachman *et al*., 2013; Ramaseshadri *et al*., 2013). The domestic natural pest enemy, *C. septempunctata,* is an important species for risk assessment because of the possibility of *Snf7* gene transfer from herbivorous insects (aphids), which infect LM crops, to their predators. Therefore, we first identified the morphological features which can be used to identify *C. septempunctata* collected for *Snf7* dsRNA risk assessment. Three species of ladybugs were collected and insect specimens were prepared and compared (Fig. 1). Unlike the other two ladybug species (*H. axyridis* and *C. montruzieri*), the wing cases of *C. septempunctata* were red with three spots clearly visible on each side. There was also one spot on the boundary of the wing, resulting to a total of seven spots. The results confirmed that the 12S-3 rRNA (211 bp) primer specifically amplified the *C. septempunctata* gene (Fig. 2F). In addition, nucleotide sequence analysis of the 12S-3 rRNA PCR product revealed the sequence of 12S rRNA derived from *C. septempunctata* (data not shown). Based on these results, we devised a method to identify *C. septempunctata* using morphological and molecular markers for LMO risk assessment in South Korea.

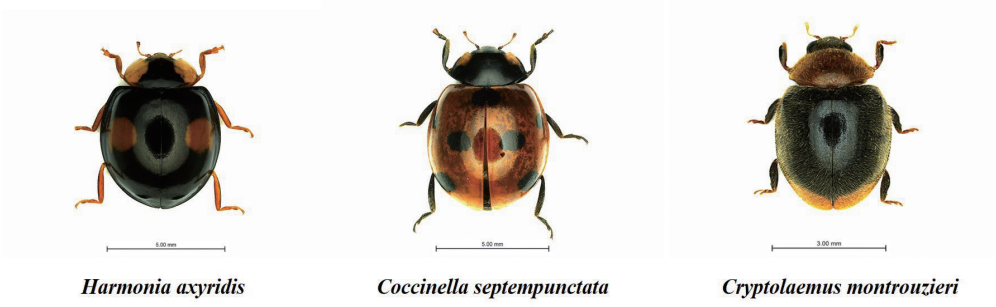

Fig. 1. Morphological features of (A) *Harmonia axyridis,* (B) *Coccinella septempunctata,* and (C) *Cryptolaemus montruzieri.*

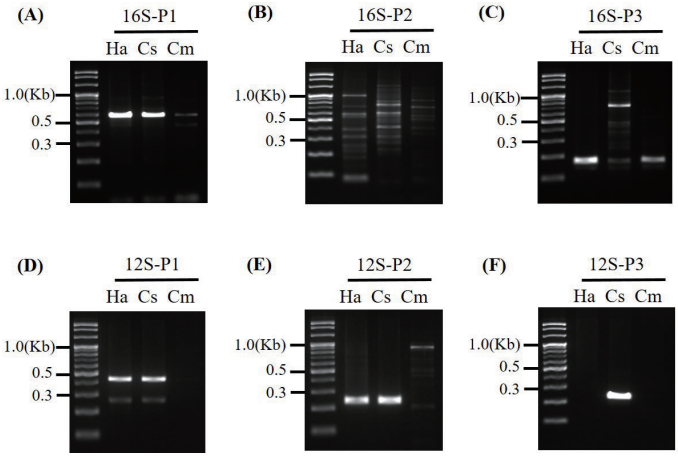

Fig. 2. Establishment of specific PCR methods for identifying *Harmonia axyridis* (Ha), *Coccinella septempunctata* (Cs), and *Cryptolaemus montruzieri* (Cm) using *16S rRNA* and *12S rRNA*. PCR results using *16S rRNA* and *12S rRNA* gene-specific primers. (A, B, C) *16S rRNA* PCR data; (D, E, F) 12S rRNA PCR data. M: 100 bp marker; lane 1: Ha; lane 2: Cs; lane 3: Cm.

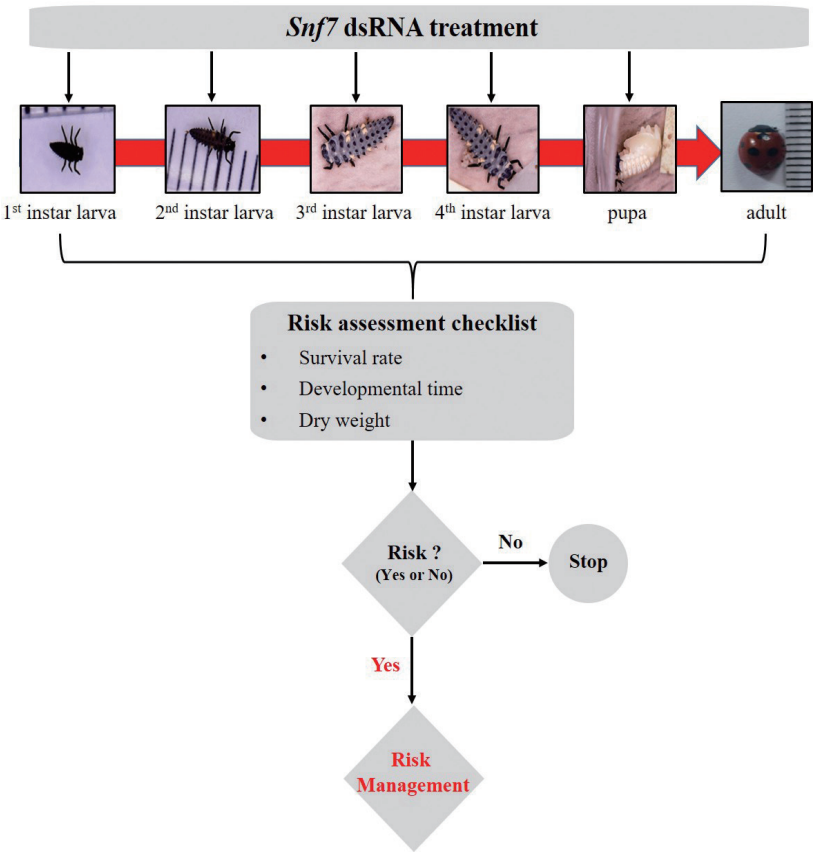

Fig. 3. Schematic diagram of the *C. septempunctata* risk assessment strategy. Risk assessment was performed starting with *C. septempunctata* larvae. All dsRNA treatments were performed daily, and all experiments were repeated three times. The dsRNA was mixed into *Ephestia kuehniella* eggs. The risk to *C. septempunctata* was measured using survival rate, development time, and dry weight. If a risk was found, we proceeded to the next step.

Establishment of risk assessment method using *Snf7* dsRNA

To conduct risk assessments for selected domestic insect species, specific risk assessment methods were developed based on the growth characteristics of each species. Therefore, we devised the following dietary risk assessment to determine the risk of *Snf7* dsRNA in *C. septempunctata* (Fig. 3). Firstly, the ecology of *C. septempunctata* from the larval to adult stages was identified. Then, the first instar larvae that hatched from the eggs were treated with *Snf7* dsRNA until adulthood. The risk assessment checklist for *C. septempunctata* relied on survival rate, developmental time, and dry weight. If there are no differences in the risk assessment checklists between treated and control groups, the risk assessment could be stopped. However, if a potential risk was identified, risk assessment using LM crops was carried out for risk management. This risk assessment method could be applied in various ways depending on the domestic risk assessment species and the LMO expression products, such as, dsRNA and proteins

Synthesis of *Snf7, GFP,* and *vATPase* A dsRNA and evaluation of their expression in *C. septempunctata*

Snf7 dsRNA purity is important for conducting toxicity risk assessment for *C. septempunctata,* as it is not possible to determine the risks posed by insufficiently pure dsRNA. To confirm the purity and identity of the *Snf7* dsRNA, the dsRNAs were cloned into L4440 expression vectors (Fig. 4A) and *Snf7, GFP* (negative control), and *vATPase* A (positive control) dsRNA were synthesized using RNA polymerase. The synthesized dsRNAs were confirmed by gel electrophoresis. Each dsRNA was cloned and identified as a single band (Fig. 4B). We also confirmed that the dsRNAs were purely synthesized (Fig. 4C). These results suggest that the dsRNA synthesis method used herein can efficiently generate highly purified *Snf7* dsRNA. In addition, it can be applied to the synthesis of excess dsRNA required for the risk assessment of imported RNAi-based LMOs in domestic insect species.

Dietary *Snf7* dsRNA risk assessment for *C. septempunctata*

To evaluate the risk of the synthesized *Snf7* dsRNA against *C. septempunctata,* an experiment was first conducted to ascertain whether *Snf7* dsRNA administered to the insects was present in vivo. To confirm the presence of *Snf7* dsRNA in vivo, we sampled *C. septempunctata* on day 1, 3, and 5 after daily dietary treatment with *Snf7, GFP,* and *vATPase* A dsRNAs. RT-PCR was performed to ensure that the dsRNA showed stable levels in the body of *C. septempunctata* (Table 3). We confirmed that *Snf7, GFP,* and *vATPase* A dsRNA migrated into *C. septempunctata* and were

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f7 dsRNA

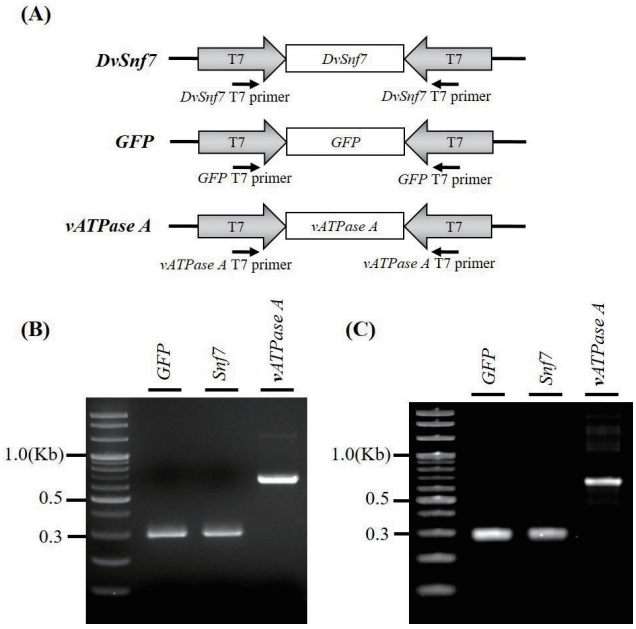

Fig. 4. *Snf7, GFP,* and *vATPase* A gene cloning and dsRNA synthesis. (A) Schematic diagrams of T7 promoter primer positions for *Snf7, GFP,* and *vATPase* A. (B) Confirmation of *Snf7, GFP,* and *vATPase* A product size after PCR using the T7 primer. (C) Confirmation of *Snf7, GFP,* and *vATPase* A levels after dsRNA synthesis.

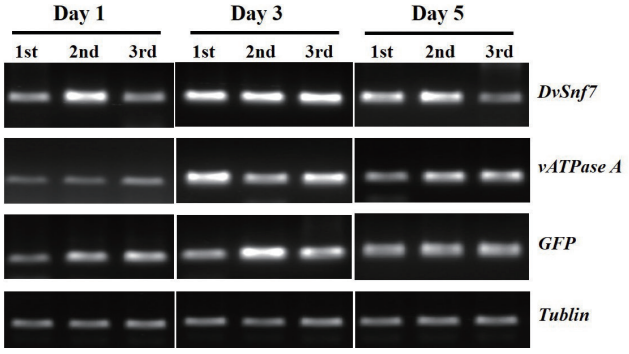

Fig. 5. Confirmation of the presence and stability of *Snf7* dsRNA in treated *C. septempunctata.* The genomic DNA was isolated from *Snf7* dsRNA-treated *C. septempunctata* on days 1, 3, and 5 post treatment. After performing RT-PCR, DNA electrophoresis was performed to confirm the expression and presence of each dsRNA. Lane identities are as follows. MW: 100 bp marker; lanes 1-3: replicate samples.

stable throughout the feeding period (Fig. 5). Based on these results, a risk assessment of the pure synthesized *Snf7* dsRNA in *C. septempunctata* was performed by modifying the previously reported RNAi dsRNA ladybug risk assessment method. The first instar larvae of *C. septempunctata* were treated with 2 μg/L of *Snf7, GFP,* or *vATPase* A dsRNA for 7, 14, and 21 days, and the experiment was repeated thrice (Fig. 6A). The test containers were observed at least once daily for insects showing symptoms and dead ones. The movement of the larvae was observed every day, and if there was no movement or response when touched with a micro-brush, the insect was considered dead. We identified non-adult individuals and calculated their survival rates. The *Snf7* dsRNA-treated group showed no differences in survival compared to the negative control (Fig. 6B). In addition, comparing the *Snf7* dsRNA-treated group with the negative control, the *Snf7* dsRNA treatment showed no effect on the development time or dry weight of *C. septempunctata* (Fig. 6C, D). In contrast, in the *vATPase* A dsRNA-treated group (the positive control), almost all the *C. septempunctata* died, confirming that the *Snf7* dsRNA risk assessment experiments were successful.

In conclusion, dietary supplementation of *Snf7* dsRNA did not pose any risk to *C. septempunctata.* However, considering the potential domestic environmental and ecological impacts, the risk assessment of *C. septempunctata* using high concentrations of *Snf7* dsRNA requires further research. Therefore, methods for assessing the risks of un intentional spread of LM crops on herbivore predators must be designed and specific RNAi-based LM crops must be developed for this purpose. In the future, we plan to develop RNAi-based LM crops which will select herbivorous insects eaten by *C. septempunctata* and develop a food chain-based (LM crops–herbivorous insects–predators) LMO risk assessment method suitable for domestic conditions. The development of risk assessment methods for LMOs for domestic insect species will enable active responses to the risks posed by new LMOs which are rapidly developed and imported to the ecosystem.

Conflict of Interest

The authors declare that they have no competing interests.

Acknowledgments

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References

Alvarez-Alfageme, F., Pálinkás, Z., Bigler, F., and Romeis, J. (2012). Development of an early-tier laboratory bioassay for assessing the impact of orally-active insecticidal compounds on larvae of Coccinella septempunctata (Co-

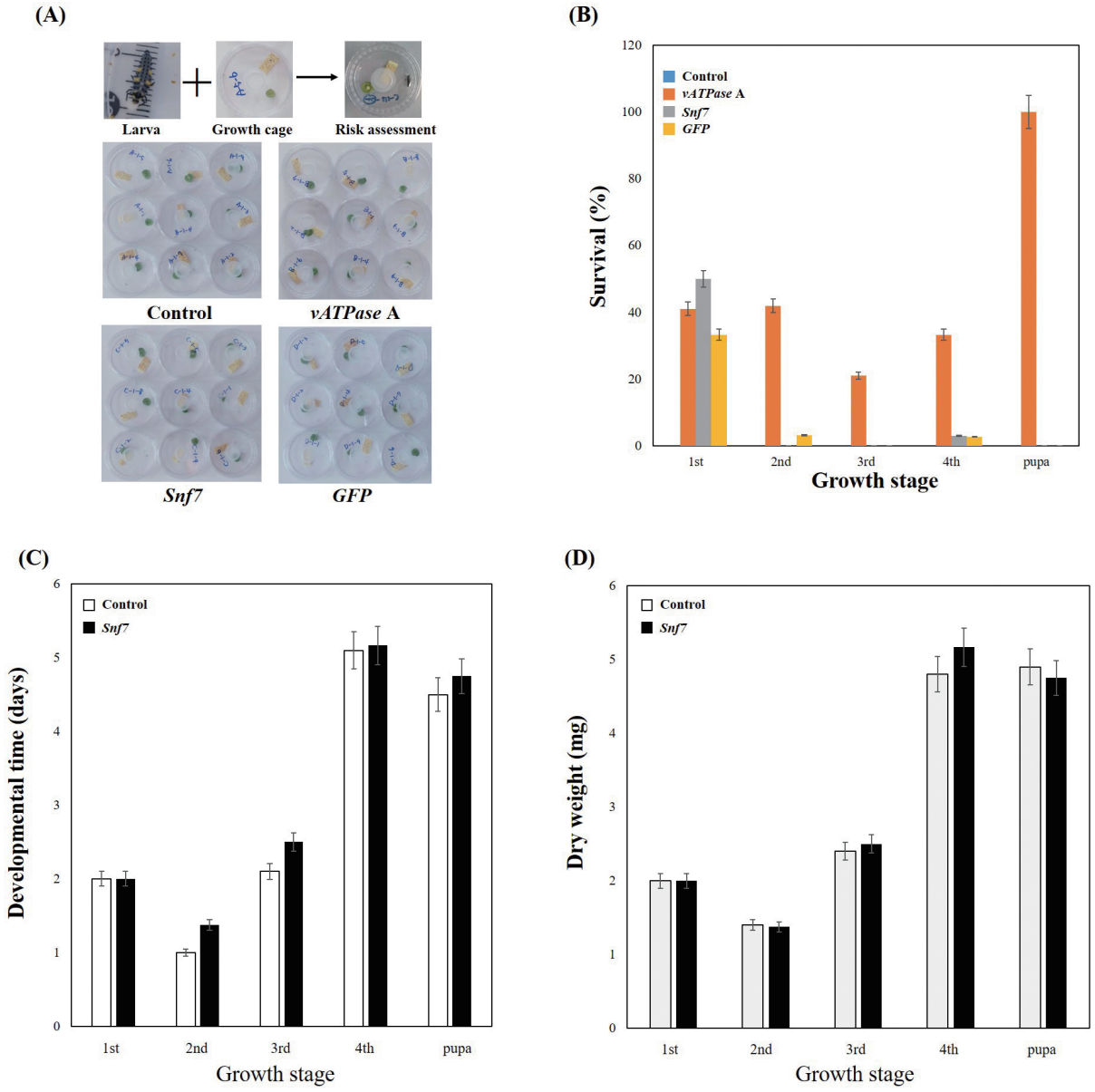

Fig. 6. Dietary *Snf7* dsRNA risk assessment against *C. septempunctata.* (A) Schematic of risk assessment. (B) The *Snf7* dsRNA risk was assessed using ten *C. septempunctata* larvae of similar size and activity, placed in separate breeding cages and treated with 2 μg/μL *Snf7, GFP,* or *vATPase* A dsRNA mixed 1:1 in 50% sucrose. The survival rate of *C-septempunctata* was measured at each growth stage. Each experiment was repeated thrice. (C, D) After treatment with the dsRNAs for 5 days, the developmental time and dry weight of *C. septempunctata* were measured and compared between the three groups. Each experiment was repeated thrice.

leoptera: Coccinellidae). *Environmental Entomology,* 41, 1687–1693. doi:10.1603/EN12032

Bachman, P.M., Bolognesi, R., Moar, W.J., Mueller, G.M., Paradise, M.S., Ramaseshadri, P., *et al*. (2013). Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (Diabrotica virgifera virgifera LeConte). *Transgenic Research,* 22, 1207-1222. doi:10.1007/s11248-013-9716-5

Bachman, P., Fridley, J., Mueller, G., Moar, W., and Levine,S.L. (2020). Sequence-activity relationships for the *Snf7* insecticidal dsRNA in Chrysomelidae. *Frontiers in Plant Science,* 11, 1303. doi:10.3389/fpls.2020.01303

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann,

P., Ilagan, O., *et al*. (2007). Control of coleopteran insect pests through RNA interference. *Nature Biotechnology,* 25, 1322–1326. doi:10.1038/nbt1359

Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flannagan, R., *et al*. (2012). Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (Diabrotica virgifera virgifera LeConte). *PLoS One,* 7, e47534. doi:10.1371/journal.pone.0047534

Burand, J.P., and Hunter, W.B. (2013). RNAi: future in insect management. *Journal of Invertebrate Pathology,* 112, S68–S74. doi:10.1016/j.jip.2012.07.012

Dolling, W.R. (1991). *The Hemiptera.* London: Oxford University Press, p. 274.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature,* 391, 806-811. doi:10.1038/35888

Fischer, J.R., Zapata, F., Dubelman, S., Mueller, G.M., Jensen, P.D., and Levine, S.L. (2016). Characterizing a novel and sensitive method to measure dsRNA in soil. *Chemosphere,* 161, 319–324. doi:10.1016/j.chemosphere.2016.07.014

Haller, S., Widmer, F., Siegfried, B.D., Zhuo, X., and Romeis, J. (2019). Responses of two ladybird beetle species (Coleoptera: Coccinellidae) to dietary RNAi. *Pest Management Science,* 75, 2652–2662. doi:10.1002/ps.5370

Harwood, J.D., Samson, R.A., and Obrycki, J.J. (2007). Temporal detection of Cry1Ab-endotoxins in coccinellid predators from fields of Bacillusthuringiensis corn. *Bulletin of Entomological Research,* 97, 643-648. doi:10.1017/S000748530700524X

Harwood, J.D., Wallin, W.G., and Obrycki, J.J. (2005). Uptake of Bt endotoxins by nontarget herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. *Molecular Ecology,* 14, 2815–2823. doi:10.1111/j.1365-294X.2005.02611.x

Hunter, W.B., Glick, E., Paldi, N., and Bextine, B.R. (2012). Advances in RNA interference: dsRNA treatment in trees and grapevines for insect pest suppression. *Southwestern Entomologist,* 37, 85–87. doi:10.3958/059.037.0110

Kalushkov, P., and Hodek, I. (2004). The effects of thirteen species of aphids on some life history parameters of the RNAi for insect control:current perspective and future ladybird Coccinella septempunctata. *Biological Control,* 49, 21-32. doi:10.1023/B:BICO.0000009385.90333.b4

Katoch, R., Sethi, A., Thakur, N., and Murdock, L.L. (2013). challenges. *Applied Biochemistry and Biotechnology,*171,847–873. doi:10.1007/s12010-013-0399-4

Kim, D.W., Sung, H., Shin, D., Shen, H., Ahnn, J., Lee, S.K., *et al*. (2011). Differential physiological roles of ESCRT complexes in Caenorhabditis elegans. *Molecules and Cells*, 31, 585–592. doi:10.1007/s10059-011-1045-z

Kunte, N., McGraw, E., Bell, S., Held, D., and Avila, L-A. (2020). Prospects, challenges and current status of RNAi through insect feeding. *Pest Management Science,* 76, 26–41. doi:10.1002/ps.5588

Le, Z. (2015). Population dynamic of Henosepilachna vigintioctopunctata in different host plants in Jianghan plain. *Northern Horticulture,* 11, 103–105.

Lee, J-A., Beigneux, A., Ahmad, S.T., Young, S.G., and Gao, F-B. (2007). ESCRT-III dysfunction causes autophagosome accumulation and neurodegeneration. *Current Biology,* 17, 1561–1567. doi:10.1016/j.cub.2007.07.029

Li, H., Guan, R., Guo, H., and Miao, X. (2015). New insights into an RNAi approach for plant defence against piercing-sucking and stem-borer insect pests. *Plant Cell & Environment,* 38, 2277–2285. doi:10.1111/pce.12546

Liang, C., Han, S., Han, H., Zhao, F., and He, Y. (2019). Selection of reference genes for Harmonia axyridis (Coleoptera:Coccinellidas) feeding on different diets. *Journal of Asia-Pacific Entomology,* 22, 1115-1122. doi:10.1016/j.aspen.2019.07.011

Lim, H.S., Jung. Y.J., Kim, I.R., Kim, J., Ryu, S., Kim, B., *et al*. (2017). Acute oral toxicity of dsRNA to honey bee, Apis mellifera. *Korean Journal of Environmental Agriculture,* 36, 241-248. doi:10.5338/KJEA.2017.36.4.36

Liu, F., Yang, B., Zhang, A., Ding, D., and Wang, G. (2019). Plant-mediated RNAi for controlling Apolygus lucorum. *Frontiers in Plant Science,* 10, 64. doi:10.3389/fpls.2019.00064

Lu, J., Guo, W., Chen, S., Guo, M., Qiu, B., Yang, C., *et al*. (2020). Double-stranded RNAs targeting HvRPS18 and HvRPL13 reveal potential targets for pest management of the 28-spotted ladygbeetle, Henosepilachna vigintioctopunctata. *Pest Management Science,* 76, 2663-2673. doi:10.1002/ps.5809

Niu, J., Shen, G., Christiaens, O., Smagghe, G., He, L., and Wang, J. (2018). Beyond insects: current status, achievements and future perspectives of RNAi in mite pests. *Pest Management Science,* 74, 2680–2687. doi:10.1002/ps.5071

Pan, H., Yang, X., Romeis, J., Siegfried, B., and Zhou, X. (2020). Dietary RNAi toxicity assay exhibits differential responses to ingested dsRNAs among lady beetles. *Pest Management Science,* 76, 3606-3614. doi:10.1002/ps.5894

Pang, X.F., and Mao, J.L. (1979). *Economic Insects of China, 14, Coleoptera-Coccinellidae, II.* Beijing: Science Press, pp. 108–112.

Peck, J.W., Bowden, E.T., and Burbelo, P.D. (2004). Structure and function of human Vps20 and *Snf7* proteins. *Biochemical Journal,* 377, 693–700. doi:10.1042/BJ20031347

Ramaseshadri, P., Segers, G., Flannagan, R., Wiggins, E., Clinton, W., Llagan, O., *et al*. (2013). Physiologicaland

cellular responses caused by RNAi-Mediated suppression of *Snf7* Orthologue in Western Corn Rootworm (Diabrotica virgifera virgifera) Larvae. *Plos One,* 8, 54270. doi:10.1371/journal.pone.0054270

Romeis, J., and Widmer, F. (2020). Assessing the risks of topically applied dsRNA-Based Products to non-target arthropods. *Frontiers in Plant Science,* 11. doi:10.3389/fpls.2020.00679

San Miguel, K., and Scott, J.G. (2016). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest Management Science,* 72, 801–809. doi:10.1002/ps.4056

Sweeney, N.T., Brenman, J.E., Jan, Y.N., and Gao, F.B. (2006). The coiled-coil protein shrub controls neuronal morphogenesis in Drosophila. *Current Biology,* 16, 1006–1011. doi:10.1016/j.cub.2006.03.067

Tu, J., Vallier, L.G., and Carlson, M. (1993). Molecular and genetic analysis of the *SNF7* gene in Saccharomyces cerevisiae. *Genetics,* 135, 17–23.

Vogel, E., Snatos, D., Mingels, L., Verdonckt, T.-W., and Broeck, J.V. (2019). RNA interference in insects: protecting beneficials and controlling pests. *Frontiers in Physiology,* 9, 1912. doi:10.3389/fphys.2018.01912

Winter, V., and Hauser, M.T. (2006). Exploring the ESCRTing machinery in eukaryotes. *Trends in Plant Science,* 11, 115–123. doi:10.1016/j.tplants.2006.01.008

Yao, D.B., Chi, D.F., Wu, Q.Y., Li, X.C., and Yu, J. (2011). Molecular phylogenetic relationships of differ forms within Harmonia axyridis Pallas (Coleoptera: Coccinellidae) based on sequences of 12S rRNA and 16S rRNA gene. *Advanced Materials Research,* 183-185, 757-767. doi:10.4028/www.scientific.net/AMR.183-185.757

Yoon, J.S., Mogilicherla, K., Gurusamy, D., Chen, X., Chereddy, S.C.R.R., and Palli, S.R. (2018). Doublestranded RNA binding protein, Staufen, is required for the initiation of RNAi in coleopteran insects. *Proceedings of National Academy of Science of the United States of America,*115, 8334–8339. doi:10.1073/pnas.1809381115

Yu, C., Fu, M., Lin, R., Zhang, Y., Yongguan, L., Jiang, H., *et al*. (2014). Toxic effects of hexaflumuron on the development of *Coccinella septempunctata. Environmental Science and Pollution Research International,* 21, 1418–1424. doi:10.1007/s11356-013-2036-8

Zhang, J., Khan, S.A., Heckel, D.G., and Bock, R. (2017). Next-generation insect-resistant plants: RNAimediated crop protection. *Trends in Biotechnology,* 35, 871-882. doi:10.1016/j.tibtech.2017.04.009

Zhang, X., Zhang, J., and Zhu, K.Y. (2010). Chitosan/double-stranded RNA nanoparticle-mediated RNA interference to silence chitin synthase genes through larval feeding in the African malaria mosquito (Anopheles gambiae). *Insect Molecular Biology,* 19, 683–693. doi:10.1111/j.1365-2583.2010.01029.x

Zhang, Y-y., Li, H-X., Shu, W-B., Zhang, C-J., and Ye, Z-B. (2011). RNA interference of a mitochondrial APX gene improves vitamin C accumulation in tomato fruit. *Scientia Horticulturae,* 129, 220-226. doi:10.1016/j.scienta.2011.03.025

Zhou, X., Wheeler, M.M., Oi, F.M., and Scharf, M.E. (2008). RNA interference in the termite Reticulitermes flavipes through ingestion of double-stranded RNA. *Insect Biochemistry and Molecular Biology,* 38, 805–815. doi:10.1016/j.ibmb.2008.05.005

Zhu, F., Xu, J., Palli, R., Ferguson, J., and Palli, S.R. (2011). Ingested RNA interference for managing the populations of the Colorado potato beetle. Leptinotarsa decemlineata. *Pest Management Science,* 67, 175–182. doi:10.1002/ps.2048

Zhu, K.Y., and Palli, S.R. (2019). Mechanisms, applications, and challenges of insect RNA interference. *Annual Review of Entomology,* 65, 293–311. doi:10.1146/annurev-ento-011019-025224

Zotti, M., Dos Santos, E.A., Cagliari, D., Christiaens, O., Taning, C.N.T., and Smagghe, G. (2018). RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Management Science,* 74, 1239–1250. doi:10.1002/ps.4813