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A chitinase structurally related to the glycoside hydrolase family 48 is indispensable for the hormonally induced diapause termination in a beetle

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Abstract

Two proteins (APAP I and II) of the glycoside hydrolase family 48 (Family GH48) were isolated from the active adults of the leaf beetle *Gastrophysa atrocyanea*. Full-length and cDNAs were sequenced. APAP I expression and function were examined in detail. The protein has a chitinase but not a glucanase and cellobiohydrolase activity. It is expressed in the feeding stages, including beetles whose diapause was terminated with a juvenile hormone agonist. Suppression of the APAP I expression by means of RNA interference prevented the hormonal termination of diapause.

Keywords: Glycoside hydrolase; GH48; Chitinase; Feeding behavior; Insect reproductive diapause

Many insects extend their lifespan by entering developmental arrest known as diapause. Diapause is typically programmed by changes of the daylength that signal the advent of adverse conditions [1]. In the case of the leaf beetle *Gastrphysa atrocyanea*, however, imaginal diapause is obligatory and controlled genetically without input from environmental cues [2]. Diapausing adults cease feeding and bore into the soil where they rest from the summer over the winter. They resume feeding and initiate reproduction in the following spring.

Insect hormonal systems direct the onset and termination of diapause. Most cases of adult diapause can be attributed to the absence of the juvenile hormone (JH). A diapause-like state can be induced by surgically extirpating the corpus allatum (CA) and conversely, diapause can be terminated by implantation of CA or by application of JH [1]. Thus, these hormonal signals are capable of evoking or suppressing the patterns of gene expression noted during diapause, but little is known about our understanding of the molecular regulation of diapause response [3–5]. Application of exogenous JH or a synthetic JH agonist to diapausing females of *G. atrocyanea* elicits behavioral changes (abandoning the soil, identification and movement to the food plant), feeding, egg development, mating readiness, and oviposition [6]. In search for the mechanisms of these JH effects, we have analyzed changes in the protein spectrum that occur before diapause initiation and after diapause termination. In the present paper, we describe two "active phase-associated proteins" (APAP I and II) that are characteristic of developmental and non-diapausing stages. The proteins belong to the glucoside hydrolase family 48 but are functionally unique.

Materials and methods

Insects and hormonal treatments. A laboratory culture of the leaf beetle *G. atrocyanea* (Chrysomelidae) was established from adults collected outdoors in the Iwate Prefecture (Japan) in April 2003. The insects were

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fed fresh host leaves of the dock, *Rumex obtusifolius*, and reared in the laboratory at 25 °C and a 12:12 h (light:darkness) photoperiod [6]. Newly oviposited eggs, larvae (to the 3rd-instar), pupae, and emerged adults (pre-diapausing, diapausing, and post-diapausing) were reared under the same conditions. The diapause was in some cases interrupted artificially with the JH agonist (pyriproxyfen, supplied by Sumitomo Chemical Co. Ltd.). Adults one month after the emergence, which have finished pre-diapause feeding and bore into the soil, were each treated topically with 0.05 μg pyriproxyfen in 1 μL acetone for 7 days. This procedure terminated diapause in 100% of treated insects [7].

Purification and amino acid sequencing. Beetles were frozen in liquid nitrogen and stored at −80 °C until use. Samples of 5 g were homogenized in 15 mL sample buffer (10 mM Tris–HCl, pH 7.3) and centrifuged at 10,000*g* for 20 min at 4 °C. The supernatant was precipitated with 40% and after a repeated centrifugation with 60% saturated ammonium sulfate. The pellet left after the second precipitation was dissolved in 3 mL of the sample buffer and used for FPLC carried out at 4 °C on a column packed with Superdex200 HR10/30 (GE Healthcare) and equilibrated with the sample buffer. Fractions were collected at a flow rate of 0.5 mL/min and analyzed by SDS–PAGE. The fractions containing the proteins of interest (APAP I and II) were absorbed on the MonoQ HR5/5 column (GE Healthcare) equilibrated with the sample buffer and eluted with a linear gradient of 0–1 M NaCl at a flow rate of 1 mL/min. Eluted samples were examined by SDS–PAGE and the N-terminal amino acid sequence of selected proteins was determined by automated Edman degradation on the protein sequencer (PPSQ-21, Shimadzu).

cDNA cloning. Total RNA was prepared from pre-diapausing adults (usually 0.1 g; about 6 adults) with guanidine isothiocyanate and subjected to Oligo dT30-Latex (TaKaRa) to isolate poly(A)$^+$ RNA. The cDNA was synthesized from 5 μg mRNA using a Smart cDNA synthesis kit (Clontech) according to the provided protocol. PCR products obtained with degenerate primers (designed on the basis of amino acid sequence of identified proteins) and the Smart PCR primers (Clontech) were subcloned into pCR2.1 vector using the Original TA cloning kit (Invitrogen). The nucleotide sequence of cloned fragments was determined by a dyeterminator cycle sequencing on the DNA sequencer DSQ-1000 (Shimadzu). Gene-specific primers (Supplementary Table 1) were designed from the internal sequence of the analyzed DNA fragments. The entire APAP I and II cDNA sequences (GenBank Accession Nos. AB241611 and AB241612) were obtained by 3′ RACE and 5′ RACE using gene-specific primers in combination with the Smart PCR primers (Clontech).

Northern blot analysis. Total RNAs were isolated with the Isogen kit (Wako) from the eggs, 1st to 3rd-instar larvae, pupae, pre-diapausing adults, diapausing adults, and reproducing (post-diapausing) adults, and adults that had been injected with dsRNA (as described below). About 100 mg of insects at each developmental stage were used for the RNA isolation. Total RNAs (10 μg) were separated by electrophoresis on 1.0% agarose gels containing formaldehyde and transferred to Hybond-N$^+$ membranes (GE Healthcare). The membranes were pre-treated with the hybridization buffer {5× SSC, 1.0% blocking reagent (Roche), 0.1% *N*-lauroylsarcosine, and 0.02% SDS} for 2 h at 50 °C. The RNAs were hybridized with a DIG-11-dUTP-labeled probe (in hybridization buffer) for 12 h at 50 °C. The 181 bp-long probe corresponded to the full-length cDNA of APAP I and was prepared from 100 ng of pCR2.1-*apap1* template by PCR with the APAP1fP and APAP1rP primer (Supplementary Table 1).

Production of the antiserum. Polyclonal antiserum was generated in rabbit against a synthetic peptide containing 15 N-terminal amino acid residues of APAP I. This synthetic peptide was conjugated to keyhole limpet hemocyanin (KLH) as a carrier protein. About 100 μg of the synthetic polypeptide in phosphate-buffered saline was mixed with Freund's complete adjuvant (Nacalai) and injected into a New Zealand white rabbit. The resulting antiserum recognized protein of expected size on the Western blots.

Western blot analysis. About 100 mg of insects at each developmental stage frozen in liquid nitrogen and stored ar −80 °C were homogenized in 300 μL of extraction buffer (62.5 mM Tris–HCl, pH 7.5, 5 mM EDTA). The concentration of proteins in the supernatant (10,000*g* for 20 min at 4 °C) was determined with the BCA protein assay kit (Pierce). Aliquots of the protein extracts (20 μg) were separated by SDS–PAGE on the 7.5% gels and transferred to the PVDF membranes (Millipore) and hybridized with the antibody specific to APAP I. The immunogenic reaction was detected by enhanced chemiluminescence (GE Healthcare).

Cellobiohydrolase assay. Aliquots (10 μL, 10 μg) of the purified APAP I were incubated with 10 μL of 60 mM solutions of cellotriose, cellotetraose, and cellopentaose, respectively, or 2% (w/v) sodium carboxymethylcellulose (CMC) in sodium acetate buffer at 25 °C for 6 h. The hydrolysis products were separated by thin layer chromatography (TLC). TLC was performed according to the method of Scrivener and Slaytor [8].

Chitinase assay. Chitinase activity was measured using colloidal chitin as the substrate. Colloidal chitin was prepared essentially by the method of Hirano and Nagano [9] from the practical-grade crab shell chitin (Sigma). The reaction mixture consisted of 10 μL (10 μg) of purified APAP I or *Serratia marcescens* chitinase (Sigma) as a positive control, and 1 mL of 0.5% (w/v) colloidal chitin in 0.1 M phosphate buffer, pH 7.0. After incubation at 25 °C for 2 h, the reducing power of the reaction mixture was measured colorimetrically at 420 nm with ferri-ferrocyanide reagent by the method of Imoto and Yagishita [10]. Enzyme activity was corrected with controls of boiled enzyme preparation and expressed as *N*-acetylglucosamine liberated (nmol/mL). Values are means ± SE from three experiments.

dsRNA preparation. A slightly modified method of Fire et al. [11] was used to generate the *apap1*-specific dsRNAs. Briefly, the *apap1* cDNA was amplified by PCR as described above for the preparation of the probe for the Northern blotting, except that DIG-11-dUTP was omitted in the reaction. The PCR product was purified using a Mag Extractor PCR & Gel Clean Up kit (Toyobo) and inserted into the pGEM-T Easy Vector (Promega) between the T7 and SP6 promoters. M13 forward (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers were used to PCR-amplify the *apap1* sequence with the flanking T7 and SP6 promoters. This PCR product served as a linear template for the in vitro generation of single-stranded RNA using T7 and SP6 RNA polymerases (Roche). After phenol/chloroform-extraction, the sense and antisense RNA strands were annealed by heating to 65 °C for 10 min, followed by cooling to 37 °C over 30 min. The dsRNA (181 bp) was ethanol-precipitated and suspended in double distilled water to a 1.0 mg/mL.

RNA interference and the statistical analysis of adult viability. Diapausing female adults one month after emergence were injected with 0.2 μL (200 ng) of the *apap1* dsRNA. The injection needle was inserted into the intersegmental membrane between the metathorax and 1st abdominal segment as described previously [12]. Control insects were injected with 0.2 μL (200 ng) of *lacZ* dsRNA. The injected adults were treated with JH agonist pyriproxyfen as described above and incubated at 25 °C for 7 days. Statview 5.0 (SAS) software (Kaplan–Meier) was used for the statistical analysis and to determine survival means and percentiles. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal. *P* values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time.

Results and discussion

Identification and enzymatic assay of APAPs

Two active phase-associated proteins (APAP I and II) were isolated from the pre-diapausing *G. atrocyanea* adults 3–4 days post-eclosion (Supplementary Fig. 1). None of the proteins were detected in the diapausing adults. Two fragments of APAP I and II cDNAs were isolated by RT-PCR from the mRNA obtained from the pre-diapausing adults. The deduced amino acid sequences of these cDNAs are shown in Supplementary Fig. 2. In search for homologous

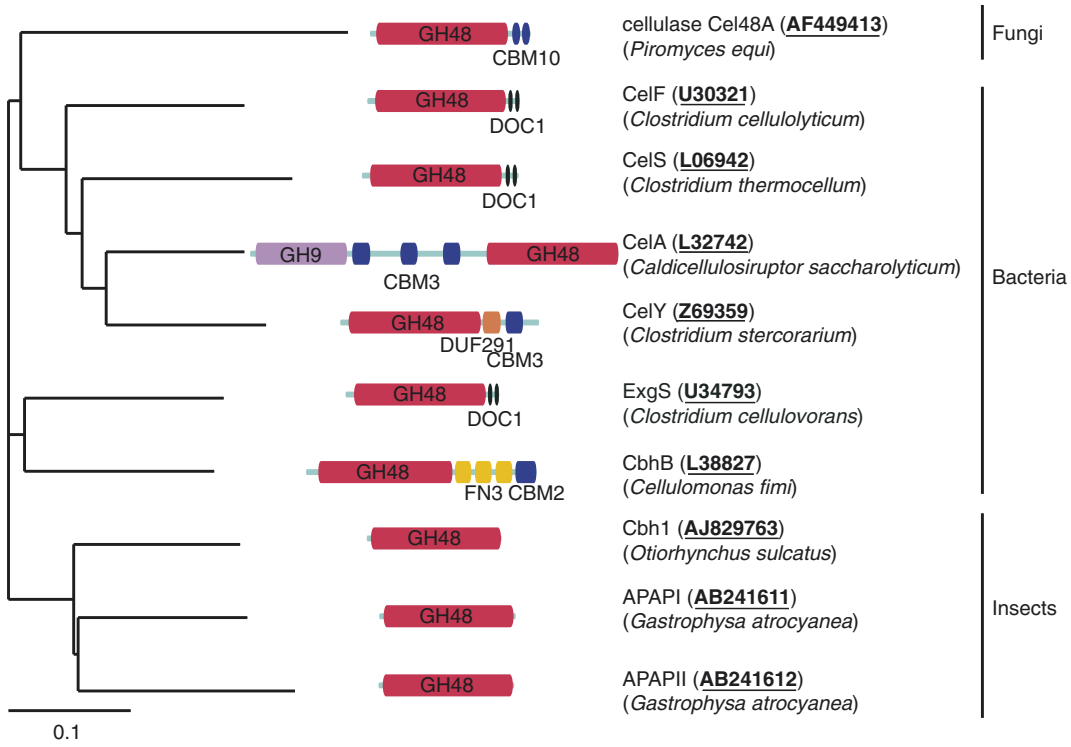

Fig. 1. Comparison of amino acid sequences and domain structures of active phase-associated proteins (APAP I and II) in the leaf beetle and glycosyl hydrolase family 48 (Family GH48) members. A phylogenetic tree was constructed using the catalytic domain sequences. The scale indicates distance (number of amino acid substitutions per site). Members of fungi, bacteria, and insects were temporarily grouped. GH48, catalytic domain of glycosyl hydrolase family 48; GH9, catalytic domain of glycosyl hydrolase family 9; CBM 2, 3, 10, cellulose binding domain; DOC1, dockerin 1; DUF291, domain of unknown function; FN3, fibronectin type III domain.

proteins in the GenBank database (BLAST and FASTA) we found that APAP I and II apparently belong to the glycoside hydrolases Family 48 (GH48). APAP I and II share considerable amino acid sequence homology with GH48 members from bacteria, fungi, and insects (Fig. 1). However, GH48 are in most cases components of complex proteins that include additional functional domains. For example, the CelA protein of *Caldicellulosiruptor saccharolyticum* is composed of the catalytic domains of GH48 and GH9 and a cellulose binding domain (CBM 3) [13]. In other proteins, GH48 domain is composed with dockerin, a domain of unknown function (DUF29) and fibronetin III domain (Pfam: http://www.sanger.ac.uk/Software/Pfam/). Strikingly, APAP I and II, and a gene encoding a novel eukaryotic cellobiohydrolase in the vine weevil (*Otiorkynchus sulcatus*) (AJ829763) share only the catalytic GH48 domain and lack the other elements. Using purified APAP I we investigated its cellulose-digesting ability as an endo- or exo-glucanase but no enzyme activity was detected (Fig. 2A). However, in the assay with colloidal chitin, APAP I exhibited chitinase activity reaching nearly half of the level of the *S. marcescens* chitinase (Fig. 2B). No sequence similarity was found between APAP I and insect chitinases: this is the first report on a chitinase activity in a GH48 protein. Insect cellulases belong mainly to families GH9 [14,15] and GH45 [16] and insect chitinases to family 18 of the gylcohydrolase superfamily [17]. The analysis of APAP II function is now in progress and necessary to understand a new member in comparison with the known chitinases.

Stage-dependent expression of APAP I

Being present in the actively reproducing beetles that survive about 2 weeks and absent in the diapausing ones that live for more than 6 months, APAP I and II may play a role in lifespan regulation. Alternatively, it may be important in digestion and nutrient absorption. We thus examined changes in APAP I expression throughout the beetle development. Northern and Western blotting analyses confirmed APAP I expression in the larvae, pre-diapausing and reproducing adults (3 days post-eclosion and 5 days after diapause termination, respectively), but not in the non-feeding stages such as eggs, pupae, and diapausing adults (Fig. 3). It seems that APTP I is linked to feeding.

APAP I-less adults and their behaviors

For the functional relationships between APAP I, juvenile hormone action, and the feeding behavior, we generated APAP I-less individuals by the injection of double-strand *apap1* RNA (dsRNA). Gene silencing caused by dsRNA has already proved a useful tool in the analysis of a diapause-specific peptide [12] and two small

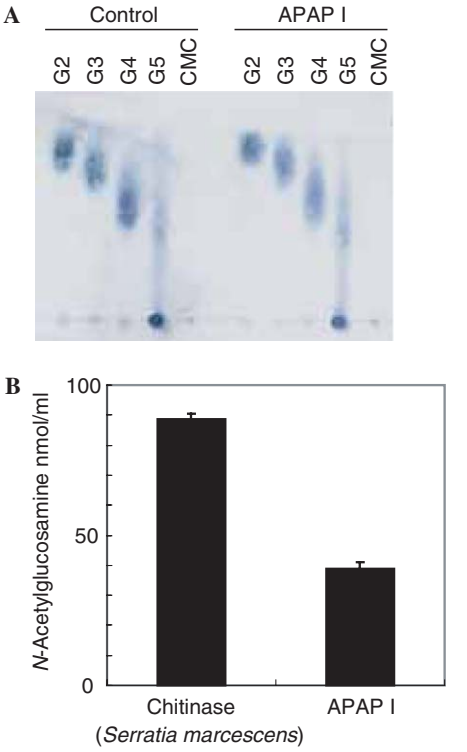

Fig. 2. Enzymatic properties of APAP I. (A) Detection of cellulases. Enzyme solution (10 μL) of the purified APAP I (10 μg) was incubated with 10 μL of cello-oligosaccharides (cellobiose, G2; cellotriose, G3; cellotetraose, G4; cellopentaose, G5) and 2% (w/v) sodium carboxymethyl-cellulose (CMC) in sodium acetate buffer at 25 °C for 6 h. Hydrolytic products from these substrates were analyzed by TLC. TLC was performed using a solvent system (2-propanol/acetone/0.1 M lactic acid 2:2:1). Sugars were visualized by incubating the plate at 100 °C for 10 min, after spraying with DPAP reagent (1 g diphenylamine, 7.5 mL of 85% phosphoric acid, 50 mL acetone, and 1 mL aniline). As a result, cellulase digesting ability of APAP I was not detected. (B) Detection of chitinases. The reaction mixture (1.01 mL) containing 10 μg APAP I or *S. marcescens* chitinase (Sigma) and 0.5% (w/v) colloidal chitin was incubated for 2 h at 25 °C. The activity was measured at 420 nm with ferri-ferrocyanide reagent and expressed as *N*-acetylglucosamine liberated (nmol/mL). Values are means ± SE from three experiments.

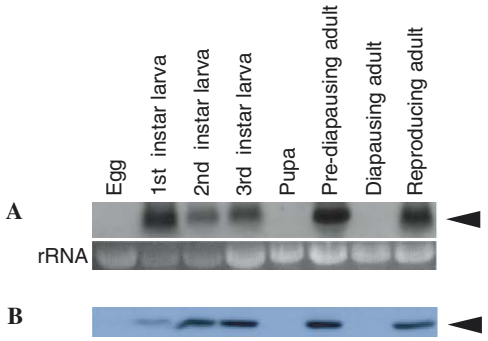

Fig. 3. Northern (A) and Western (B) blot analyses of *apap1* expression at each developmental stage of the leaf beetle. Total RNA (10 μg) and protein (20 μg) were prepared from newly oviposited eggs, first-instar larvae, second-instar larvae, third (last)-instar larvae, pupae, pre-diapausing adults (at 3 days post-eclosion), diapausing adults (20 days post-eclosion), and reproducing adults (7 days post-diapause). The arrowhead in (A) indicates 2 kb *apap1*-specific transcripts. Ethidium bromide-stained rRNA is shown below the Northern blot in (A). The arrowhead in (B) indicates a 71 kDa protein recognized by an APAP I-specific antibody.

heat shock proteins [18] in *G. atrocyanea*. To monitor possible APAP I interaction with JH action, *apap1* dsRNA was injected into the diapausing adults that were subsequently treated with the JH agonist (Fig. 4). The *lacZ* gene was injected as a control. The control beetles responded to the JH agonist by resuming feeding and reproduction after diapause termination. At 2–3 days after JH agonist treatment followed for 7 days (i.e., after 9–10 days of the dsRNA injection), the results of Western blot analysis showed a marked decrease (Fig. 4A). The behavior patterns of beetles also greatly varied. About 40% of APAP I-less beetles remained on the soil and their food consumption ranged from the normal voracious feeding to moderate feeding, but some did not feed. Surprisingly, the feeding behavior of APAP I-less adults was clearly distinguishable from that of the control insects and their ultimate behavior was a pattern of non-feeding and soil-dwelling (Fig. 4B). In adults at 7 days observation after JH agonist treatment, the control and APAP I-less insects did not show a significant difference among the adult viability (Fig. 4C), but the behavior patterns were distinctly different: the control females on the soil consumed food and developed eggs, while those injected with dsRNA burrowed into the soil, did not feed, and developed no eggs (Fig. 4D).

Since the APAP I-less adults show non-feeding and soil-dwelling behavior, APAP I seems to be necessary for the diapause termination by JH. Based on the CAZy-carbohydrate-active enzymes server at URL: http://www.cazy.org/CAZY/, APAP I is the first chitin-digesting member of the GH48 family [19]. To our knowledge, insect epidermal cells secrete cuticle even during the intermolting period [20]. We have evidence that APAP I is localized mainly in the hemolymph (data not shown) and may be linked to chitin metabolism in the integument, trachease, and also in the digestive tract. This might explain its presence in the feeding stages and feeding inhibition in the APAP I-less adults. The role of APAP I in the control of feeding is apparently different from the newly discovered *takeout* gene that links feeding behavior to the circadian clock in *Drosophila melanogaster* [21,22]. APAP I and TAKEOUT exhibit no sequence similarity. The expression of *takeout* is probably upstream or simultaneous to the expression of neurohormones that promote feeding and development, whereas APAP I seems to affect feeding secondarily after its JH stimulation. A possible scenario is that *apap1* silencing interferes with chitin metabolism and cuticle deposition in the mouth parts, foregut lining, peritrophic membrane, and possibly other body parts involved in the feeding and digestion. The cessation of feeding and interruption of reproduction are secondary effects. Thus, the further elucidation of APAP I and II will provide significant advances in understanding the molecular mechanism of extending insect lifespan through diapause, although APAP II remains to be analyzed.

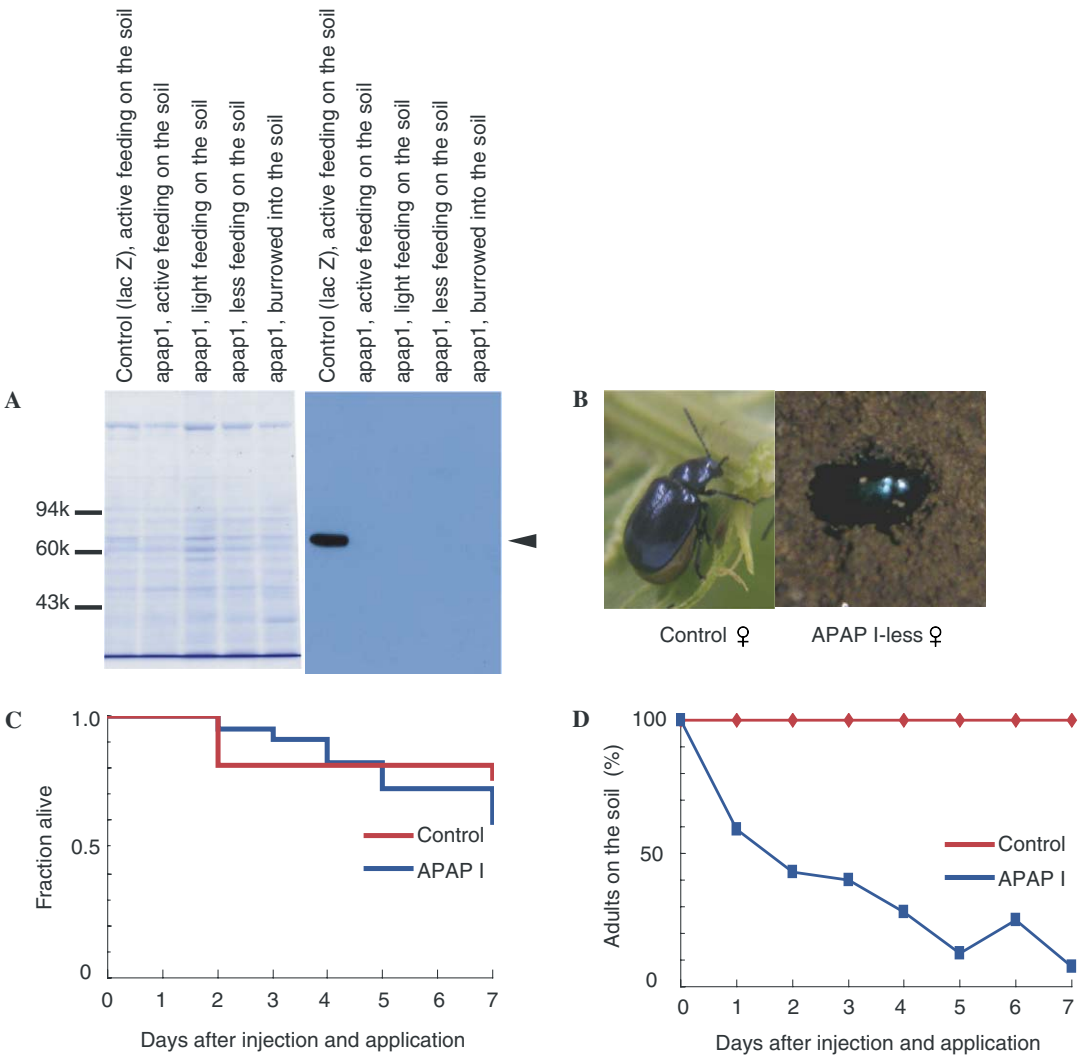

Fig. 4. Effects of APAP I on feeding behavior and soil-dwelling. (A) SDS–PAGE (left side) and Western blot analysis (right side) of diapausing adult samples injected with *apap1*-specific dsRNA and treated with 0.05 μg of a juvenile hormone agonist (JHA) for 7 days (see Materials and methods). Thereafter these adults made to progress to the reproductive stage were observed and analyzed 2–3 days. During this short period, their behavior was divided into four patterns: active feeding, light feeding, less feeding, non-feeding, and burrowing into the soil. The arrow indicates the immunologically specific protein. (B) Control beetle injected with *LacZ* dsRNA and APAP I-less beetle injected with *apap1*-specific dsRNA. After JHA application as well as in (A), the control female beetles on the soil began feeding and showed ovarian development within the observation of a few days. In contrast, the APAP I-less beetles burrowed into the soil. (C) Viability of the leaf beetles following RNAi. Diapausing adults were injected with *apap1* dsRNA ($n = 22$ females) or *lacZ* dsRNA ($n = 21$ females). JHA-applied beetles were observed for 7 days. Statview 5.0 (SAS) software (Kaplan–Meier) was used for the statistical analysis and for determining the survival means. (D) Soil-dwelling behavior of leaf beetles following RNAi. Insects on the soil were assumed to be showing soil-dwelling behavior.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.04.126.

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