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Author(s)	Yamamoto, Kohji; Higashiura, Akifumi; Hirowatari, Aiko et al.
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Characterisation of a diazinon-metabolising glutathione S-transferase in the silkworm *Bombyx mori* by X-ray crystallography and genome editing analysis

Kohji Yamamoto¹, Akifumi Higashiura^{2,4}, Aiko Hirowatari¹, Naotaka Yamada¹, Takuya Tsubota³, Hideki Sezutsu³ & Atsushi Nakagawa²

Previously, we found an unclassified glutathione S-transferase 2 (bmGSTu2) in the silkworm *Bombyx mori* that conjugates glutathione to 1-chloro-2,4-dinitrobenzene and also metabolises diazinon, an organophosphate insecticide. Here, we provide a structural and genome-editing characterisation of the diazinon-metabolising glutathione S-transferase in *B. mori*. The structure of bmGSTu2 was determined at 1.68 Å by X-ray crystallography. Mutation of putative amino acid residues in the substrate-binding site showed that Pro13, Tyr107, Ile118, Phe119, and Phe211 are crucial for enzymatic function. *bmGSTu2* gene disruption resulted in a decrease in median lethal dose values to an organophosphate insecticide and a decrease in acetylcholine levels in silkworms. Taken together, these results indicate that bmGSTu2 could metabolise an organophosphate insecticide. Thus, this study provides insights into the physiological role of bmGSTu2 in silkworms, detoxification of organophosphate insecticides, and drug targets for the development of a novel insecticide.

Glutathione (GSH) S-transferases (GSTs, EC 2.5.1.18) are cytosolic enzymes that are present in both prokaryotes and eukaryotes¹. Seven GSTs are classified in mammals, alpha, mu, pi, omega, sigma, theta, and zeta, and can be distinguished based on their amino acid sequences. Sequence identity in one class covers approximately 50% and less than 30% distributed between the other classes^{2,3}. Six GST classes (delta, epsilon, omega, sigma, theta, and zeta) have been reported in dipteran insects, including *Anopheles gambiae*⁴ and *Drosophila melanogaster*^{5,6}. GSH conjugation is essential for the detoxification of xenobiotics^{7,8}. GSTs for insects can influence their sensitivity in insecticides^{4,9}, and as the Lepidoptera comprises major agricultural pests, it is important to study lepidopteran GSTs. We have characterised diverse GSTs (delta, epsilon, omega, sigma, theta, zeta, and an unclassified GST) in the silkworm *Bombyx mori*, a lepidopteran model animal^{10–16}; a sigma-class GST in the fall webworm *Hyphantria cunea*, one of the most serious lepidopteran pests of broad-leaved trees¹³; and a delta-class GST in *Nilaparvata lugens*, a rice crop pest¹⁷. Previously, we identified a novel GST obtained from *B. mori* (bmGSTu2)¹⁸.

In the present paper, we provide a structural and genome-editing characterisation of a diazinon-metabolising glutathione S-transferase in *B. mori*. Moreover, the crystal structure of bmGSTu2 as well as *bmGSTu2* gene disruption analysis helps clarify xenobiotic agents affect insects and contributes to a more detailed understanding of the GST system.

¹Department of Bioscience and Biotechnology, Kyushu University Graduate School, 744 Motooka, Nishi-ku, Fukuoka, 819-0395, Japan. ²Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka, 565-0871, Japan. ³Transgenic Silkworm Research Unit, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 1-2 Owashi, Tsukuba, Ibaraki, 305-8634, Japan. ⁴Department of Virology, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima, 734-8551, Japan. Correspondence and requests for materials should be addressed to K.Y. (email: yamamok@agr.kyushu-u.ac.jp)

	Native	Hg derivative
Wavelength (Å)	0.9000	1.0070
Space group	$P4_1$	$P4_1$
Unit cell parameters (Å)	$a = b = 86.26, c = 58.77$	$a = b = 85.47, c = 58.58$
Resolution range (Å)	42.3–1.68 (1.71–1.68)	48.3–2.48 (2.52–2.48)
Total number of reflections	363,111	226,847
Number of unique reflections	48,961 (2,294)	15,172 (722)
Multiplicity	7.4 (7.1)	15.0 (14.4)
R_{merge} (%)	4.9 (>100)	6.3 (68.2)
R_{pim}	2.6 (48.5)	2.0 (20.2)
$CC_{1/2}$ highest-resolution shell	0.696	0.907
$\langle I \rangle / \langle \sigma(I) \rangle$	25.2 (1.64)	70.7 (4.37)
Completeness (%)	99.3 (94.1)	99.8 (97.0)
Refinement statistics		
Resolution range (Å)	42.3–1.68	
Number of reflections	44,320	
R_{work} (%) / R_{free} (%)	18.65/22.10	
Root-mean-square deviations		
Bond lengths (Å) / Bond angles (°)	0.007/0.832	
Average B-factors (Å²) / Number of atoms		
Protein (Chain A, B)	15.6/1,672, 15.7/1,665	
Small molecules	15.78/24	
Water	29.6/461	
Ramachandran plot		
Favored region (%)	98.74	
Allowed region (%)	0.76	
Outliers (%)	0.50	

Table 1. Data collection and refinement statistics. (values in parentheses indicate the highest-resolution shell).

^a $R_{\text{merge}} = \sum(I - \langle I \rangle) / \sum \langle I \rangle$, where I is the intensity measurement for a given reflection and $\langle I \rangle$ is the average intensity for multiple measurements of this reflection. ^b $R_{\text{work}} = \sum |F_{\text{obs}} - F_{\text{cal}}| / \sum F_{\text{obs}}$, where F_{obs} and F_{cal} are the observed and calculated structure-factor amplitudes. ^cThe R_{free} value was calculated using only an unrefined, randomly chosen subset of reflection data (5%) that were excluded from refinement. ^dSmall molecules include acetate ion, and ethylene glycol.

Results

X-ray structural analysis of bmGSTu2. We already overexpressed recombinant bmGSTu2 in bacteria, and purified it¹⁸. The purified protein was crystallised in a space group of $P4_1$ with unit cell dimensions $a = b = 86.26$ Å and $c = 58.77$ Å. The structure was solved and the phases were refined; Table 1 includes relevant data. The final R_{work} and R_{free} factors were 18.7% and 22.1% for resolutions of 42.3–1.68 Å, respectively, with root-mean-square deviations for bond lengths and angles of 0.007 Å and 0.832°, respectively. The Ramachandran plot data showed that 98.74% of the main-chain dihedral angles were in the preferred regions, 0.74% in the allowed regions, and 0.50% in the outlier regions.

Structural characteristics of bmGSTu2. The bmGSTu2 amino acid sequence indicated 34% and 33% identity with *N. lugens* delta-class GST (nIGSTD, PDB ID: 3WYW) and *B. mori* delta-class GST (bmGSTD, PDB ID: 3VK9), respectively (Fig. 1A). The crystal structure of bmGSTu2 was determined at 1.68 Å resolution and solved by the single-wavelength anomalous diffraction (SAD) method using the Hg-derivative. The resulting structure revealed a homodimer of the bmGSTu2 molecule after analysis by PISA program for investigation of macromolecular complexes¹⁹ and gel filtration elution profile (data not shown). Structural elements, characterised by the STRIDE program for protein secondary structure assignment²⁰, showed that bmGSTu2 contains 8 α -helices and 4 β -strands (Fig. 1A). Two discrete domains, N-terminal (residues 1–78) and C-terminal (residues 89–233), were connected by a linker region (residues 79–88) (Fig. 1B). The N-terminal domain included 4 β -strands ($\beta 1$ [residues 3–7], $\beta 2$ [residues 29–32], $\beta 3$ [residues 56–59], and $\beta 4$ [residues 62–64]) and 3 α -helices ($\alpha 1$ [residues 12–24], $\alpha 2$ [residues 43–48], and $\alpha 3$ [residues 67–78]). The C-terminal domain consisted of $\alpha 4$ (residues 89–114), $\alpha 5$ (residues 126–146), $\alpha 6$ (residues 159–173), $\alpha 7$ (residues 177–193), and $\alpha 8$ (residues 195–208). Similar to other GSTs, the bmGSTu2 structure adopted the canonical GST fold. Screening the predicted 3D model of bmGSTu2 in Protein Data Bank (<https://www.rcsb.org>) showed highest similarity to nIGSTD (PDB ID: 3WXW) with an E-value of 2.26E-29. The structure of bmGSTu2 reveals root-mean square-deviation of 1.50 Å to that of nIGSTD.

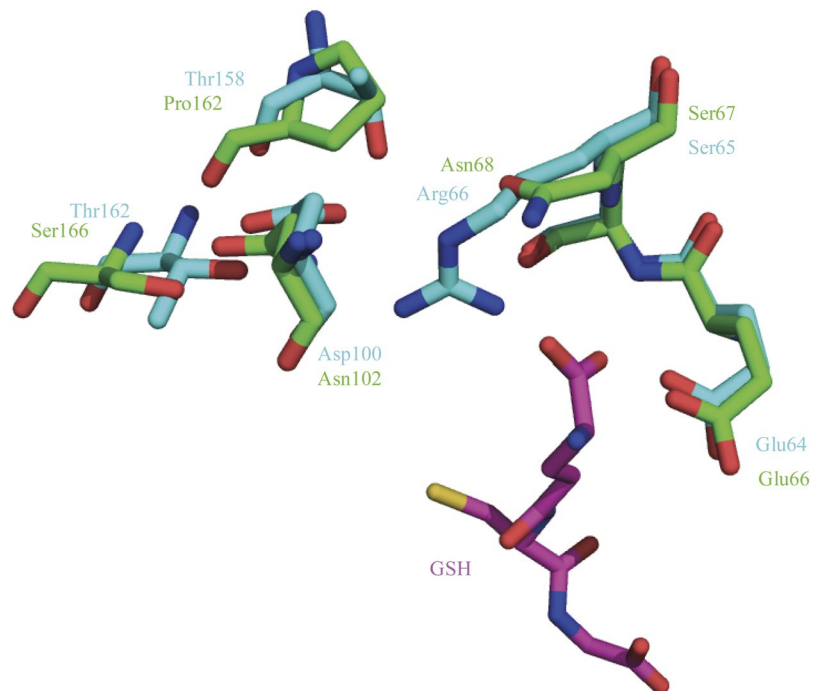


Figure 2. Amino acid residues of the electron-sharing network. Carbon atoms for bmGSTu2, agGSTd3-3, and GSH are represented by green, cyan, and magenta, respectively. Atoms of oxygen, nitrogen, and sulphur are red, blue, and yellow, respectively. Amino acid residues for bmGSTu2 and agGSTd1-6 are described in green and cyan, respectively.

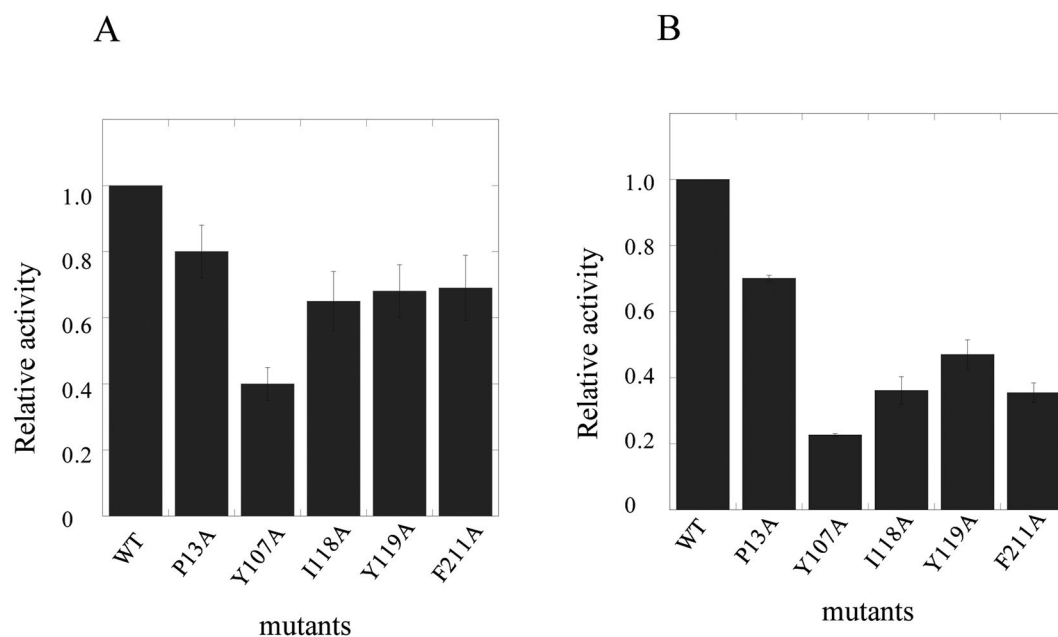


Figure 3. Specific activities of bmGSTu2 mutants in reactions with CDNB (A) and diazinon (B). The activities of wild-type (WT) and mutants (P13A, Y10A, I118A, Y119A, and F211A) are shown. Data represent the mean values of three independent experiments. Statistics were performed using one-way ANNOVA. Significant level is at $P < 0.05$.

knock-in system^{24,25}. This enables the discrimination of the wild-type and mutant allele easily. In the present study, we disrupted the *bmgstu2* gene and inserted the GFP sequence as a reporter (Fig. 4). TALEN vectors designed against the coding region of the *bmgstu2* gene were microinjected into 300 silkworm embryos using a mixture of the donor vector (PITCh vector), resulting in a number of GFP-positive G1 individuals. The genotyping analysis

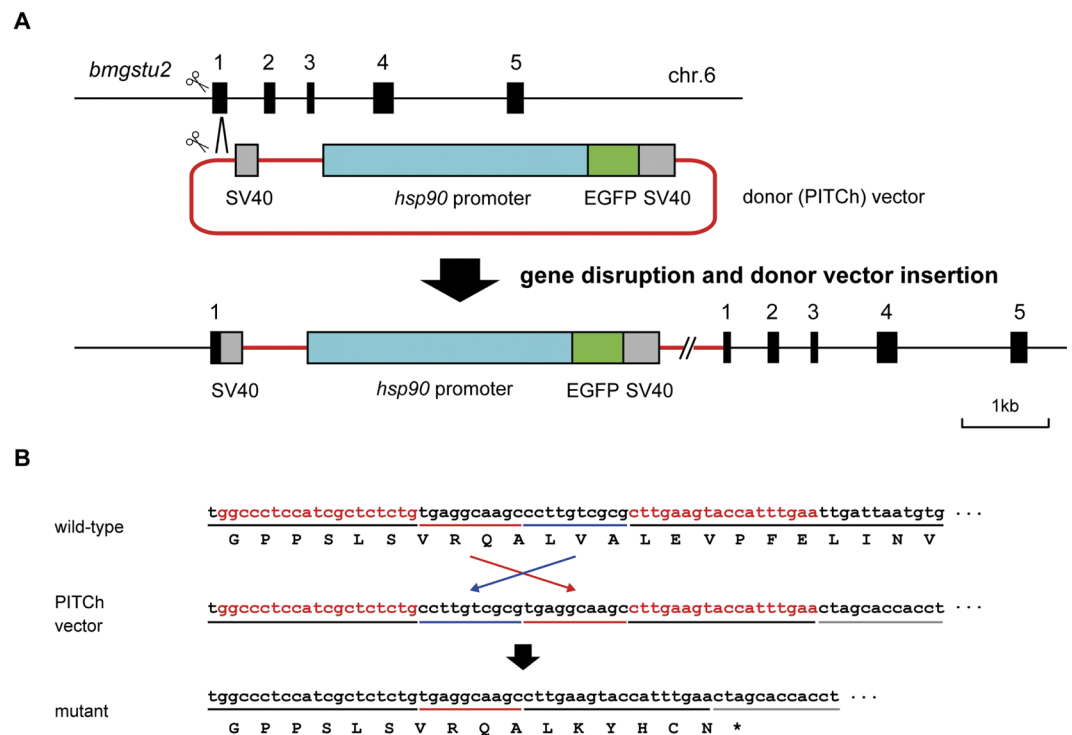


Figure 4. Establishment of the mutant strain. (A) Schematic representation of *bmgstu2* mutant allele. The structure of the *bmgstu2* gene is shown at the top. Black bars indicate exons of *bmgstu2*. The scissors indicate TALENs that target both the genome and PITCh vector including SV40, the *hsp90* promoter, and EGFP. (B) The resulting sequence of the mutant allele. The red character indicates the TALEN target site. The black underline represents the sequence derived from *bmgstu2*; red and blue lines represent microhomology sequences, and the grey line indicates a partial sequence of SV40. The precise donor (PITCh) vector insertion was present in the established strain.

revealed that 15 individuals showed targeted gene disruption (Fig. S1). The sequencing analysis revealed that one of these individuals showed precise integration for both 5' and 3' junctions (data not shown), and we utilised this mutant strain for further functional analysis of *bmgstu2*.

Median lethal dose (LD50) and acetylcholine (ACh) measurements. We measured the LD50 levels for diazinon, a widely used organophosphate insecticide, in the mutant and control *B. mori* strains. The LD50 value in the mutant was decreased to 70% of that in the control strain of *B. mori* (Fig. 5). Acetylcholine (ACh) levels were also estimated by using acetylcholinesterase (AChE) and choline oxidase. Binding of organophosphate insecticide to AChE results in inactivation of AChE, which indicates that AChE is unable to metabolise ACh. Organophosphate insecticides exert their toxicity by allowing ACh to overact at its receptors in the central and peripheral nervous systems. Notably, ACh levels in the control strain were decreased to 70% of that in the knock-in strain (Fig. 6).

Discussion

In our previous study, we identified and characterised bmGSTu2¹⁸. We also found that bmGSTu2 was capable of metabolising diazinon. Mutation of putative amino acid residues in the G-site showed that Ile54, Glu66, Ser67, and Asn68 are crucial for enzymatic function.

In the present study, we focused on the crystal structure of bmGSTu2 to examine amino acid residues that contribute to the conjugation reaction of diazinon on GSH, as well as the physiological role of bmGSTu2 by genome editing analysis using TALEN. Additionally, we solved the crystal structure of bmGSTu2. Superposition of the structure of bmGSTu2 onto that of nGSTD demonstrated that the α -helices and β -strands of bmGSTu2 were conserved across the structures (Fig. 1A,B).

Although amino acid residues in the G-site are conserved in *B. mori* GSTs^{11,26–30}, the sequence diversity of the H-site is attributed to substrate specificity³¹; furthermore, this diversity may be the reason why substrate specificities of *B. mori* GSTs are varied. Our mutagenesis experiments indicate that the residues Pro13, Tyr107, Ile118, Phe119, and Phe211 in bmGSTu2 play important roles in its enzymatic functions. Among all the mutants tested, the Y107A mutation showed the most apparent decrease in activity. Another critical residue, Tyr107, is located in the H-site and is highly conserved among delta-class GSTs, whereas the Phe residue is conserved instead of Tyr among epsilon-class GSTs³². Similar results were obtained for *A. dirus* delta-class GST4-4 (adGST4-4). Tyr111 of adGST4-4, the corresponding residue to Tyr107 of bmGSTu2, was reported to contribute to substrate-binding and stabilisation of GSH³³. Among the GSTs, the H-site of delta-class *A. gambiae* GSTs (adGSTd1-6) was the most

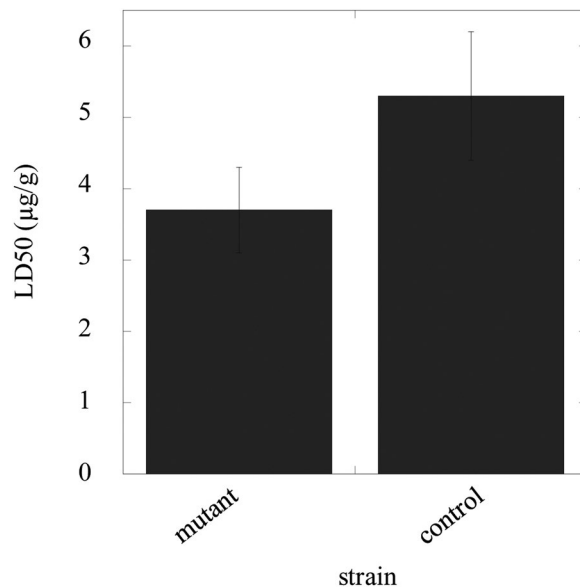


Figure 5. The effect of diazinon on LD50 values. Day-1 fifth-instar larvae of the mutant (homozygote) and control (heterozygote) were exposed to diazinon solutions via direct contact with the larval abdomen. At 24 h post-treatment (on day 2), LD50 values were determined. Relationships between two variables were examined by one-way ANOVA, with a significance level at $P < 0.05$.

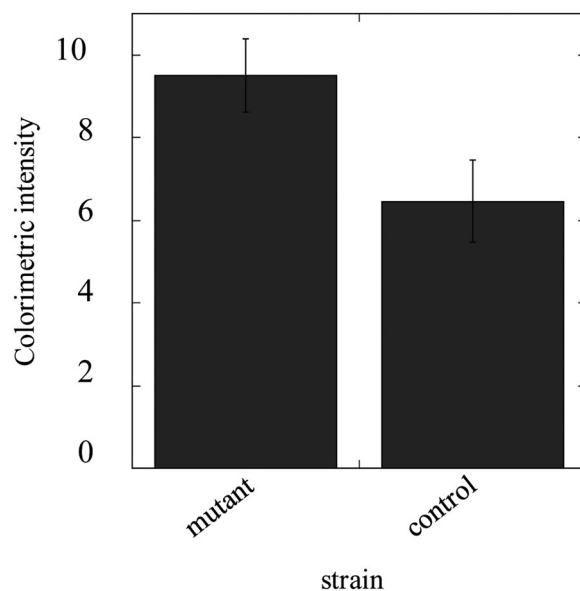


Figure 6. The effect of diazinon on ACh content. Crude extracts were prepared from the whole bodies of mutant (homozygote) and control (heterozygote). ACh production was determined by a colorimetric assay described in the Methods section. The colorimetric intensity was normalised to body weight, in grams. The intensities are expressed as the means of triplicate experiments. The significance of differences between each group was calculated based on one-way ANOVA analysis ($P < 0.05$).

similar. The H-site of agGSTd1-6 (PDB ID: 1PN9) is composed of residues that are mostly hydrophobic: Leu6, Ala10, Pro11, Leu33, Met34, Tyr105, Phe108, Tyr113, Ile116, Phe117, Phe203, and Phe207³⁴. In the bmGSTu2 sequence, 5 of the 12 residues (Pro13, Tyr107, Ile118, Phe119, and Phe211) are equivalent to the above agGST1-6 residues, and the remainder (Val8, Phe35, and Ile110) exhibit similarity to those in agGSTd1-6.

Other sites proposed to be requisite for bmGSTu2 function are the lock-and-key motif, the small hydrophobic core, and an ionic bridge^{21,22,35,36}. The lock-and-key motif is important for stabilising hydrophobic interactions of GST monomers^{22,27}. An intersubunit motif (lock-and-key) was found in the dimer interface of bmGSTD, a delta-class GST²⁷, wherein the ‘key’ residues (Glu66, Arg68, and Tyr100) are introduced into a hydrophobic

Measurements of enzyme activity. GST was assayed spectrophotometrically using 1-chloro-2,4-dinitrobenzene (CDNB) and 5 mM GSH as standard substrates⁴⁷. GST activity was expressed as mol CDNB conjugated with GSH per min per mg of protein. The ability of bmGSTu2 to metabolise diazinon was determined by HPLC according to previous reports. The eluate was monitored at 246 nm for the detection of metabolites. Specific activity toward diazinon was determined on the basis of the corresponding peak area identified per mg of protein.

Construction of TALEN and PITCh vectors. TALEN vectors were constructed as described by Takasu *et al.* (2014, 2016)^{48,49}. The target site was selected within the coding region of the *bmgstu2* gene, and the sequence around the target site was determined in the *w1-pnd* strain. TALEN was assembled using the Golden Gate TALEN and TAL Effector kit (Addgene, Cambridge, USA) and the TALEN backbone vector pBlue-TAL²³. The mRNA was *in vitro* synthesised using the mMACHINE mMESSAGE mMACHINE T7 kit (Ambion, Carlsbad, USA).

PITCh vector construction was carried out following the method described in Tsubota and Sezutsu (2017)⁵⁰. Inverse PCR was carried out using the primers 5'-TGAGGCAAGCCTTGAAGTACCATTTGAACTAGCACCTGTTCTGTAG-3' and 5'-CGCGACAAGGCAGAGCGATGGAGGGCCACTCGAA TTAGATCTTTGG-3' against the pBachsp90GFP-3xP3DsRed plasmid^{24,51}. The PCR product was self-ligated, and the inserted sequence was checked using Applied Biosystems 3130xl (Life Technologies) after cycle sequencing with BigDye Terminator V3.1 (Life Technologies, Carlsbad, USA).

Microinjection. Microinjection was carried out following the method described in Tamura *et al.*⁵². The TALEN mRNAs and PITCh vector were injected into *w1-pnd* embryos at the syncytial preblastoderm stage. The TALEN mRNA concentration was 125 ng/μL each, and the PITCh vector concentration was 500 ng/μL.

Genotyping of the mutant individuals. Genomic DNA was extracted using the DNeasy kit (QIAGEN, Hilden, Germany) for each G1 adult from the crosses. PCR amplification was carried out using primers 5'-CACGAGTACAGAGAATATGG-3' and 5'-ATTTGTTGGCAGCACTGCTT-3' for the 5' junction and 5'-ATAACGACCGCTGAGTCAA-3' and 5'-GGTAGTACTCGTTAGCTAGC-3' for the 3' junction. The amplicons were sequenced using Applied Biosystems 3130xl after cycle sequencing with BigDye Terminator V3.1.

Tissue dissection. Each tissue was isolated on ice from day-3 fifth-instar larvae and kept at -80 °C until use. Day-1 fifth-instar larvae were exposed to various concentrations of diazinon solutions via direct contact with the larval abdomen. At 24 h post-treatment (on day 2), LD50 values were recorded. For genome-editing experiments, the *w1-pnd* (non-diapausing) strain was used to establish the mutant strains. The established strains were crossed with the *w-c* (diapausing) strain to maintain the stocks. These strains were reared using an artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) at 25 °C under a 12-h light/dark photoperiod.

Acetylcholine (ACh) measurements. The ACh content of silkworms was measured using a colorimetric acetylcholine assay kit (Cell Biolabs Inc., San Diego, CA, USA). Briefly, whole bodies of the silkworms were homogenised in chloroform/methanol (2:1, v/v). After centrifugation, the lower phase was collected and dried completely. The resulting extract was dissolved in chloroform/methanol/water (86:14:1, v/v/v) and used as crude extract. Acetylcholinesterase and choline oxidase were added to detect acetylcholine. Acetylcholine content was measured at a wavelength of 540 nm after incubation and estimated as the arbitrary colorimetric units normalised to the milligrams of body weight used.

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Author Contributions

Conceived the project: K.Y., T.T., H.S., A.N. Performed the experiments: K.Y., A.k.H., A.i.H., N.Y., T.T. Analysed the experiments: K.Y., A.k.H., N.Y., T.T., H.S., A.N. Wrote the paper: K.Y., A.k.H., T.T.

Additional Information

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