Title: Acute feeding disorder after oral RNAi exposure results in efficient pest control

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ABSTRACT

Oral feeding of RNA interference (f-RNAi) is an innovative technology in the protection of crops from herbivorous pests. The strength of this technology lies in choosing optimal f-RNAi targets. Conventional targets have been chosen on due to their induction of lethality or infertility in target pests. However, conventional targets are slow-acting (c.a. one week) and crop damage can progress until the induction of lethality due to f-RNAi. Here, we show that f-RNAi of *death-associated inhibitor of apoptosis protein 1* (*diap1*) induces acute feeding cessation. In the solanaceous pest, *Henosepilachna vigintioctopunctata*, f-RNAi of *diap1* caused a decrease in feeding leaf area during the first 24 hours after treatment. Feeding cessation in f-RNAi individuals was achieved during 24-48 hours. We compared our targets with conventional targets by analyzing the influence of frequently used target genes, *vacuolar ATPase subunits A* and *E*, and showed that unlike *diap1* f-RNAi, conventional targets did not induce acute feeding disorder. Feeding experiments of *diap1* double-stranded RNA (dsRNA) from other insects against *H. vigintioctopunctata* did not induce feeding cessation and showed high species-specificity of *diap1* dsRNA. We also showed that 1.6 ng *diap1* dsRNA is sufficient to induce acute feeding cessation in *H. vigintioctopunctata*. These results suggest that *diap1* is an optimal target gene for rapid reduction of crop damage by herbivorous insects. In addition, our findings suggest that acute feeding cessation should be assessed as well as conventional criteria in selecting target genes for pest control by f-RNAi.

KEYWORDS: RNAi, pest control, Inhibitor of apoptosis protein

INTRODUCTION

Techniques for manipulating endogenous essential genes in pests using the RNA interference (RNAi)[1,2,3] or genome editing such as CRISPR/Cas9 system[4,5] have recently attracted attention alternative strategies for pest management to conventional chemical pesticides. Pest control mediated by endogenous gene manipulation can be achieved by inducing lethality[6,7] or infertility[5,8], which are essential for eradication of pests. However, since pests continue to feed on crops until the effects of lethality or infertility appears, such target genes are not necessarily optimal for preventing crop damage. Therefore, it would be more desirable for pest control to identify genes that can inducing acute feeding cessation in pests.

We found that the *death-associated inhibitor of apoptosis protein 1* (*diap1*) gene can be an effective target gene for pest control meeting the criterion. Diap1 is one of insect paralogues of *inhibitor of apoptosis protein* genes of vertebrates and essential for maintaining cell survival by inhibiting the apoptotic pathway through inactivating caspase proteins[9,10]. For an optimal target of pest control by oral feeding of RNAi (f-RNAi), *diap1* has ideal properties such as an evolutionarily conserved protein with moderate diverged sequences, an extremely short half-life of protein molecules in cells (40 minutes)[11] and higher expression in the midgut (Fig. S1). We investigated the effects of f-RNAi for *diap1* in the 28-spotted ladybird, *Henosepilachna vigintioctopunctata,* which is a pest for Solanaceae (Fig. S2) and compared its pest control efficacy with those of *vacuolar ATPase subunit* (*v-ATPase*) *A* and *E* which were previously reported as effective target genes[6,12] and frequently used.

RESULTS AND DISCUSSIONS

diap1, v-ATPase A and *v-ATPase E* orthologs cloned from *H. vigintioctopunctata* (Figs. S3, S4) were expressed in whole body including the gut (Fig. 1a). For f-RNAi, we fed 50 ng of the double-stranded (ds) RNA targeting each gene to the 3rd instar larvae (Fig. S5). Quantitative RT-PCR analysis revealed that the expression of each target gene in the larvae at two-days after f-RNAi treatment significantly decreased as compared to the negative control larvae fed with *egfp* dsRNA (Fig. 1b). This result indicated that the knockdown of each target gene by f-RNAi was effective in *H. vigintioctopunctata*.

We found that the body weight significantly decreased in the larvae fed with 50 ng of *diap1* dsRNA as compared to the larvae fed with *egfp* dsRNA (Fig. 1c and Table S1). We further found that potato leaves were hardly eaten by *diap1* f-RNAi treated larvae (Fig. 1d). In order to evaluate the influence on crop damage after RNAi treatment, we measured the area of potato leaves consumed by the larvae during 0-24 and 24-48 hours after dsRNA ingestion (Fig. 1e). *diap1* RNAi treated larvae showed a significantly narrower area of consumed leaves than negative control larvae within 48 hours after dsRNA ingestion (Fig. 1f and Tables S1, S2), whereas no significant difference was observed in the *v-ATPase A* and *E* RNAi treatment. These results indicate that the knockdown of *diap1* by f-RNAi induces rapid feeding cessation in the larvae after RNAi treatment, although all larvae treated with f-RNAi in our experiments did not show lethality at least until 48 hours after f-RNAi. Previous reports evaluated lethality and growth-inhibition in pest treated with injection- or f-RNAi targeting several genes including *v-ATPase*[6,12] and *diap1*[13] in longer periods (c.a., a week). However, the acute effects of f-RNAi on feeding cessation have not been evaluated thus far. Our findings provide the first report on effective target genes which

induce rapid feeding cessation, an essential phenotype for crop protection. Since *diap1* f-RNAi induced almost complete feeding cessation that is not observed in conventional target gene RNAi treatments (Table S1), *diap1* seems to be the most desirable target gene for the alleviation of crop damage.

The moderately diverged sequence in the *diap1* gene is useful for designing species-specific RNAi pesticides. The nucleotide sequences of *diap1* when compared among 10 insect species showed 37.3-53.7% identities (Fig. 2a). Notably, the nucleotide sequences between *H. vigintioctopunctata* and *Harmonia axyridis,* which belong to the same family showed lower identity in *diap1* (Fig. 2a) than those of *v-ATPase A* and *E* (Fig. S6). This finding suggested that dsRNA targeting the homologous region in the *diap1* ortholog genes can use for species-specific RNAi pesticide reagents, and allowed us to examine RNAi specificity on *H. vigintioctopunctata* larvae using 320 ng dsRNAs targeting *diap1* sequences cloned from 4 other insects (Fig. 2b). Of the RNAi treatments, only *H. vigintioctopunctata diap1* RNAi treatment showed reduction in feeding damage in 0-24 hours after RNAi treatment, and feeding cessation in 24-48 hours after treatment (Fig. 2c). In contrast, f-RNAi effects under the cross-species treatment of the *v-ATPase* genes were previously reported[6]. These results indicate that *diap1* is also an optimal target for designing highly species-specific RNAi reagents.

We also investigated the dose-sensitivity of *diap1* f-RNAi. Larvae fed with 1.6 and 8 ng of *diap1* dsRNA showed significant decreases in the area of consumed leaves (Fig. 2d). This result suggests that 1.6 ng dsRNA is sufficient to induce feeding cessation in the 3rd instar larva of *H. vigintioctopunctata*. Since many previous reports of f-RNAi for pest management require an amount ranging from several hundred nano-grams to a few micro-

grams of dsRNA[14], *diap1* is also the most efficient dsRNA target for f-RNAi to date.

The technologies for application of RNAi pesticide have been developed[3]. It has also been reported that combination of RNAi and Bt pesticide produces a stronger insecticide effect than either alone[15]. However, these studies did not investigate genes with either acute affects or high species- and dose-sensitivity, which are essential to the practice of RNAi as a pesticide. In the future, targeting *diap1* may allow the application of highly efficient RNAi pesticide treatments.

Furthermore, our results indicate the representative target genes in terms of lethality and growth-inhibition are not necessarily the optimal target for stopping crop damage. We propose the quantitative evaluation of acute feeding disorders should be required for selecting target genes in the application of RNAi pesticides, in addition to the conventional criteria.

MATERUALS AND METHODS

Insects

Henosepilachna vigintioctopunctata were collected from potato leaves at either Nagoya University or National Institute for Basic Biology, in Japan and used these animals to establish a laboratory line. Laboratory stocks of *Harmonia axyridis* were derived from field collections in Aichi, Japan. They were reared as described by Niimi *et al.*[17] *Helicoverpa armigera* was kindly provided by Dr. Chie Goto (NARO Agricultural Research Center). *Blattela lateralis* were purchased from Remix. The larvae of *Oxya yezoensis* were collected from rice field at Nagoya University.

139 **Cloning of partial *diap1* sequences from insects**

140 Total RNA was extracted from the gonad of *H. vigintioctopunctata*, the embryos of *H.*
141 *axyridis*, the anterior wing primordia of *H. armigera*, the posterior wing primordia of *B.*
142 *lateralis* or the larval posterior legs of *O. yezoensis* using TRIzol (Invitrogen, California,
143 USA) according to the manufactural protocol. The first-strand cDNA was synthesized
144 using the SuperScript II Reverse Transcriptase (Life Technologies Japan Ltd., Tokyo,
145 Japan) with SMART RACE cDNA Amplification kit (Clontech, Mountain View, California,
146 USA) from 1 μg total RNA. *diap1* cDNA fragment of each species was amplified using
147 degenerated primer sets designed from conserved amino acid sequences among insects
148 (diap1_F1 or diap1_F2/diap1_R in Table S7). The RT-PCR were performed using
149 AmpliTaq Gold DNA polymerase (Perkin Elmer, Boston, USA). PCR fragments were
150 separated with electrophoresis using 1% agarose gel in tris-borate-EDTA (TBE) buffer and
151 extracted using MagExtractor (TOYOBO Co. Ltd., Osaka, Japan). Each PCR fragment was
152 subcloned into the EcoRV recognition site of pBluescript KS (+) vector (Stratagene, La
153 Jolla, CA, USA) using DNA Ligation kit Ver. 2 (TaKaRa bio. Inc., Shiga, Japan). The
154 vector was transformed into XL1-Blue *Escherichia coli* competent cells (GMbiolab Co.,
155 Ltd, Taichung, Taiwan). The plasmid was extracted from the transformed colony using
156 FlexiPrep Kit (Amersham Pharmacia Biotech Inc., New Jersey, USA). The nucleotide
157 sequences of the PCR fragment inserted into vectors were confirmed using the dideoxy
158 chain-termination method using an automatic DNA sequencer (CEQ 2000XL; Beckman
159 Coulter, California, USA). Sequence analysis was carried out using a DNASIS system
160 (Hitachi Software Engineering, Tokyo, Japan). The partial length *diap1* nucleotide
161 sequences of the insects were deposited in DNA Data Bank of Japan (DDBJ) (accession

162 numbers: LC473085 in *H. axyridis*, LC473088 in *H. armigera*, LC473087 in *B. lateralis*
163 and LC473086 in *O. yezoensis*).
164
165 **Rapid amplification of cDNA end (RACE) of *diap1* in *H. vigintioctopunctata***
166 To obtain a full-length *diap1* cDNA in *H. vigintioctopunctata*, the 5'- and 3'- rapid
167 amplification of cDNA end (RACE) were performed according to the manufacturer's
168 protocol of the SMART RACE cDNA Amplification kit (Clontech, Mountain View,
169 California, USA). The gene-specific primers are described in Table S7 (1st PCR:
170 Hvig_diap1_1 for 5'-RACE and Hvig_diap1_3 for 3'-RACE; nested PCR: Hvig_diap1_2
171 for 5' RACE and Hvig_diap1_4 for 3'RACE). These primers were designed from the *diap1*
172 cDNA fragment cloned above. The cloning and confirmation of nucleotide sequences of
173 RACE fragment were performed according to the same way as the above method. The full
174 length *diap1* nucleotide sequences of *H. vigintioctopunctata* was deposited in DDBJ
175 (accession numbers: LC473084). The *diap1* full-length nucleotide sequence is shown in
176 Supplementary Sequences.
177
178 **Transcriptomic analysis**
179 In order to obtain *diap1*, *v-ATPase A* and *v-ATPase E* sequences of *H. vigintioctopunctata*,
180 transcriptomic analysis was performed using total RNA from the whole body of *H.*
181 *vigintioctopunctata* 3rd instar larvae. Total RNAs were extracted using the RNeasy Mini
182 kit (QIAGEN, Tokyo, Japan). The integrity of extracted RNAs were confirmed using a
183 Bioanalyzer 2000 (Agilent Technologies) and these RNAs were used to construct cDNA
184 libraries using the Truseq™ RNA Sample Prep Kit (Illumina) according to the

185 manufacture's instruction. Pair-end sequences with 200 bp were obtained from these cDNA
186 libraries through Hiseq2000 (Illumina). The raw reads were deposited in DDBJ Sequence
187 Read Archive (accession number: #### [the deposition of data is now proceeding, and the
188 accession number will be obtained during editorial process]). We rearranged pair-end reads
189 in order and excluded unpaired reads using cmpfastq_pe
190 (http://compbio.brc.iop.kcl.ac.uk/software/cmpfastq_pe.php). Low quality reads and
191 adapter sequences were trimmed from obtained short-reads using Cutadapt v1.15[18]. Then,
192 *de novo* assembly of trimmed short-read sequence was performed using Trinity-v4.2.0
193 (https://github.com/trinityrnaseq/trinityrnaseq/)[19]. We also analyzed the RNA-seq data of
194 *H. axyridis* obtained from National Center for Biotechnology Information (NCBI)
195 Sequence Read Archive database (run number.: ERR1309559) in the same way as *H.*
196 *vigintioctopunctata*.
197

198 **BLAST search and phylogenetic analysis of target genes**

199 BLAST databases of the assembled transcriptomic sequences in *H. vigintioctopunctata* and
200 *H. axyridis* were established by makeblastdb program in BLAST+ ver. 2.7.1[20]. We searched
201 homologous genes of *v-ATPase A, v-ATPase E* and *diap1* in the *H. vigintioctopunctata*
202 BLAST database with the orthologues in *Drosophila melanogaster* (accession number:
203 Diap1, NP_001261916.1; V-ATPase A, NP_652004.2; V-ATPase E, NP_524237.1)
204 obtained from NCBI database as query sequences using the tblastn program in BLAST+
205 ver. 2.7.1. Then, we confirmed the orthology of these obtained sequences by molecular
206 phylogenetic analysis (Fig. S4). The homologous sequences of V-ATPase A, V-ATPase E
207 and Diap1 in other animals were obtained from NCBI database (Table S6). All sequences

208 including *H. vigintioctopunctata* homologues were aligned using MAFFT ver. 7[21] with the
209 L-INS-i program. The using sites for phylogenetic construction were selected by trimAl
210 v1.2[22] with a gap threshold value of 0.7. The multiple alignments were shown in Figs. S7-
211 S9. The selection of best-hit substitution model and construction of maximal-likelihood
212 phylogeny were performed using MEGA X[23]. Bootstrap values were calculated after 1000
213 replications. BLAST search and multiple alignment were performed under Super Computer
214 Facilities of National Institute of Genetics. The *diap1*, *v-ATPase A* and *v-ATPase E*
215 nucleotide sequences of *H. vigintioctopunctata* and *H. axyridis* are shown in
216 Supplementary Sequences.
217
218 **Expression profile analysis of target genes in larval tissues**
219 Total RNA was extracted from nerve cord, legs, wing discs fat body, Malpighian tubules,
220 gut and carcass of 4th instar larvae of *H. vigintioctopunctata* which is the dissectable stage
221 for each tissue using the RNeasy Mini kit (QIAGEN, Tokyo, Japan). The first-strand cDNA
222 was synthesized from the 1 μg total RNA using SuperScript III Reverse Transcriptase (Life
223 Technologies Japan Ltd., Tokyo, Japan). RT-PCR for the target genes and the internal
224 control (*ribosomal protein 49* (*rp49*), accession number: AB480201) was performed using
225 the first-strand cDNA and Q5 High-Fidelity DNA Polymerase (New England Biolabs
226 Japan Inc., Tokyo, Japan) with 30 cycles. Primers for RT-PCR were designed by
227 Primer3web version 4.1.0[24] and are shown in Table S7 (Hvig_diap1_RT-PCR_F/
228 Hvig_diap1_RT-PCR_R, Hvig_v-ATPase_A_RT-PCR_F/Hvig_v-ATPase_A_RT-PCR_R,
229 Hvig_v-ATPase_E_RT-PCR_F/ Hvig_v-ATPase_E_RT-PCR_R and Hvig_rp49_RT-
230 PCR_F/Hvig_rp49_RT-PCR_R). The annealing temperatures for RT-PCR were calculated

231 by NEB Tm Calculator (https://tmcalculator.neb.com/). The expression pattern was
232 confirmed by agarose gel electrophoresis of the PCR products with 2% Agarose S
233 (NIPPON GENE Co. Ltd., Toyama, Japan).
234
235 **Double strand RNA synthesis**
236 Comparison analysis of RNAi efficiency of target genes
237 The total RNA was extracted from 3rd instar larvae of *H. vigintioctopunctata* using TRI
238 Reagent (Molecular Research Center Inc., Ohio, USA) according to the manufactural
239 protocol. The first-strand cDNA was synthesized from 1 µg total RNA using SuperScript
240 III Reverse Transcriptase (Life Technologies Japan Ltd., Tokyo, Japan). The templates of
241 dsRNA were initially amplified from the first-strand cDNA and secondary amplified from
242 the initial PCR products by RT-PCR using Q5 High-Fidelity DNA Polymerase (New
243 England Biolabs Japan Inc., Tokyo, Japan). The initial PCR primers are described as
244 Hvig_diap1_F/Hvig_diap1_R, Hvig_v-ATPase_A_F/Hvig_v-ATPase_A_R and Hvig_v-
245 ATPase_E_F/ Hvig_v-ATPase_E_R in Table S7. The nested primers were flanked with T7
246 promoter sequences on the 5' ends (Hvig_T7-diap1_F/Hvig_T7-diap1_R, Hvig_T7-v-
247 ATPase_A_F/Hvig_T7-v-ATPase_A_R and Hvig_T7-v-ATPase_E_F/Hvig_T7-v-
248 ATPase_E_R in Table S7). The nested PCR products were purified using MagExtractor
249 (TOYOBO Co. Ltd., Osaka, Japan). The sequences of interest were confirmed by sanger
250 DNA sequencing service at FASMAC Co. Ltd. (Kanagawa, Japan). The dsRNAs of target
251 genes were synthesized from the purified PCR products using AmpliScribe T7-Flash
252 Transcription Kit (Epicentre Technologies, Co., Wisconsin, USA).
253 Species-specificity and dose analysis of *diap1* in *H. vigintioctopunctata*

254 The templates of dsRNA were amplified from the above pBluescript KS (+) vectors
255 inserted *diap1* sequences in each insect by PCR using AmpliTaq Gold (Perkin Elmer,
256 Boston, USA). The PCR primers are described in Table S7 (T7-KS/T7-SK). The PCR
257 products were purified by the same ways to the above method. The dsRNAs were
258 synthesized using MEGAscript T7 Transcription Kit (Ambion, Texas, USA).
259

260 **Feeding RNAi assay**

261 Synthesized dsRNA was artificially fed to 3rd instar larvae of *H. vigintioctopunctata* within
262 24 hours after molting (Fig. S5). The 0.5 μl solution including dsRNA of each gene was
263 dropped on slide glasses in front of the larvae which had been starved after molting to the
264 3rd instar. Larvae starved for this amount of time readily consumed the dsRNA solution.
265 After the larvae had consumed the entire droplet, each larva was put on a fresh potato leaf
266 and kept in a plastic container at room temperature. Body weight of the larvae after 48
267 hours were measured using a precision electronic balance (Table S1). The potato leaves
268 were collected and exchanged for new potato leaves up to 48 hours every 12 or 24 hours
269 (Fig. 1e). dsRNA of *enhanced green fluorescent protein* (*egfp*) were fed to 3rd instar larvae
270 as the negative control.
271

272 **Evaluation of knockdown of mRNAs expression by quantitative (q) RT-PCR**

273 The total RNA was extracted from the whole-body of *H. vigintioctopunctata* 3rd instar
274 larvae 48-hours after f-RNAi treatment using TRI Reagent (Molecular Research Center
275 Inc., Ohio, USA). The first-strand cDNA was synthesized from 1 μg total RNA using
276 SuperScript III Reverse Transcriptase (Life Technologies Japan Ltd., Tokyo, Japan). qRT-

277 PCR was performed on LightCycler 96 instrument (Roche, Basel, Swizerland) using
278 THUNDERBIRD SYBR qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan) according to the
279 manufactural protocol with the first-strand cDNA as the template. The same primer sets as
280 the above expression profile analysis of each gene were used. The expression level of target
281 genes relative to *rp49* was calculated by the $2^{-\Delta\Delta Ct}$ method[25].
282

283 **Measurements of leaf area eaten by feeding RNAi larvae**

284 We measured the potato leaf area eaten by f-RNAi larvae using the following method with
285 the digital microscope system (VHX-5000, KEYENCE, Osaka, Japan). A leaf was put on
286 the glass plate on the stage and was covered with a plastic wrap. Then, the region of leaf
287 eaten was manually bordered with a black pen. After focusing on the leaf using 10x
288 magnification, we automatically binarized the image and measured the area of the region
289 eaten by larvae with the digital microscope system.
290

291 **Statistical analysis**

292 We performed the Welch's *t*-test to evaluate the differences of effects of f-RNAi assay. We
293 adjusted *P*-value with the Holm's methods for the multiple-comparisons. The significance
294 level used in our analysis was P-value < 0.05. All statistical analyses were performed by
295 R-3.5.0 (https://cran.r-project.org/).
296

297 **Sequence analysis of target genes among insects**

298 The nucleotide and amino acid sequences of target genes in 9 insect species were obtained
299 from NCBI's database (Table S6). Then, multiple alignments were performed for each pair

300 of species using MAFFT ver.7 with L-INS-i program. The proportions of identical sites for
301 length of coding sequence region of each gene were manually calculated. The identical
302 sites of partial sequences of *diap1* between *H. vigintioctopunctata* and other insects using
303 for f-RNAi assay were identified in the same way (alignment: Fig. S10).
304

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315

316 **Contributions**

317 Y.C., H.Y., Y.S., T.Y. and T.N. conceived and designed the study. Y.C., H.K. and T.N.
318 performed the majority of experiments. Y.C., T.S. and Y.S. analyzed RNA-seq data. H.K.
319 maintained *H. vigintioctopunctata* and potato in laboratory. T.N. supervised the study. Y.C.
320 and T.N. wrote the manuscript. All authors discussed the manuscripts.
321

322 **Competing interests**

323 The authors declare that no competing interests exist.

324

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409

410 **Figure Legends**

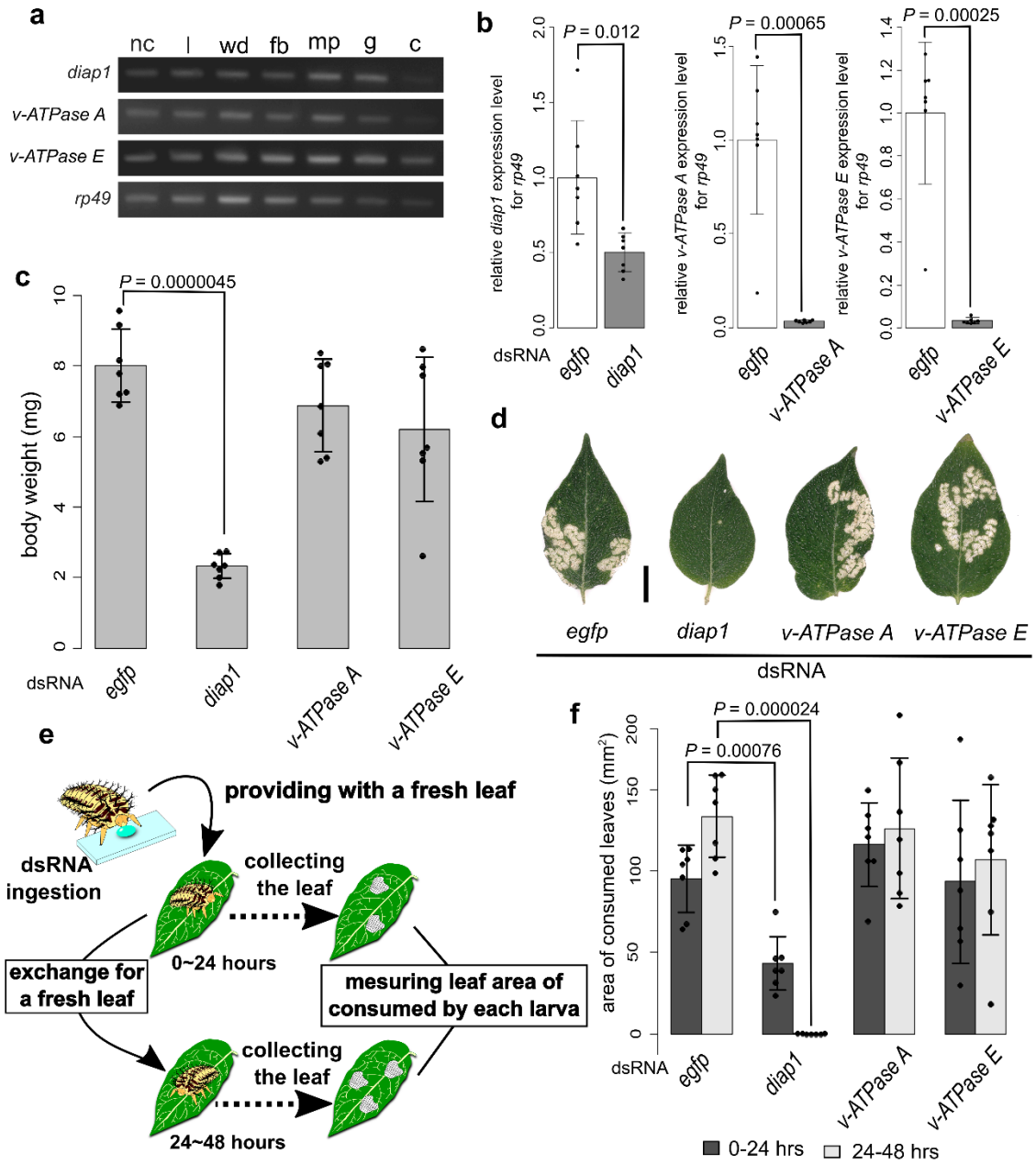
411 Figure 1: Effects of f-RNAi of *diap1, v-ATPase A* and *v-ATPase E* in *Henosepilachna*
412 *vigintioctopunctata*. (a) Tissue expression profile in 4th instar larva. nc, nerve cord; l, legs;
413 wd, wing discs; fb, fat body; mp, Malpighian tube; g, gut; c, carcass. (b) Quantitative RT-
414 PCR in the larvae two-days after f-RNAi. (c) Body-weight of the larvae two-days after f-

415 RNAi. (d) The leaves consumed by larvae two-days after f-RNAi. Scale, 1 cm. (e) Scheme
416 of f-RNAi experiment to evaluate feeding disorders. (f) Leaf area consumed by the larvae
417 for 0-24 hours (dark gray) and 24-48 hours (right gray) after f-RNAi. *P*-values were
418 calculated by Welch's *t*-test and adjusted by Holm's method for multiple comparisons.
419 Adjusted *P*-values were shown in c and f, and non-significances were not shown. Data of
420 b, c and f are means ± s.d., *n*=7 each gene RNAi larvae. Information are provided in Table
421 S1 for c and f.
422
423 Figure 2: Species-specificity and efficiency of *diap1* dsRNA. (a) Identity of *diap1*
424 nucleotide among insects. Numbers in each column indicates the identity between species.
425 Branches show the phylogenetic relationship[16]. (b) Image of sequence identity of *diap1*
426 dsRNAs among insects. Blue bars indicate the identical nucleotide sites between *H.*
427 *vigintioctopunctata* and each species. UTR, untranslated region; CDS, coding sequence;
428 BIR, baculovirus inhibitior of apoptosis protein repeat; RING, really interesting new gene.
429 (c) Area consumed by the larvae during 12-24 (upper) and 24-48 (lower) hours after various
430 insect-derived *diap1* dsRNAs ingestion. (d) Area consumed by the *diap1* f-RNAi larvae
431 treated with different amounts of dsRNA. Data of c and d are means ± s.d., *n*=3 in c and
432 *n*=4 in d. *P*-values were calculated by Welch's *t*-test and adjusted by Holm's method.
433 Adjusted *P*-values were shown in c and d, and non-significances were not shown. Amel,
434 *Apis melifera*; Blat, *Blatta lateraris*; Bmor, *Bombyx mori*; Caqu, *Catajapyx aquilonaris*;
435 Dmel, *Drosophila melanogaster*; Hvig, *Henosepilachna vigintioctopunctata*; Harm,
436 *Helicoverpa armigera*; Haxy, *Harmonia axyridis*; Ldec, *Leptinotarsa decemlineata*; Lmig,
437 *Locusta migratoria*; Ofas, *Oncopeltus fasciatus*; Oyez, *Oxya yezoensis*. Information for a,

438 c and d is provided in Tables S3, 4 and 5, respectively.

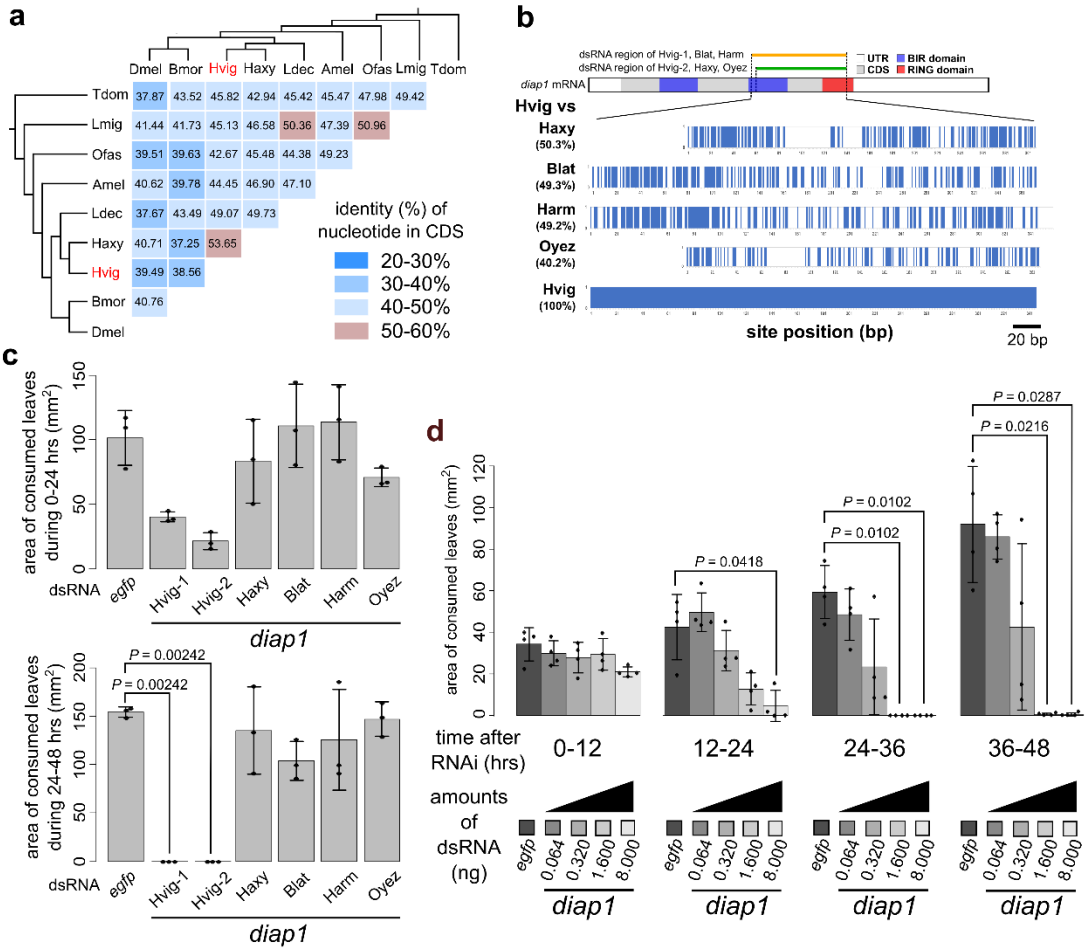
439

440 **FIGURES**



441

442 **Figure 1**


a
Dmel Bmor Hvig Haxy Ldec Amel Ofas Lmig Tdom
Tdom 37.87 43.52 45.82 42.94 45.42 45.47 47.98 49.42
Lmig 41.44 41.73 45.13 46.58 50.36 47.39 50.96
Ofas 39.51 39.63 42.67 45.48 44.38 49.23
Amel 40.62 39.78 44.45 46.90 47.10
Ldec 37.67 43.49 49.07 49.73
Haxy 40.71 37.25 53.65
Hvig 39.49 38.56
Bmor 40.76
Dmel
identity (%) of nucleotide in CDS
20-30%
30-40%
40-50%
50-60%
b
dsRNA region of Hvig-1, Blat, Harm
dsRNA region of Hvig-2, Haxy, Oyez
UTR
CDS
BIR domain
RING domain
diap1 mRNA
Hvig vs
Haxy (50.3%)
Blat (49.3%)
Harm (49.2%)
Oyez (40.2%)
Hvig (100%)
site position (bp)
20 bp
c
area of consumed leaves during 0-24 hrs (mm²)
dsRNA egfp Hvig-1 Hvig-2 Haxy Blat Harm Oyez
diap1
area of consumed leaves during 24-48 hrs (mm²)
P = 0.00242
P = 0.00242
d
area of consumed leaves (mm²)
P = 0.0287
P = 0.0216
P = 0.0102
P = 0.0102
P = 0.0418
time after RNAi (hrs)
0-12
12-24
24-36
36-48
amounts of dsRNA (ng)
egfp 0.064 0.320 1.600 8.000
diap1

443 **Figure 2**