Title: Oral RNAi of *diap1* in a pest results in rapid reduction of crop damage

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Abstract

Selecting an appropriate target gene is critical to the success of feeding RNA interference (f-RNAi)-based pest control. Gene targets have been chosen based on their ability to induce lethality. However, lethality induction by f-RNAi is slow-acting and crop damage can progress during this time. Here, we show that f-RNAi of *death-associated inhibitor of apoptosis protein 1* (*diap1*), but not two conventional targets *vacuolar ATPase subunit A* and *E*, induces acute feeding cessation in the solanaceous pest, *Henosepilachna vigintioctopunctata* during 24–48 hours. We also found that the feeding cessation by *diap1* f-RNAi has species-specificity and occurs with only 1.6 ng dsRNA. Our results suggest that *diap1* is an appropriate target in the context of rapid reduction of crop damage. We propose that acute feeding disorder should be assessed as a novel criterion for selecting appropriate target genes for RNAi-based pest control in addition to the conventional criterion based on lethality.

Introduction

Techniques for manipulating endogenous essential genes in pests using RNA interference (RNAi) have become popular as alternative strategies to conventional chemical pesticides for use in pest management in the last decade[1,2,3].

Innovative and practical applications of the technology are currently being developed, such as the use of transgenic plants that produce large amounts of double-stranded RNA (dsRNA) in the chloroplasts[4] or environmental RNAi applications, such as spraying dsRNA[5] on crops. Regardless of application method, the selection of an appropriate target gene is essential to achieve effective pest control[6]. The most target genes reported so far are housekeeping genes such as the *v-ATPase* genes and metabolic genes such as the chitin synthase genes (representative targets listed in Baum et al.[2]).

Conventionally, the criterion for selecting target genes is a combination of induction of lethality or growth-inhibition by gene silencing with RNAi[6]. For example, silencing of *v-ATPase subunit A* (*v-ATPase A*) or *v-ATPase subunit E* (*v-ATPase E*) by oral delivery of the dsRNA causes increased mortality in various pests of the order Coleoptera[7,8,9], Lepidoptera[7,10], Diptera[7], Hemiptera[11,12] and Orthoptera[13]. Such induction of lethality or growth-inhibition is extremely effective in terms of pest reduction and eradication. However neither lethality nor growth-inhibition are phenotype that are quick to induce, and it takes a certain amount of time before maximal effect of treatment is achieved. During the long-time span between treatment and phenotype, insect pests can continue to damage crops. For example, it takes more than a week for the RNAi effect of *v-ATPase A* or *v-ATPase E* to occur. Silencing of *v-ATPase E* requires 9 days to induce 100% mortality through injected and 25 days to induce 100% mortality through oral RNAi in *Tribolium castaneum*[14]. Therefore, in order to achieve effective RNAi-based control of

63 herbivorous pests, it would be desirable to evaluate and search for target genes from the
64 viewpoint of rapid termination of crop damage in addition to the induction of lethality
65 and growth-inhibition.

66 In this regard, we found that the gene silencing of *death-associated inhibitor of*
67 *apoptosis protein 1* (*diap1*) gene by oral-feeding RNAi (f-RNAi) causes acute feeding
68 disorder. *diap1* is an insect homolog of the *iap* genes and is known as a suppressor of
69 apoptosis in the fruit fly, *Drosophila melanogaster*[15]. Diap1 has E3 ubiquitin-ligase
70 activity[16] and strongly suppresses apoptosis via ubiquitination and degradation of the
71 caspase protein[17]. Silencing of the *diap1* gene promotes apoptosis activity, leading to
72 lethality. In addition, the Diap1 protein has an extremely short half-life (c.a. 40 min) in
73 *Drosophila* S2 cells[18]. In general, short protein half-life is one of the keys for the success
74 of RNAi targeting protein-coding genes[19]. Therefore, RNAi of the *diap1* gene is expected
75 to have a powerful effect. In some insects, it is known that *diap1* mRNA highly expresses
76 in the midgut[20] (Fig. S1 in *D. melanogaster*). For these reasons, *diap1* is one of the
77 candidate target gene for RNAi pesticides and has been investigated in various pests. So
78 far, RNAi of the *diap1* gene by injecting the dsRNA into the hemocoel of pest has been
79 shown to induce over 70% mortality in *T. castaneum* (Coleoptera)[14], *Anoplophora*
80 *glabripennis* (Coleoptera)[20], *Musca domestica* (Diptera)[21], *Delia radicum* (Diptera)[21],
81 *Heliothis virescens* (Lepidoptera)[22], *Lygus lineolaris* (Hemiptera)[23] and *Apolygus*
82 *lucorum* (Hemiptera)[24]. Diap1 silencing by f-RNAi has also been reported to induce 30–
83 78% of mortality in *Agrilus planipennis*[24], *T. castaneum*[14], and *Diaphorina citri*[25]. In these
84 reports, the maximum lethal effect of *diap1* silencing by f-RNAi takes 5–30 days. This
85 lethal effect of *diap1* by f-RNAi is also dose-dependent. For example, in *A. planipennis*,
86 the mortality of *diap1* f-RNAi using 1, 6 and 10 μg/μl dsRNA is 30, 35 and 78%,

respectively[24]. In *M. domestica* and *D. radicum*, it was reported that *diap1* gene silencing by injection of the dsRNA induced lethality but the f-RNAi assay did not[21]. The comparative f-RNAi assay of *diap1* and *v-ATPase E* using *T. castaneum* shows that silencing of *diap1* causes less mortality than that of *v-ATPase E*[14]. Although there are differences depending on the species, it seems that lethal effects of *diap1* f-RNA require a large amount of dsRNA and relatively long time before the effect appears. In the present study, we evaluated a novel effect of the *diap1* gene as a target of f-RNAi-based pest control. Our work investigated whether *diap1* rapidly induce cessation of feeding in addition to the conventional criterion based on lethality.

In this dsRNA feeding assay, we used the 28-spotted ladybird, *Henosepilachna vigintioctopunctata,* which is a representative solanaceous pest in Asia (Fig. S2). This species eats solanaceous plants in both the larval and adult stages. The larvae molts about once every 3–5 days and pupates at the fourth molting. In this study, we fed *diap1* dsRNA to 3rd instar larvae, which are easy to bioassay in terms of body size and amount of feeding. We also compared the effects of *diap1* f-RNAi on feeding disorder with *v-ATPase A* and *v-ATPase E*. For the effect of *diap1* f-RNAi, we also evaluated the species-specificity and dose-sensitivity, which are generally challenges to select target genes for RNAi-based pest control.

Results

Identification and expression of target genes in *H. vigintioctopunctata.* We isolated the homologous sequences of three target genes, *diap1*, *v-ATPase A* and *v-ATPase E*, from cDNA of *H. vigintioctopunctata* (Fig. S3). The *diap1* homologous sequence of *H.*

111 *vigintioctopunctata* was 2012 bp and 391 amino acids. Similar to the report in *D.*
112 *melanogaster*[17], the Diap1 homologous sequences had three domains, two baculoviral
113 inhibitor of apoptosis protein repeats (BIR) and one really interesting new gene (RING)
114 domain. The *v-ATPase A* and *v-ATPase E* homologous sequence of *H. vigintioctopunctata*
115 had 2503 bp and 1282 bp, respectively. The V-ATPase A and V-ATPase E homologous
116 sequence had 614 and 226 amino acids, respectively. We cannot detect any in-frame
117 isoforms in these target genes.

118 To confirm the orthology of these genes, we performed molecular phylogenetic
119 analysis for amino acid sequence of each target gene. All of these genes were located in
120 the robust supported clade of each gene (Fig. S4). Therefore, the isolated homologous
121 sequences were identified as the ortholog of each target gene in *H. vigintioctopunctata*.

122 To observe the expression profile of each target gene, we performed RT-PCR using
123 cDNA from the nerve cord, legs, wing disc, fat body, Malpighian tube, gut and carcass of
124 *H. vigintioctopunctata* larvae. We confirmed that the *diap1, v-ATPase A* and *v-ATPase E*
125 orthologs were transcribed in whole larval body including the gut (Fig. 1a).

126

127 **Knockdown effect of f-RNAi of target genes in *H. vigintioctopunctata***. For the oral
128 feeding RNA interference (f-RNAi) assay, we designed and synthesized the 300–400 bp
129 double-stranded RNA (dsRNA) targeting each gene (yellow lines in Supplementary
130 Sequence). We fed 3rd instar larvae with a droplet containing 50 ng of the dsRNA (Fig.
131 S5, $n = 7$ larvae for each gene). As the negative control, we fed 3rd instar larvae with the
132 same dose of *egfp* dsRNA. To evaluate the knockdown effect of each gene, we performed
133 quantitative (q) RT-PCR using cDNA of the whole-body larvae at two-days after f-RNAi
134 treatment. The qRT-PCR analysis revealed that the expression level of each target gene

in the f-RNAi treated larvae significantly decreased as compared to the control larvae (Welch's *t* test, $P = 0.01$, 0.0007 and 0.0002 in *diap1*, *v-ATPase A* and *v-ATPase E,* respectively) (Fig. 1b). Compared to the control larvae, the mean expression level of *diap1*, *v-ATPase A* and *v-ATPase E* in the f-RNAi treated larvae reduced to 50.2, 3.6, 3.4%, respectively. The knockdown effect of *diap1* f-RNAi seems to be lower than that of *v-ATPase* genes, although, the reduced rate of *diap1* mRNA level is nearly equivalent to that in previous *diap1* f-RNAi studies[14,20,22,25] showing the obvious phenotype. Considering these facts, we determined that our knockdown effect of *diap1* is reasonable. Therefore, our results indicate that the knockdown of each target gene by f-RNAi was effective in *H. vigintioctopunctata*.

Acute feeding disorder effect of *diap1* silencing in *H. vigintioctopunctata*. To observe the f-RNAi effect in *H. vigintioctopunctata*, we provided the 3rd instar larvae with 50 ng (100 ng/µl) of the *diap1, v-ATPase A* or *v-ATPase E* dsRNA ($n = 7$ in each target gene). Then, we provided the treated larvae with potato leaves as food.

At 2 days after f-RNAi treatment, we found that the body weight significantly decreased in the *diap1* f-RNAi treated larvae compared to the control larvae fed with *egfp* dsRNA (Welch's *t* test, $P < 0.001$) (Fig. 1c and Table S1). The mean body weight of the *diap1* f-RNAi larvae was 2.32 mg, which was 3.5-fold lower than the control larvae. In contrast to the *diap1* f-RNAi treatment, we cannot detect significant reduction of body weight in *v-ATPase* genes treated larvae compared to the control larvae (Fig. 1c and Table S1). We further found that the consumed area of potato leave was reduced in *diap1* f-RNAi treatment (Fig. 1d). These results suggested that silencing of the *diap1* gene but not the *v-ATPase* genes could cause feeding disorder in the *H. vigintioctopunctata* larvae.

159 Therefore, in order to evaluate the influence on crop damage after RNAi treatment, we
160 measured the area of potato leaves consumed by the larvae during 0-24 and 24-48 hours
161 after dsRNA ingestion (Fig. 1e). As the result, the *diap1* f-RNAi treated larvae showed a
162 significantly narrower area of consumed leaves than the control larvae within 24 hours
163 after dsRNA ingestion (mean area: 43.24 mm^2 in *diap1* f-RNAi and 95.54 mm^2 in *egfp* f-
164 RNAi, Welch's *t* test, $P < 0.001$) (Fig. 1f and Tables S1, S2). The mean leaf area consumed
165 by the *diap1* f-RNAi treated larvae was 2.2-fold narrower than the *egfp* f-RNAi treated
166 larvae. Surprisingly, we detected almost complete cessation of crop damage by the *diap1*
167 f-RNAi treated larvae during 24–48 hours after dsRNA ingestion (mean area: 0.15 mm^2
168 in *diap1* f-RNAi and 133.90 mm^2 in *egfp* f-RNAi, Welch's *t* test, $P < 0.001$) (Fig. 1f and
169 Tables S1, S2). In contrast to the *diap1* f-RNAi, we cannot observe any significant effects
170 on the consumed leaf area in the *v-ATPase A* and *v-ATPase E* RNAi treatment during both
171 0–24 and 24–48 hours (Fig. 1f and Tables S1, S2). In *egfp*, *v-ATPase A* and *v-ATPase E*
172 f-RNAi treatment, the consumed leaf area increased to 108–140% during 24–48 hours
173 compared to 0–24 hours. In contrast, the leaf area consumed by the *diap1* f-RNAi treated
174 larvae decreased to 0.35% during 24–48 hours compared to 0–24 hours. All f-RNAi
175 larvae did not show lethality within 48 hours in our experiments. These results showed
176 that the f-RNAi of *diap1* but not *v-ATPase* genes induces acute feeding disorder in the
177 larvae within 24 hours.
178
179 **Species-specificity of *diap1* f-RNAi effect in *H. vigintioctopunctata***. Species-specificity
180 is one of the advantages of RNAi-based pest control compared to conventional chemical
181 pesticides. However, since evolutionary and widely conserved genes generally have an
182 overall strongly conserved sequence, there is a concern that it is difficult to design a

183 species-specific dsRNA. Therefore, we evaluated species-specificity in the acute feeding
184 disorder effect by *diap1* silencing.

185 We compared the nucleotide sequences of *diap1* among 10 insect species belonging to
186 7 orders of Zygentoma, Orthoptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera
187 and Diptera. It showed that the *diap1* sequence had 37.3-53.7% identities among the
188 insects (Fig. 2a). The identity of *diap1* was lower than that of the *v-ATPase* genes in all
189 comparative combinations except for the comparisons with *Locusta migratoria* (Figs. 2a,
190 S6). Notably, the sequences between *H. vigintioctopunctata* and *Harmonia axyridis*
191 belonging to the same family (Coccinellidae) showed only 53.65% identity in *diap1,*
192 which was about 1.5-fold lower than those of *v-ATPase A* and *v-ATPase E* (Figs. 2a, S6).
193 This result showed that *diap1* gene has a moderately diverged sequence compared to *v-*
194 *ATPase* genes, and suggested that dsRNA targeting the homologous region in *diap1* is
195 usable for species-specific RNAi pesticide reagents.

196 Then, we validated species-specificity of *diap1* f-RNAi effect on *H.*
197 *vigintioctopunctata* larvae using 320 ng dsRNAs targeting *diap1* of 4 other insects, *H.*
198 *axyridis* (Coleoptera), *Blatta lateraris* (Blattodea), *Helicoverpa armigera* (Lepidoptera),
199 *Oxya yezoensis* (Orthoptera) (n = 3 in each treatment) (Fig. 2b). The dsRNA region of
200 *diap1* of *H. vigintioctopunctata* identified 50.3, 49.1, 49.3 and 40.1% with *H. axylidis*, *B.*
201 *lateraris*, *H. armigera* and *O. yezoensis*, respectively. This region did not show
202 consecutive matches over 16 bp between *H. vigintioctopunctata* and each insect. We
203 detected that the *diap1* f-RNAi using dsRNA of *H. axyridis* and *O. yezoensis* caused weak
204 feeding disorder effect in *H. vigintioctopunctata* larvae during 0–24 hours after the
205 treatment (Fig. 2c). The mean leaf area during 0–24 hours after f-RNAi treatment was 2.5,
206 4.8, 1.2 and 1.4 -folds lower than the *egfp* f-RNAi larvae in *H. vigintioctopunctata* region

1, *H. vigintioctopunctata* region 2, *H. axyridis* and *O. yezoensis* dsRNA treatment, respectively. The *diap1* f-RNAi also showed the slightly lower consumed leaf area in all f-RNAi treatments with *diap1* dsRNA of the other species during 24–48 hours after treatment (Fig. 2c). In contrast, of the f-RNAi treatments, only *H. vigintioctopunctata diap1* dsRNA treatment showed complete feeding cessation during 24-48 hours after the treatment (Fig. 2c). We also detected the significant reduction of the consumed leaf area between 0–24 and 24–48 hours after f-RNAi treatment in only *H. vigintioctopunctata diap1* dsRNA treatment (Welch's *t* test, $P = 0.003$ and 0.03 in region 1 and 2, respectively). The *B. laterais diap1* dsRNA treatment showed the reduction of the consumed area between 0–24 and 24–48 hours after the treatment, although, the reduction was only 6.3% in rate and did not show significant difference (Welch's *t* test, $P = 0.8$) (Table S5). These results show that the *diap1* dsRNA used here cause weak cross-species effect in the context of reduction of leaf consuming but not in the context of the feeding cessation and inhibition of increase in appetite of *H. vigintioctopunctata* larvae.

Dose-sensitivity of *diap1* f-RNAi effect in *H. vigintioctopunctata*. Dosage of dsRNA is one of the key factors of success in RNAi-based pest control, which is involved in cost and efficiency of RNAi. Therefore, we investigated the dose-sensitivity of *diap1* f-RNAi. We provided the *H. vigintioctopunctata* larvae with 0.064, 0.32, 1.6 or 8.0 ng of *diap1* dsRNA and 1000 ng of *egfp* dsRNA as the negative control ($n = 4$ in each treatment). We found that the 8.0 ng (16 ng/μl) dsRNA treatment caused significant decline of the consumed leaf area during 12–24 hours after f-RNAi (Welch's *t* test, $P = 0.04$) (Fig. 2d, Table S5). The mean of consumed area of 8.0 ng dsRNA treatment was 9.3-fold narrower than that of the control. Notably, during both 24–36 and 38–48 hours, almost complete

231 feeding cessation was detected in the 1.6 (3.2 ng/μl) and 8.0 ng dsRNA treated larvae
232 (Fig. 2d, Table S5). The consumed area of 0.32 ng (0.64 ng/μl) dsRNA treated larvae was
233 narrower to 39.2–73.5% than the control larvae during 12–48 hours after treatment,
234 although, this treatment did not show any significant and feeding cessation effect. The
235 0.016 ng (0.032 ng/μl) dsRNA treated larvae showed up to only 97% less consumed area
236 than the control larvae and no feeding cessation effect. This result shows that the feeding
237 disorder effect of *diap1* f-RNAi has dose-sensitivity and only 1.6 ng dsRNA is sufficient
238 to induce acute feeding cessation effect in the 3rd instar larva of *H. vigintioctopunctata*
239 during 24–48 hours.
240
241 **Non-recoverability, lethality and growth-inhibition of *diap1* f-RNAi effect in *H.***
242 ***vigintioctopunctata*.** To verify recoverable, lethal and/or growth-inhibitory effect of
243 *diap1* f-RNAi in *H. vigintioctopunctata* larvae after the feed cessation effect, we observed
244 the effects from 48 hours after the treatment with the 8 or 50 ng of *diap1* dsRNA ($N = 7$
245 in each target). Following our previous results, the *diap1* f-RNAi larvae exhibited
246 feeding-cessation and non-lethality within 48 hours after the treatment with both 8 and
247 50 ng of dsRNA (Fig. 3a–c, Table S6). We found that the 50 and 8 ng of *diap1* dsRNA
248 caused death of larvae from 48–72 hours (2–3 days) and 72–96 hours (3–4 days) after the
249 treatment, respectively (Fig. 3c). In addition, all of the treated larvae exhibited lethal
250 effects up to 4–5 and 5–6 days after the treatment with 50 and 8 ng of dsRNA, respectively
251 (Fig. 3c). The *diap1* f-RNAi larvae did not show molting into the 4th instar (Fig. 3d). In
252 contrast, the control larvae fed with 50 ng of *egfp* dsRNA molted into 4th instar larvae
253 and pupae in 2–4 and 8–10 days after the treatment, respectively (Fig. 3d). We also found
254 that almost all of the *diap1* f-RNAi larvae continued to exhibit feeding-cessation up to

255 their death (Fig. 3a, b, Table S6). Only one larva fed with 8 ng *diap1* dsRNA showed
256 slight feeding (0.38 mm^2) during the day 3–4, but was dead by day 5 (Fig. 3a–c, Table
257 S6). These results demonstrated that the f-RNAi of *diap1* causes lethality and growth-
258 inhibition, but not conspicuous recovery of crop damage, in *H. vigintioctopunctata* larvae.
259

260 **Discussion**

261 Searching for and selecting appropriate target gene is the keystone for effective RNAi-
262 based pest control. In this study, we evaluated the potential of *diap1* gene as a target of
263 RNAi-based pest control in the context of rapid reduction of crop damage by *H.*
264 *viginctiopunctata*.

265 Our f-RNAi assay reveals that silencing of the *diap1* gene causes acute feeding
266 disorder/cessation in *H. vigintioctopunctata* after only 24–48 hours f-RNAi (Fig. 1). In
267 this study, the mechanism of the acute feeding disorder by *diap1* silencing is unclear.
268 However, it is known that Diap1 promotes regeneration of gut via intestinal stem cell
269 proliferation when intestinal tissue is injured in *Drosophila melanogaster*[26,27]. In addition,
270 solanaceous plants produce glycoalkaloids, such as the solanine produced by potatoes,
271 which induces cell damage and apoptosis[28]. Considering this, one possibilities is that
272 *diap1* silencing by f-RNA suppresses intestinal stem cell proliferation via promotion of
273 apoptosis and increases the damage caused by the defensive compounds such as solanine
274 in *H. vigintioctopunctata* larvae. Further research is needed to elucidate the mechanism
275 of induction of acute feeding disorders by *diap1* f-RNAi.

276 We clarified the species-specificity of the significant feeding disorder effect by *diap1*
277 f-RNAi (Fig. 2). Cross-species assay using *diap1* dsRNA of other species showed a weak
278 cross-species effect in the feeding disorder effect. Previous studies using dipteran insects

have reported that *diap1* dsRNA with an identical 15 bp sequence region exhibits a cross-species effect in lethality[21]. In this study, the region of the *diap1* dsRNA had a contiguous identical 16 bp sequence (Figs. 2b, S10). Based on these facts, the cross-species effect in the feeding disorder observed in this study is considered to be due to this short identical sequence. On the other hand, the cross-species effect in this study was not significant and did not cause any feeding cessation (Fig. 2c). Therefore, we consider that *diap1* f-RNAi effect has species-specificity in the view of feeding cessation. In the practical application, the cross-species effect in feeding disorder may be completely eliminated by selecting the *diap1* dsRNA regions without >15 bp sequence identical among the target pest and the non-target insects in surrounding habitat.

Similar to the induction of the lethal effect of *diap1* f-RNAi in previous studies[21,23,24,25], we showed that in *H. vigintioctopunctata*, the effect of *diap1* f-RNAi on feeding disorder has dose-sensitivity for dsRNA (Fig. 2d). We found that only 1.6 ng (3.2 ng/µl) of dsRNA is sufficient to induce feeding cessation in *H. vigintioctopunctata* larvae within 48 hours after the f-RNAi treatment (Fig. 2d). In our experiment, the cost of *diap1* dsRNA *in vitro* synthesis was about $0.05/µg dsRNA. Therefore, the lowest cost of feeding cessation effect of *diap1* f-RNAi is theoretically about ¢1 per a thousand *H. viginctiopunctata* larvae in our protocol. These suggest that by *diap1* silencing, induction of acute feeding disorder is achieved using very small amounts of dsRNA, and thus can be utilized as a low cost and high efficiency of RNAi-based pest control.

We showed that all of the *diap1* f-RNAi larvae lead to death during the days 2–6 days (Fig. 3). This lethal effect is rapid compared to that of the previous study in *A. planipennis* (10 days for 78% mortality)[24]. Since all of the *diap1* f-RNAi larvae were dead within 1-4 days after the feeding cessation, this rapid lethality may have occurred as a result of the

303 acute feeding cessation, either indirectly or directly.

304 We compared the feeding disorder effect between *diap1* and *v-ATPase A* and *v-ATPase*
305 *E.* The *v-ATPase* genes are reported as efficient targets of f-RNAi in various pests[8,14].
306 Significantly, we show that the acute feeding cessation can be caused by ingestion of 50
307 ng *diap1* dsRNA, but not that of the same dose of both *v-ATPase A* and v-*ATPase E*, in
308 *H. vigintioctopunctata* (Fig. 1). In addition, the feeding cessation effect persists until
309 death in the larvae fed with 50 or 8 ng *diap1* dsRNA. While we have not analyzed feeding
310 disorder and lethality of silencing of *v-ATPase* genes from 48 hours after f-RNAi, our
311 results suggest that the crop damage of *H. vigintioctopunctata* larvae would be more
312 reduced by f-RNAi of *diap1* than *v-ATPase* genes and *diap1* is a more appropriate target
313 of RNAi-based pest control in the view of crop damage reduction. We also propose that
314 the selection of target genes for RNAi-based pest control should be evaluated not only for
315 induction of lethality/growth-inhibition but also for induction of acute feeding disorders.

316 In conclusion, we show that *diap1* f-RNAi in *H. vigintioctopunctata* causes the species-
317 specific and highly efficient at inducing acute feeding disorder/cessation, which is a
318 crucial aspect for crop damage reduction. To date, innovative application technologies for
319 the practice of RNAi-based pest control have been developed[29]. The main application
320 technologies are as follows: 1) Utilization of *in vitro* synthesized dsRNA: for example, a
321 method of drying industrially produced dsRNA and spraying it[5], 2) Utilization of *in planta*
322 synthesized dsRNA: for example, a method for producing transgenic plants that
323 synthesizes dsRNA targeting the pest genes in cell[8,11,12] or chloroplast[4], 3) Use of *in vivo*
324 synthesized dsRNA: for example, spraying a large amount of dsRNA synthesized
325 efficiently by bacteria[30,31]. In this study, we provided the larvae with a droplet containing
326 dsRNA as an experimental model, but it is our methods are applicable for any of the

technologies described above. Recently, it has been clarified that silencing of a dsRNA binding protein-coding gene, *Staufen* homolog, improves RNAi efficiency via reducing resistance for RNAi in the Colorado potato beetle[32]. Combining *Staufen* homolog and *diap1* silencing may cause the feeding disorder effect more efficiently in some pests.

It is expected that searching and selecting target genes inducing acute feeding disorder/cessation by f-RNAi in various herbivorous pests will realize efficient RNAi-based pest control. This will be able to solve problems such as the dsRNA synthesis cost, or persistence of crop damage until lethal or growth-inhibitory effect appears.

Material 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.*[34] *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.

Cloning of partial *diap1* sequences from insects. Total RNA was extracted from the gonad of *H. vigintioctopunctata*, the embryos of *H. axyridis*, the anterior wing primordia of *H. armigera*, the posterior wing primordia of *B. lateralis* or the larval posterior legs of *O. yezoensis* using TRIzol (Invitrogen, California, USA) according to the manufactural protocol. The first-strand cDNA was synthesized using the SuperScript II Reverse

Transcriptase (Life Technologies Japan Ltd., Tokyo, Japan) with SMART RACE cDNA Amplification kit (Clontech, Mountain View, California, USA) from 1 μg total RNA. *diap1* cDNA fragment of each species was amplified using degenerated primer sets designed from conserved amino acid sequences among insects (diap1_F1 or diap1_F2/diap1_R in Table S8). The RT-PCR were performed using AmpliTaq Gold DNA polymerase (Perkin Elmer, Boston, USA). PCR fragments were separated with electrophoresis using 1% agarose gel in tris-borate-EDTA (TBE) buffer and extracted using MagExtractor (TOYOBO Co. Ltd., Osaka, Japan). Each PCR fragment was subcloned into the EcoRV recognition site of pBluescript KS (+) vector (Stratagene, La Jolla, CA, USA) using DNA Ligation kit Ver. 2 (TaKaRa bio. Inc., Shiga, Japan). The vector was transformed into XL1-Blue *Escherichia coli* competent cells (GMbiolab Co., Ltd, Taichung, Taiwan). The plasmid was extracted from the transformed colony using FlexiPrep Kit (Amersham Pharmacia Biotech Inc., New Jersey, USA). The nucleotide sequences of the PCR fragment inserted into vectors were confirmed using the dideoxy chain-termination method using an automatic DNA sequencer (CEQ 2000XL; Beckman Coulter, California, USA). Sequence analysis was carried out using a DNASIS system (Hitachi Software Engineering, Tokyo, Japan). The partial length *diap1* nucleotide sequences of the insects were deposited in DNA Data Bank of Japan (DDBJ) (accession numbers: LC473085 in *H. axyridis*, LC473088 in *H. armigera*, LC473087 in *B. lateralis* and LC473086 in *O. yezoensis*).

Rapid amplification of cDNA end (RACE) of *diap1* in *H. vigintioctopunctata*. To obtain a full-length *diap1* cDNA in *H. vigintioctopunctata*, the 5'- and 3'- rapid amplification of cDNA end (RACE) were performed according to the manufacturer's

375 protocol of the SMART RACE cDNA Amplification kit (Clontech, Mountain View,
376 California, USA). The gene-specific primers are described in Table S8 (1st PCR:
377 Hvig_diap1_1 for 5'-RACE and Hvig_diap1_3 for 3'-RACE; nested PCR: Hvig_diap1_2
378 for 5' RACE and Hvig_diap1_4 for 3'RACE). These primers were designed from the
379 *diap1* cDNA fragment cloned above. The cloning and confirmation of nucleotide
380 sequences of RACE fragment were performed according to the same way as the above
381 method. The full length *diap1* nucleotide sequences of *H. vigintioctopunctata* was
382 deposited in DDBJ (accession numbers: LC473084). The *diap1* full-length nucleotide
383 sequence is shown in Supplementary Sequences.
384
385 **Transcriptomic analysis**. In order to obtain *v-ATPase A* and *v-ATPase E* sequences of *H.*
386 *vigintioctopunctata*, transcriptomic analysis was performed using total RNA from the
387 whole body of *H. vigintioctopunctata* 3rd instar larvae. Total RNAs were extracted using
388 the RNeasy Mini kit (QIAGEN, Tokyo, Japan). The integrity of extracted RNAs were
389 confirmed using a Bioanalyzer 2000 (Agilent Technologies) and these RNAs were used
390 to construct cDNA libraries using the Truseq™ RNA Sample Prep Kit (Illumina)
391 according to the manufacture's instruction. Pair-end sequences with 200 bp were obtained
392 from these cDNA libraries through Hiseq2000 (Illumina). The raw reads were deposited
393 in DDBJ Sequence Read Archive (accession number: DRA008720). We rearranged pair-
394 end reads in order and excluded unpaired reads using cmpfastq_pe
395 (http://compbio.brc.iop.kcl.ac.uk/software/cmpfastq_pe.php). Low quality reads and
396 adapter sequences were trimmed from obtained short-reads using Cutadapt v1.15[35]. Then,
397 *de novo* assembly of trimmed short-read sequence was performed using Trinity-v4.2.0
398 (https://github.com/trinityrnaseq/trinityrnaseq/)[36]. We also analyzed the RNA-seq data of *H.*

399 *axyridis* obtained from National Center for Biotechnology Information (NCBI) Sequence
400 Read Archive database (run number.: ERR1309559) in the same way as *H.*
401 *vigintioctopunctata*.
402
403 **BLAST search and phylogenetic analysis of target genes**. BLAST databases of the
404 assembled transcriptomic sequences in *H. vigintioctopunctata* and *H. axyridis* were
405 established by makeblastdb program in BLAST+ ver. 2.7.1[37]. We searched homologous
406 genes of *v-ATPase A, v-ATPase E* and *diap1* in the *H. vigintioctopunctata* BLAST
407 database with the orthologues in *Drosophila melanogaster* (accession number: Diap1,
408 NP_001261916.1; V-ATPase A, NP_652004.2; V-ATPase E, NP_524237.1) obtained
409 from NCBI database as query sequences using the tblastn program in BLAST+ ver. 2.7.1.
410 Then, we confirmed the orthology of these obtained sequences by molecular phylogenetic
411 analysis (Fig. S4). The homologous sequences of V-ATPase A, V-ATPase E and Diap1 in
412 other animals were obtained from NCBI database (Table S7). All sequences including *H.*
413 *vigintioctopunctata* homologues were aligned using MAFFT ver. 7[38] with the L-INS-i
414 program. The using sites for phylogenetic construction were selected by trimAl v1.2[39]
415 with a gap threshold value of 0.7. The multiple alignments were shown in Figs. S7-S9.
416 The selection of best-hit substitution model and construction of maximal-likelihood
417 phylogeny were performed using MEGA X[40]. Bootstrap values were calculated after 1000
418 replications. BLAST search and multiple alignment were performed under Super
419 Computer Facilities of National Institute of Genetics. The *diap1*, *v-ATPase A* and *v-*
420 *ATPase E* nucleotide sequences of *H. vigintioctopunctata* and *H. axyridis* are shown in
421 Supplementary Sequences.
422

423 **Expression profile analysis of target genes in larval tissues**. Total RNA was extracted
424 from nerve cord, legs, wing discs fat body, Malpighian tubules, gut and carcass of 4th
425 instar larvae of *H. vigintioctopunctata* which is the dissectable stage for each tissue using
426 the RNeasy Mini kit (QIAGEN, Tokyo, Japan). The first-strand cDNA was synthesized
427 from the 1 μg total RNA using SuperScript III Reverse Transcriptase (Life Technologies
428 Japan Ltd., Tokyo, Japan). RT-PCR for the target genes and the internal control
429 (*ribosomal protein 49* (*rp49*), accession number: AB480201) was performed using the
430 first-strand cDNA and Q5 High-Fidelity DNA Polymerase (New England Biolabs Japan
431 Inc., Tokyo, Japan) with 30 cycles. Primers for RT-PCR were designed by Primer3web
432 version 4.1.0[41] and are shown in Table S8 (Hvig_diap1_RT-PCR_F/ Hvig_diap1_RT-
433 PCR_R, Hvig_v-ATPase_A_RT-PCR_F/Hvig_v-ATPase_A_RT-PCR_R, Hvig_v-
434 ATPase_E_RT-PCR_F/ Hvig_v-ATPase_E_RT-PCR_R and Hvig_rp49_RT-
435 PCR_F/Hvig_rp49_RT-PCR_R). The annealing temperatures for RT-PCR were
436 calculated by NEB Tm Calculator (https://tmcalculator.neb.com/). The expression pattern
437 was confirmed by agarose gel electrophoresis of the PCR products with 2% Agarose S
438 (NIPPON GENE Co. Ltd., Toyama, Japan).
439
440 **Double strand RNA synthesis**. For comparison analysis of RNAi efficiency of target
441 genes, the total RNA was extracted from 3rd instar larvae of *H. vigintioctopunctata* using
442 TRI Reagent (Molecular Research Center Inc., Ohio, USA) according to the manufactural
443 protocol. The first-strand cDNA was synthesized from 1 μg total RNA using SuperScript
444 III Reverse Transcriptase (Life Technologies Japan Ltd., Tokyo, Japan). The templates of
445 dsRNA were initially amplified from the first-strand cDNA and secondary amplified from
446 the initial PCR products by RT-PCR using Q5 High-Fidelity DNA Polymerase (New

447 England Biolabs Japan Inc., Tokyo, Japan). The initial PCR primers are described as
448 Hvig_diap1_F/Hvig_diap1_R, Hvig_v-ATPase_A_F/Hvig_v-ATPase_A_R and Hvig_v-
449 ATPase_E_F/ Hvig_v-ATPase_E_R in Table S8. The nested primers were flanked with
450 T7 promoter sequences on the 5' ends (Hvig_T7-diap1_F/Hvig_T7-diap1_R, Hvig_T7-
451 v-ATPase_A_F/Hvig_T7-v-ATPase_A_R and Hvig_T7-v-ATPase_E_F/Hvig_T7-v-
452 ATPase_E_R in Table S8). The nested PCR products were purified using MagExtractor
453 (TOYOBO Co. Ltd., Osaka, Japan). The sequences of interest were confirmed by sanger
454 DNA sequencing service at FASMAC Co. Ltd. (Kanagawa, Japan). The dsRNAs of target
455 genes were synthesized from the purified PCR products using AmpliScribe T7-Flash
456 Transcription Kit (Epicentre Technologies, Co., Wisconsin, USA).
457 For Species-specificity and dose analysis of *diap1,* the templates of dsRNA were
458 amplified from the above pBluescript KS (+) vectors inserted *diap1* sequences in each
459 insect by PCR using AmpliTaq Gold (Perkin Elmer, Boston, USA). The PCR primers are
460 described in Table S8 (T7-KS/T7-SK). The PCR products were purified by the same ways
461 to the above method. The dsRNAs were synthesized using MEGAscript T7 Transcription
462 Kit (Ambion, Texas, USA).
463
464 **Feeding RNAi assay**. Synthesized dsRNA was artificially fed to 3rd instar larvae of *H.*
465 *vigintioctopunctata* within 24 hours after molting (Fig. S5). The 0.5 µl solution including
466 dsRNA of each gene was dropped on slide glasses in front of the larvae which had been
467 starved after molting to the 3rd instar. The larvae completely consumed the dsRNA
468 solution. The elapsed time in consumed the solution was 114–542 seconds (median time
469 = 206.5 seconds). After the larvae had consumed the entire droplet, each larva was put on
470 a fresh potato leaf and kept in a plastic container at room temperature. Body weight of

471 the larvae after 48 hours were measured using a precision electronic balance (Table S1).
472 The potato leaves were collected and exchanged for new potato leaves up to 48 hours
473 every 12 or 24 hours (Fig. 1e). dsRNA of *enhanced green fluorescent protein* (*egfp*) were
474 fed to 3rd instar larvae as the negative control.
475
476 **Evaluation of knockdown of mRNAs expression by qRT-PCR**. The total RNA was
477 extracted from the whole-body of *H. vigintioctopunctata* 3rd instar larvae 48-hours after
478 f-RNAi treatment using TRI Reagent (Molecular Research Center Inc., Ohio, USA). The
479 first-strand cDNA was synthesized from 1 μg total RNA using SuperScript III Reverse
480 Transcriptase (Life Technologies Japan Ltd., Tokyo, Japan). qRT-PCR was performed on
481 LightCycler 96 instrument (Roche, Basel, Swizerland) using THUNDERBIRD SYBR
482 qPCR Mix (TOYOBO Co. Ltd., Osaka, Japan) according to the manufactural protocol
483 with the first-strand cDNA as the template. The same primer sets as the above expression
484 profile analysis of each gene were used. The expression level of target genes relative to
485 *rp49* was calculated by the $2^{-\Delta\Delta Ct}$ method[42].
486
487 **Measurements of leaf area eaten by feeding RNAi larvae**. We measured the potato leaf
488 area eaten by f-RNAi larvae using the following method with the digital microscope
489 system (VHX-5000, KEYENCE, Osaka, Japan). A leaf was put on the glass plate on the
490 stage and was covered with a plastic wrap. Then, the region of leaf eaten was manually
491 bordered with a black pen. After focusing on the leaf using 10x magnification, we
492 automatically binarized the image and measured the area of the region eaten by larvae
493 with the digital microscope system.
494

Statistical analysis. We performed the Welch's *t*-test to evaluate the differences of effects of f-RNAi assay. We adjusted *P*-value with the Holm's methods for the multiple-comparisons. The significance level used in our analysis was *P*-value < 0.05. All statistical analyses were performed by R-3.6.0 (https://cran.r-project.org/).

Expression level of *diap1* in tissues of *Drosophila melanogaster*. We obtained the expression dataset of *diap1* (FBgn0260635) in tissue of *Drosophila melanogaster* from FlyAtlas Anatomical Expression Data[43]. Then, we created the histogram based on the expression data (Supplementary Fig. 1).

Sequence analysis of target genes among insects. The nucleotide and amino acid sequences of target genes in 9 insect species were obtained from NCBI's database (Table S7). Then, multiple alignments were performed for each pair of species using MAFFT ver.7 with L-INS-i program. The proportions of identical sites for length of coding sequence region of each gene were manually calculated. The identical sites of partial sequences of *diap1* between *H. vigintioctopunctata* and other insects using for f-RNAi assay were identified in the same way (alignment: Fig. S10).

References

1. Price, D. R. G. & Gatehouse, J. A. RNAi-mediated crop protection against insects. *Trends Biotechnol.* 26, 393–400 (2008).

2. Baum, J. A. & Roberts, J. K. Progress Towards RNAi-Mediated Insect Pest Management. *Adv. Insect Physiol.* 47, 249–295 (2014).

519 3. Mamta, B. & Rajam, M. V. RNAi technology: a new platform for crop pest control.
520 *Physiol. Mol. Biol. Plants* 23, 487–501 (2017).
521 4. Zhang, J. et al. Full crop protection from an insect pest by expression of long double-
522 stranded RNAs in plastids. *Science* 347, 991-994 (2015).
523 5. Wang, Y., Zhang, H., Li, H. & Miao, X. Second-generation sequencing supply an
524 effective way to screen RNAi targets in large scale for potential application in pest insect
525 control. *PLoS ONE* 6, e18644 (2011).
526 6. Katoch, R., Sethi, A., Thakur, N. & Murdock, L. L. RNAi for insect control: current
527 perspective and future challenges. *Appl. Biochem. Biotechnol.* 171, 847–873 (2013).
528 7. Whyard, A., Singh, A. D. & Wong, S. Ingested double-stranded RNAs can act as
529 species-specific insecticides. *Insect Biochem. Mol. Biol.* 39, 824–832 (2009).
530 8. Baum, J. A. et al. Control of coleopteran insect pests through RNA interference. *Nat.*
531 *Biotechnol.* 25, 1322-1326 (2007).
532 9. Wu et al. Lethal RNA interference response in the pepper weevil. *J. Appl. Entomol.*
533 143, 699–705 (2019).
534 10. Burke, W. G. et al. RNA Interference in the Tobacco Hornworm, *Manduca sexta*,
535 Using Plastid-Encoded Long Double-Stranded RNA. *Front. Plant Sci.* 10,
536 https://doi.org/10.3389/fpls.2019.00313 (2019).
537 11. Thakur, N. et al. Enhanced Whitefly Resistance in Transgenic Tobacco Plants
538 Expressing Double Stranded RNA of *v-ATPase A* Gene. *PLoS ONE* 9, e87235 (2014).
539 12. Liu, F. et al. Plant-Mediated RNAi for Controlling *Apolygus lucorum*. *Front. Plant*
540 *Sci.* 10, https://doi.org/10.3389/fpls.2019.00064 (2019).
541 13. Luo, Y. et al. Differential responses of migratory locusts to systemic RNA interference
542 via double-stranded RNA injection and feeding. *Insect Mol. Biol.* 22, 574–583 (2013).

543 14. Cao, M., Gatehouse, J. A. & Fitches, E. C. A Systematic Study of RNAi Effects and
544 dsRNA Stability in *Tribolium castaneum* and *Acyrthosiphon pisum*, Following Injection
545 and Ingestion of Analogous dsRNAs. *Int. J. Mol. Sci.* 19, 1079 (2018).
546 15. Hay, B. A., Wassarman, D. A. & Rubin, G. M. Drosophila homologs of baculovirus
547 inhibitor of apoptosis proteins function to block cell death. *Cell* 7, 1253–1262 (1995).
548 16. Herman-Bachinsky, Ryoo, H. D., Ciechanover, A. & Gonen, H. Regulation of the
549 *Drosophila* ubiquitin ligase DIAP1 is mediated via several distinct ubiquitin system
550 pathways. *Cell Death Diff.* 14, 861–871 (2007).
551 17. Vasudevan, D. & Ryoo, H. D. Regulation of cell death by IAPs and their antagonists.
552 *Curr. Topic. Dev. Biol.* 114, 185–208 (2015).
553 18. Yoo, S, J. et al. Hay, Hid, Rpr and Grim negatively regulate DIAP1 levels through
554 distinct mechanisms. *Nat. Cell Biol.* 4, 416–424 (2002).
555 19. Scott, J. G. et al. Towards the elements of successful insect RNAi. J. Insect. Physiol.
556 59, 1212–1221 (2013).
557 20. Rodrigues, T. B., Dhandapani, R. K., Duan, J. J. & Palli, S. R. RNA interference in
558 the Asian Longhorned Beetle: Identification of Key RNAi Genes and Reference Genes
559 for RT-qPCR. *Sci. Rep.* 7, 8913 (2017).
560 21. Powell, M. et al. Insecticidal effects of dsRNA targeting the *Diap1* gene in dipteran
561 pests. *Sci. Rep.* 7, 15147 (2017).
562 22. Mogilicherla, K., Howell, J. L. & Palli, S. R. Improving RNAi in the Brown
563 Marmorated Stink Bug: Identification of target genes and reference genes for RT-qPCR.
564 *Sci. Rep.* 8, 3720 (2018).
565 23. Walker III, W. B. & Allen, M. L. RNA interference-mediated knockdown of IAP in
566 *Lygus lineolaris* induces mortality in adult and pre-adult life stages. *Entomol Exp. Appl.*

138, 83–92 (2011).

24. Rodrigues, T. B. et al. Development of RNAi method for screening candidate genes to control emerald ash borer, *Agrilus planipennis*. *Sci. Rep.* 7, 7379 (2017).

25. Galdeano, D. M. et al. Oral delivery of double-stranded RNAs induces mortality in nymphs and adults of the Asian citrus psyllid, *Diaphorina citri*. *PLoS ONE*. 12, e0171847 (2017).

26. Karpowicz, P., Perez, J. & Perrimon, N. The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration. *Development*. 138, 4135–4145 (2010).

27 Hong, A. W., Meng, Z. & Guan, K. L. The Hippo pathway in intestinal regeneration and disease. *Nat. Rev. Gastroenterol. Hepatol.* 13, 324–337 (2016).

28. Gao, S. Y., Wang, Q. J. & Ji, Y. B. Effect of solanine on the membrane potential of mitochondria in $HepG_2$ cells and $[Ca^{2+}]_i$ in the cells. *World J. Gastroenterol.* 7, 3359–3367 (2006).

29. Palli, S. R. RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide. *Curr. Opin. Insect. Sci.* 6, 1–8 (2014).

30. Tian, H. et al. Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. *PLoS ONE* 13, e6225 (2009).

31. Li, X., Zhang, M. & Zhang, H. RNA Interference of Four Genes in Adult *Bactrocera dorsalis* by Feeding Their dsRNAs. *PLoS ONE* 6, e17788 (2011).

32. Yoon, J. S. et al. Double-stranded RNA binding protein, *Staufen*, is required for the initiation of RNAi in coleopteran insects. *Proc. Natl. Acad. Sci. USA.* 115, 8334–8339 (2018).

33. Misof, B. et al. Phylogenomics resolves the timing and pattern of insect evolution.

591 *Science* 346, 763–767 (2014).

592 34. Niimi, T., Kuwayama, H. & Yaginuma, T. Larval RNAi applied to the analysis of
593 postembryonic development in the ladybird beetle, *Harmonia axyridis*. *J. Insect*
594 *Biotechnol. Sericol.* 74, 95-102 (2005).

595 35. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing
596 reads. *EMBnet. journal* 17, 10-12 (2011).

597 36. Grabherr, M. G. et al. Trinity: reconstructing a full-length transcriptome without a
598 genome from RNA-Seq data. *Nat. Biotechnol.* 29, 644-652 (2011).

599 37. Camacho, C. et al. BLAST+: architecture and applications. *BMC Bioinform.* 10, 421
600 (2009).

601 38. Katoh, K. & Standley, D.M. MAFFT Multiple Sequence Alignment Software Version
602 7: Improvements in performance and usability. *Mol. Biol. Evol.* 30, 772-780 (2013).

603 39. Capella-Gutiérrez, S., Silla-Martínez, J. M. & Gabaldón, T. trimAl: a tool for
604 automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25,
605 1972-1973 (2009).

606 40. Kumar, S. et al. MEGA X: molecular evolutionary genetics analysis across computing
607 platforms. *Mol. Biol. Evol.* 35, 1547-1549 (2018).

608 41. Untergasser, A. et al. Primer3—new capabilities and interfaces. *Nucleic Acids Res.* 40,
609 e115 (2012).

610 42. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-
611 time quantitative PCR and the 2−ΔΔCT method. *Methods* 25, 402-408 (2001).

612 43. Chintapalli, V. R., Wang, J. & Dow, J. A. Using FlyAtlas to identify better *Drosophila*
613 *melanogaster* models of human disease. *Nat. Genet.* 39, 715-720 (2007).

614

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Contributions

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

Competing interests

The authors declare that no competing interests exist.

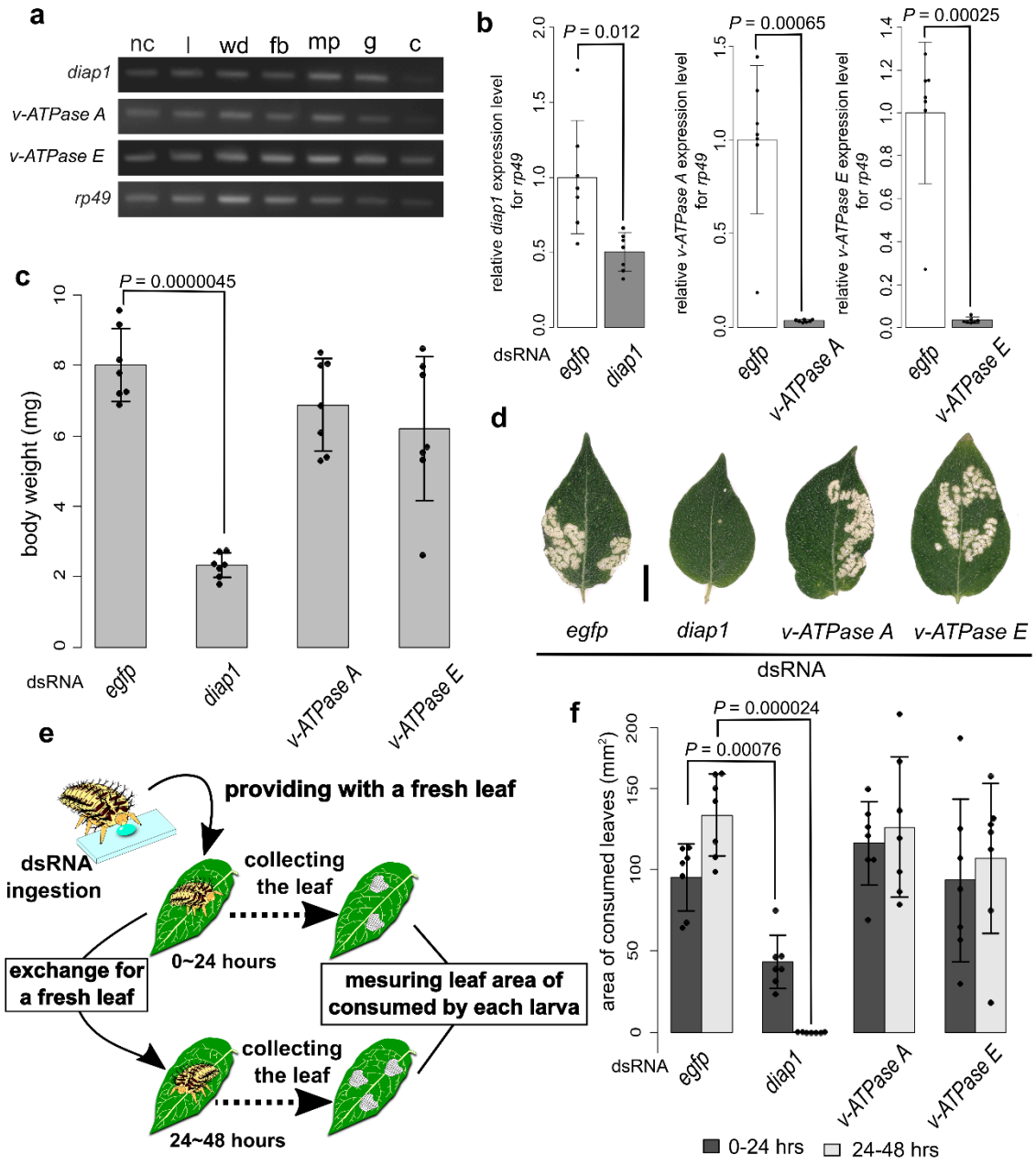
Figure Legends

Fig. 1 Effects of f-RNAi of *diap1, v-ATPase A* and *v-ATPase E* in *H. vigintioctopunctata*.

639 **a** Tissue expression profile in 4th instar larva. nc, nerve cord; l, legs; wd, wing discs; fb,
640 fatbody; mp, Malpighian tube; g, gut; c, carcass. **b** qRT-PCR in the larvae at two-days
641 after f-RNAi. **c** Body-weight of the larvae at two-days after f-RNAi. **d** The leaves
642 consumed by larvae two-days after f-RNAi. Scale, 10 mm. **e** Scheme of f-RNAi
643 experiment to evaluate feeding disorders. **f** Leaf area consumed by the larvae for 0-24
644 hours (dark gray) and 24-48 hours (right gray) after f-RNAi. *P*-values were calculated by
645 Welch's *t*-test and adjusted by Holm's method for multiple comparisons. Adjusted *P*-
646 values were shown in c and f, and non-significances were not shown. Data of b, c and f
647 are mean ± s.d., n=7 individual larvae for each f-RNAi treatment (black dot). Information
648 for c and f are provided in Table S1.
649
650 **Fig. 2** Species-specificity and efficiency of *diap1* dsRNA. **a** Identity of *diap1* nucleotide
651 among insects. Numbers in each column indicates the identity between species. Branches
652 show the phylogenetic relationship[33]. **b** Image of sequence identity of *diap1* dsRNAs
653 among insects. Blue bars indicate the identical nucleotide sites between *H.*
654 *vigintioctopunctata* and each species. UTR, untranslated region; CDS, coding sequence;
655 BIR, baculovirus inhibitior of apoptosis protein repeat; RING, really interesting new gene.
656 **c** Area consumed by the larvae during 12-24 (upper) and 24-48 (lower) hours after various
657 insect-derived *diap1* dsRNAs ingestion. **d** Area consumed by the *diap1* f-RNAi larvae
658 treated with different amounts of dsRNA. Data of c and d are mean ± s.d., n=3 in c and
659 n=4 in d (black dot). *P*-values were calculated by Welch's *t*-test and adjusted by Holm's
660 method. Adjusted *P*-values were shown in c and d, and non-significances were not shown.
661 Amel, *Apis melifera*; Blat, *Blatta lateraris*; Bmor, *Bombyx mori*; Caqu, *Catajapyx*
662 *aquilonaris*; Dmel, *Drosophila melanogaster*; Hvig, *Henosepilachna*

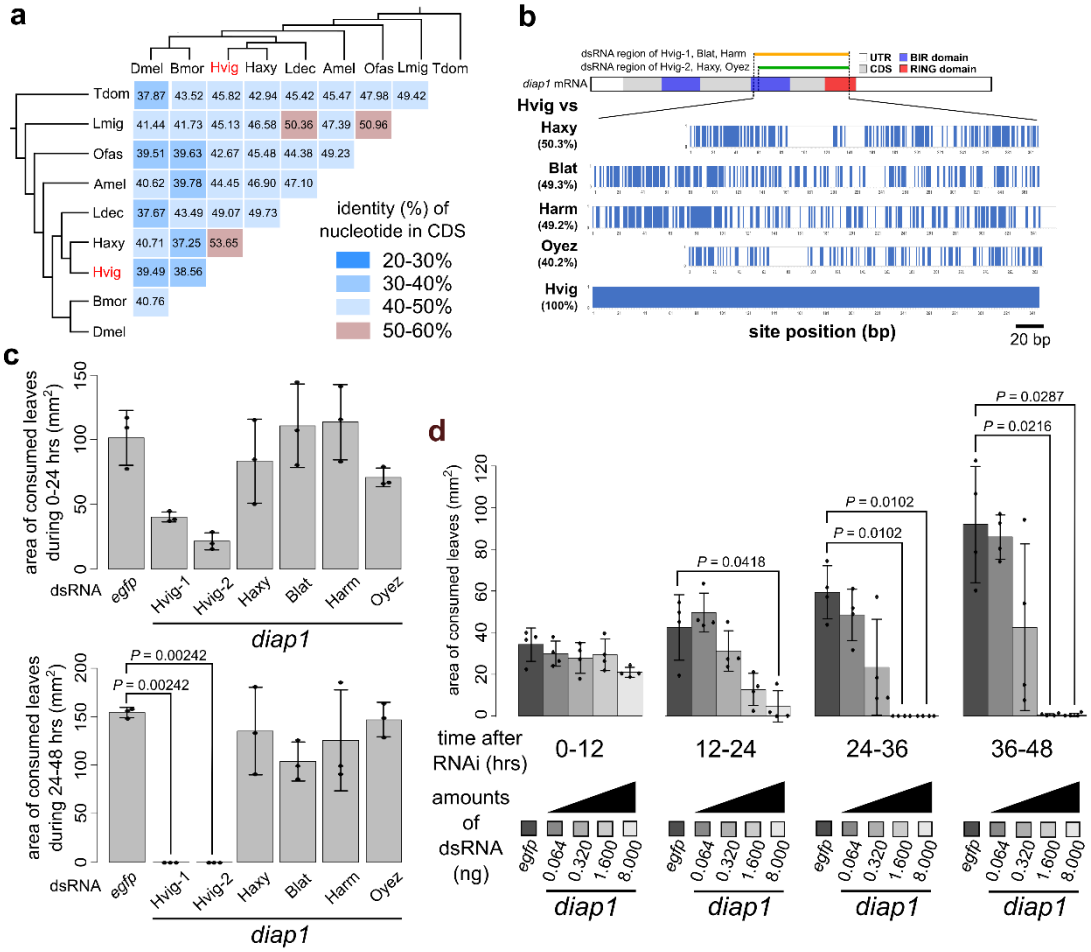
663 *vigintioctopunctata*; Harm, *Helicoverpa armigera*; Haxy, *Harmonia axyridis*; Ldec,
664 *Leptinotarsa decemlineata*; Lmig, *Locusta migratoria*; Ofas, *Oncopeltus fasciatus*; Oyez,
665 *Oxya yezoensis*. Information for a, c and d is provided in Tables S3, 4 and 5, respectively.
666
667 **Fig. 3** Recoverability, lethality and growth-inhibition of *diap1* f-RNAi in *H.*
668 *vigintioctopunctata* larva. **a** Non-recoverability of feeding cessation of the *diap1* f-RNAi.
669 The feeding cessation was caused within 48 hours after *diap1* f-RNAi with both the 50
670 and 8 ng dsRNA and persists until death of the larvae. One larva fed with the 8 ng dsRNA
671 showed slightly feeding in the day 3, but led to death in the day 4. The data shows mean
672 ± s.d. The starting number of larvae was 7 for each f-RNAi treatment. According to their
673 death, the number of larvae gradually reduced during the experiment. Black circles show
674 the number of larvae in each day. Information is provided in Table S6. **b** The leaf
675 consumed by *diap1* f-RNAi larvae. Scale, 10 mm. **c** Lethality of the *diap1* f-RNAi larvae.
676 All of the *diap1* f-RNAi larvae led to death up to the day 6, in contrast to surviving of all
677 of the control larvae fed with *egfp* dsRNA during the experiment. **d** Growth-inhibitory
678 effect of *diap1* f-RNAi. The control larvae molted into 4th instar, but not the *diap1* f-
679 RNAi larvae. The photos show the surviving larvae at the day3-4 and 4-5 in both *diap1*
680 and *egfp* f-RNAi. The *egfp* f-RNAi larvae further grew into pupal stage in the day 8–10,
681 in contrast to death of all *diap1* f-RNAi larvae up to the day 6. Scale, 3 mm.
682

683 **FIGURES**



684

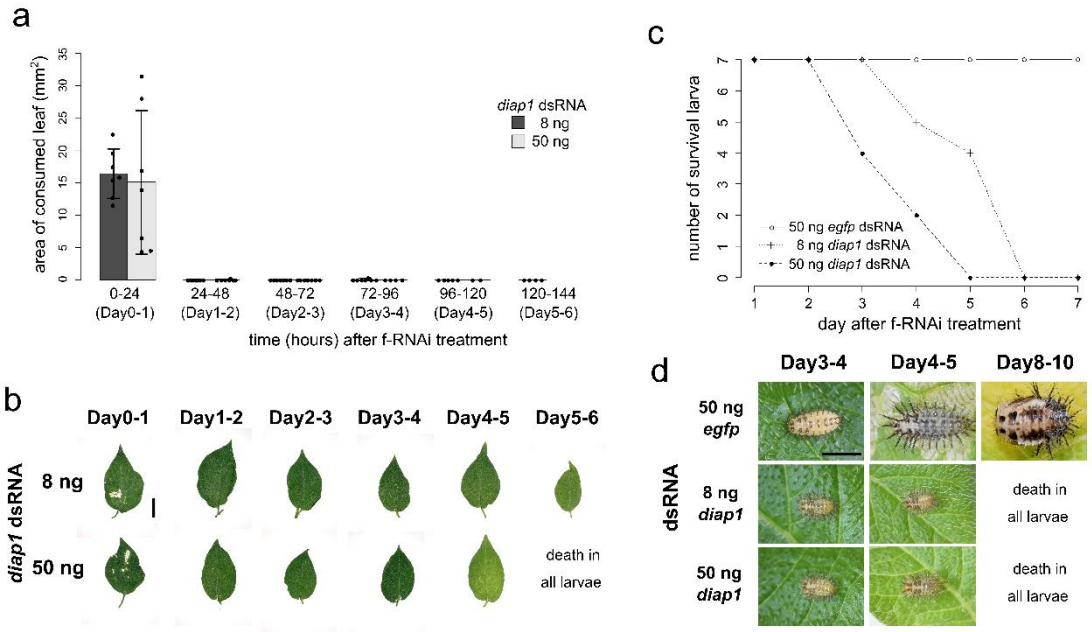
685 **Figure 1**



686

687 **Figure 2**

688



689

690 **Figure 3**

691