CRISPR/Cas9-based functional characterization of the pigmentation gene ebony in *Plutella xylostella*

X. Xu†‡, T. Harvey-Samuel§, J. Yang†‡, M. You†‡ and L. Alphey†§

*State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, Institute of Applied Ecology, Fujian Agriculture and Forestry University, Fuzhou, China; †Joint International Research Laboratory of Ecological Pest Control, Ministry of Education, Fujian Agriculture and Forestry University, Fuzhou, China; ‡Key Laboratory of Integrated Pest Management for Fujian-Taiwan Crops, Ministry of Agriculture, Fuzhou, China; and §Arthropod Genetics Group, The Pirbright Institute, Woking, UK

Abstract

Body pigmentation is an important character of insects in adapting to biotic and abiotic environmental challenges. Additionally, based on the relative ease of screening, several genes involved in insect melanization have been used in classic genetic studies or as visual markers in constructing transgenic insects. Here, a homologue of the *Bombyx mori* melanization-inhibiting gene *ebony*, associated with the conversion of dopamine to N-α-alanyl dopamine, was identified in a global pest, *Plutella xylostella*. The CRISPR/Cas9 system was applied to generate multiple *Pxebony* knockout alleles which were crossed to produce a *Pxebony* knockout strain, showing darker pigmentation in larvae, pupae and adults, compared with wildtype. Interestingly, we observed that *Pxebony* heterozygotes displayed an intermediate darkened phenotype, indicating partial dominance between the knockout and wildtype alleles. The fitness costs of *Pxebony* deficiency were also assessed in the mutant strain, indicating that embryo hatchability and larval survival were significantly reduced, while the eclosion rate was not obviously affected. Our work provides a potential target for exploring CRISPR-based genetics-control systems in this economically important pest lepidopteran.

Keywords: CRISPR/Cas9, pigmentation, ebony, *Plutella xylostella*, genetic control.

Introduction

The diamondback moth (DBM), *Plutella xylostella*, is one of the most destructive global pests of agriculture (You et al., 2013; You et al., 2020). It causes significant economic losses by feeding on *Brassica* crops and reducing crop yields. However, the control of DBM is challenged by its extreme insecticide resistance, exacerbated by the high levels of chemical-based control used against it (Furlong et al., 2013). Although genetic control techniques (e.g., gene drive systems) have been proposed as promising alternatives for agricultural pest management, proof-of-principle systems in insects are limited to dipterans, mainly built in the model *Drosophila melanogaster* and several human disease vectors (Gantz et al., 2015; Scott et al., 2017; Kryou et al., 2018; López Del Amo et al., 2020). To explore the possibility of applying genetics-based population control tools in DBM, endogenous melanization/pigmentation genes (of which null mutations cause various visual phenotypes) can be used as the assessable primary targets for testing the functional efficiency of constructed prototypes. Therefore, genes regulating insect body pigmentation may be ideal candidates as molecular markers.

There are diverse insect pigments, among which melanin is the predominant class manipulating insect cuticle colouration and sclerotization (Wittkopp and Beldade, 2009). Genes involved in melanin metabolism have been well studied in *D. melanogaster* (Wittkopp et al., 2003a). The primary precursor tyrosine is converted to dihydroxyphenylalanine (dopa) by tyrosine hydroxylase, followed by the production of dopamine from dopa by dopa-decarboxylase. Dopa and dopamine can be further used to produce black or brown melanin by yellow or phenol oxidase.
proteins. Alternatively, dopamine can be conjugated with β-alanine by β-alanyl-dopamine synthase (encoded by ebony) to generate N-β-alanyl dopamine (NBAD), which is the precursor of yellowish-tan NBAD-sclerotin, while the protein encoded by tan is involved in the reverse reaction hydrolysing NBAD back to dopamine. In addition, dopamine can be modified by arylalkylamine-N-acetyltransferase to generate N-acetyl dopamine (NADA) and the unpigmented NADA-sclerotin (Wittkopp et al., 2002). In varied field-caught D. melanogaster populations, the genomic diversity inside or flanking ebony loci can be linked to differences in its expression resulting in regional light or dark body pigmentation variations (Pool and Aquadro, 2007). Additionally, the dramatic pigmentation differences between Drosophila americana and Drosophila novamexicana has been linked to the divergence of Ebony expression levels, indicating that Ebony is also responsible for pigmentation variation between closely related insect species (Wittkopp et al., 2002). In the model lepidopteran Bombyx mori, it has been reported that ebony loss-of-function mutants showed higher levels of melanin at larval and pupal stages (Futahashi et al., 2008), while the ectopic overexpression of ebony altered wildtype black pigmentation into lighter body colour (Osanai-Futahashi et al., 2012). Related research in the butterfly, Vanessa cardui, showed that ebony knockout was sufficient to cause over-melanized wing phenotypes (Zhang et al., 2017). Based on the ease of visual screening, the ebony gene has been utilized as a molecular marker in the construction of gene drive systems in D. melanogaster by integrating sgRNA-expressing cassettes into the ebony coding sequence (Lopez Del Amo et al., 2020). However, the functions of ebony remain unclear in DBM, and the elucidation of ebony function may provide potential genomic tools for building gene drives targeting this economically important pest.

In this study, the homologue of B. mori ebony in DBM was identified and CRISPR/Cas9 was utilized to generate an ebony knockout strain for characterizing its gene functions. Although some fitness costs were observed in the ebony mutants, our work furthers the potential of applying ebony as a molecular marker in the construction and testing of gene drive systems in DBM, which may provide future population-engineering solutions.

Results

Identification and phylogenetic analysis of Pxe Ebony

To identify putative ebony orthologs in DBM, we used B. mori Ebony protein sequence as a query to blast against the DBM genome database. Protein g36009, which showed the highest score (total score = 1169.07, E value = 0), was searched for conserved domains using the CD-search programme in NCBI. The blast result confirmed the existence of an adenylation domain of nonribosomal peptide synthetases (A_NRPS, 17-509 aa) and a Phosphopantetheine attachment site (PP-binding site, 542-608 aa), which were conserved in other insect Ebony

![Figure 1](image-url)
proteins. No other predicted DBM proteins showed close similarity to the query sequences. Therefore, g360009 was considered as the Ebony ortholog in DBM, named as Pxebony hereafter. Based on the DBM genome database, the total length of Pxebony predicted primary transcript is 15 787 bp while its coding region is 2499 bp, which contains 16 exons and encodes 833 amino acids (Fig. 2A).

Ebony sequences of other insects were collected from NCBI database and aligned with Pxebony using the ClusterW tool in Mega 7. A phylogenetic tree was subsequently constructed, where Pxebony closely clustered with other lepidopteran Ebony proteins, consistent with the evolutionary relationship among insects tested in the current study. This result further confirmed that Pxebony is the Ebony ortholog in DBM and likely to exhibit conserved functions reported in other insects.

**CRISPR/Cas9-mediated knockout of Pxebony**

The sgRNA target site used in the previous report of Spodoptera litura ebony (Slebony) knockout (Bi et al., 2019) was also found in Exon 5 of Pxebony. However, the cleavage efficiency (probability of generating frameshift mutations) of this target was estimated as only 54.52% with CHOPCHOP. To explore other potential targets as well as improve editing efficiency in Pxebony, we used CHOPCHOP to search the 512 bp Pxebony genome sequence surrounding the Slebony target site. Two sgRNA targets, e-sgRNA1 and e-sgRNA2, predicted with the highest cutting efficiency (72.07% and 79.64%, respectively), were selected for subsequent CRISPR targeting.

In total, 315 eggs were microinjected with the complex of Cas9 protein and two in vitro transcribed sgRNAs, with 156 G0 pupae surviving (survival rate = 49.5%). Patchy melanization was observed in some G0 adult wings (Fig. 2B). Although this mosaic phenotype was not very distinct against a wildtype (non-mutated) background via direct visual screening, significant differences were found between them by quantifying the pigmentation intensity of the same regions on the wings with an image analysis tool ImageJ (Fig. 2C). G0 adults were subsequently inbred in pools to produce the G1 generation, where substantially melanized adults were observed. A single male and a female melanized G1 adult (G1 founders) were randomly

**Figure 2.** Germline editing of Pxebony. (A) Illustration of Pxebony gene. Exons are shown with black boxes while introns with connecting lines. Two sgRNA target sites are located in Exon 5. Scale bar is shown in the figure. (B) Mosaic phenotype observed in G0 adults. White dash rectangles represent zoomed-in photos of abnormally darker pigmentation in G0 male (left picture) and female (right picture) compared with their wildtype counterparts (WT). (C) Pigmentation intensity of the wings of G0 mosaics and WT in panel B. Five selected areas on the wings (example is shown in the left panel) were measured for mean integrated density (mean = integrated density/area). (D) Sequencing chromatograms of the PCR products of WT and G0 mosaic. Multiple peaks shown in G0 mosaic sequence confirmed the presence of varied alleles in a single individual. Data (mean ± SEM) were analysed with One-way ANOVA in the SPSS Statistics 22 programme. † † indicates P value <0.05, and ‘n.s.’ means not significant. (E) Representative mutant alleles in G0 and G1 generation. Target sites are shown in red colour while the indels are demonstrated with dash lines and the genotypes are listed on the left. PAM sites recognized by Cas9/sgRNA are underlined.

© 2021 The Authors. Insect Molecular Biology published by John Wiley & Sons Ltd on behalf of Royal Entomological Society., 30, 615–623
collected and crossed to generate a *Pxebony* knockout line, which thus contained up to four different mutant alleles. The cloning and sequencing of a mosaic G0 and the two G1 founders revealed frameshift mutations in either one or both sgRNA target sites (Fig. 2D,E), resulting in premature stop codons and likely disrupting protein function (Figure S1). Our results confirmed the editing events induced by CRISPR/Cas9 in *Pxebony*.

**Mutant phenotypes of the Pxebony knockout strain**

Darker pigmentation was observed at different developmental stages in the *Pxebony* knockout strain, including late larvae, pupae and adults, but not embryos or early larvae, compared to wildtype individuals. The head capsule and thoracic legs of the third and fourth instar larvae were brown in wildtypes but black in the *Pxebony* strain (Fig. 3A–C). There are two different, naturally occurring pigmentation patterns in DBM, that is, with (Fig. 3D) or without (Fig. 3E) vertical stripes through the whole body. However, the yellow pigmentation shown in both patterns of wildtype pupae was basically lost in *Pxebony*-deficient individuals (left most of each pair). In addition, both *Pxebony* mutant male and female adults have melanized heads, abdomens, legs and wings, which were distinctly darker than their wildtype counterparts (Fig. 3F–N).

By crossing *Pxebony* null mutants with wildtypes, an intermediate melanized phenotype was observed in the heterozygous offspring. The phenotype variation was more distinguishable in adult wings than other tissues, thus, we took photos (Fig. 4A,B) and subsequently quantified the pigmentation intensity of adult wings with ImageJ. The mean integrated density of the same areas in the three lines were then compared. Our result showed significant differences among *Pxebony* **+/−**, *Pxebony* **−/+** and WT **+/+** lines in both male and female adults, validating the existence of intermediate phenotype between dark-pigmented null-mutation and lighter wildtype colouration (Fig. 4C). Such a partially dominant marker allowing discrimination of homozygous and heterozygous mutants from each other as well as from wild type would potentially be very useful. However, the intermediate melanization of heterozygotes was not always easy to distinguish from wildtype or homozygotes in adults, due to the loss of wing scale debris during adult growth, which affects the appearance of adults. Therefore, it was not easy to count the ratios of each phenotype (wildtype, intermediate, or dark pigmentation) in a larger population, where individuals grew at inconsistent developing stages, for analysing the segregation and inheritance patterns of wildtype and mutant alleles in their progeny.

Although the *Pxebony* homozygotes selected for taking photos in Figs 3 and 4 happened to look generally smaller than wildtypes, this difference was not consistent among rearing populations and generations. It could be explained by natural variation in the genetic background or the

![Figure 3](https://example.com/figure3)

**Figure 3.** Phenotypes of the *Pxebony* mutant and wildtype diamondback moth (DBM) strains. (A): Third instar larvae. (B and C) Fourth instar larvae. (D) Pupae without stripes. (E) Pupae with stripes. White dash rectangles were zoomed-in photos of partial pupa. Blue arrows pointed out vertical stripes through the whole body, which are naturally existing in DBM pupae. (F, I and M) Male adults. (G, J and N) Female adults. (H) Adult heads. (I and J) Adult abdomens. (K) Adult hind legs. Dash box including L, M and N shows dissected adult wings. (L) Adult hind wings. (M and N) Adult forewings. *Pxebony* mutants and wildtypes were respectively shown as **ΔPxebony** and wildtype counterparts (WT). Scale bar = 0.5 mm.
existence of four mutant alleles in the knockout line (fitness of body size might be linked to some alleles). However, more experiments are needed to investigate the actual reasons contributing to this body size variation.

Fitness analysis of the Pxebony mutant strain

To detect potential fitness costs associated with the Pxebony disruption, the embryo hatchability, larval developmental period, survivability and pupal eclosion rate of the

**Figure 5.** Fitness analysis of Pxebony null mutants and wildtypes. Eggs were collected from Pxebony and wildtype counterparts (WT) moths (n = 228 and 259, respectively). The proportion of eggs hatching (‘hatchability’) was determined. For each genotype, 228 larvae (76 per replica, 3 replicas) were monitored for survival to pupation (‘larval survival rate’), with the time to pupation additionally recorded for one replica (‘larval longevity’). In addition, the survival to adult was determined (5 × 15 pupae per genotype, recorded as ‘eclosion rate’). All data were presented with mean ± SEM. The t-test analysis was conducted with GraphPad Prism 8.3.1 software to compare fitness differences, which is represented with ‘***’ (P value < 0.001), ‘**’ (P value < 0.01), ‘*’ (P value < 0.05), or ‘n.s.’ (not significant). Actual values and corresponding SEMs used in upper figure are listed in table below.
Pxebony knockout line were analysed. We found that the embryonic hatching rate and larva-to-pupa survival rate of Pxebony mutants were significantly lower than those of their wildtype progenitor strain (Fig. 5). Additionally, Pxebony individuals needed significantly more time to complete larval development than the wildtype cohorts. Nevertheless, no significant difference was observed in the pupal eclosion rates (pupa-to-adult survival) between the two lines tested. These findings indicate a role for Ebony in embryonic and larval development, potentially due to an effect on levels of dopa, dopamine or their derivatives, with loss of Ebony having an adverse effect on development (Fig. 5).

Discussion
Melanin is the major group of pigments forming insect body colour patterns, which play an important role in responding to biotic and abiotic environmental challenges. Investigating endogenous genes associated with melanin biogenesis, metabolism and transport will therefore shed light on mechanisms of insect adaptation and evolution. Additionally, these essential genes controlling observable phenotypes can be applied in insect genome engineering as valuable molecular markers to qualify or quantify editing events.

In this study, we investigated the melanin pathway gene ebony by using the genome editing tool CRISPR/Cas9 to build ebony knockout strains, showing that Pxebony null mutations caused darker pigmentation in older DBM larvae, pupae and adults. This phenotype was consistent with previous reports in D. melanogaster, Aedes aegypti, B. mori, V. cardui and S. litura (Wittkopp et al., 2002; Futahashi et al., 2008; Li et al., 2017; Zhang et al., 2017; Bi et al., 2019), revealing the conserved function of ebony in maintaining light body colouration across various insect species. In contrast to B. mori, where the ebony-mutant phenotype was less obvious in adults than in larvae and pupae (Futahashi et al., 2008) homozygous Pxebony mutant adults were clearly distinguishable from wildtypes. We also found that Pxebony disruption had minor effects on the growth and development of DBM, reducing embryonic hatchability and larval survivability as well as extending larval developmental period, without affecting adult emergence. These results indicate that Pxebony could be utilized not only as a molecular marker but also a regulator of persistence in gene drive system development. For example, the Pxbeony locus could be used as a location site for a Cas9 expressing transgene in a split/drive design, as this allele – with its associated disrupted copy of Pxebony – may eventually disappear in the target or non-target populations due to slight fitness cost of Pxebony mutation, in turn restricting the spread of the sgRNA target allele (which can only be driven in the presence of Cas9) in the population. This design is potentially beneficial for constructing confinable population suppression systems and mitigating potential ecological risks. However, further investigations (e.g., with mathematical modelling) would be needed to analyse such possibilities in detail for specific gene drive designs. In contrast, previous report of another pigmentation gene Pxyellow showed no significant impacts on moth fitness caused by Pxyellow defect (Wang et al., 2020), making it an ideal neutral marker for testing drive systems but without the additional limiting function shown in Pxebony. Therefore, our research on Pxebony provides more options and expands the genetic tool box for developing different gene drives in DBM.

It is interesting that, in contrast to the recessive ebony mutation in B. mori (Futahashi et al., 2008), but similar to D. melanogaster (True, 2003), Pxebony loss-of-function alleles caused a partial dominant phenotype, meaning that the heterozygous mutations resulted in incomplete melanization while homozygous deficiency was responsible for a completely dark pigmentation phenotype. Theoretically, such a characteristic could prove beneficial in an applied context, for example through providing a non-molecular means to estimate the allele frequency of a transgene (such as Cas9, above) inserted into that locus as part of a drive system. As described in our results, however, the intermediate phenotype was not always easily distinguishable from full knockout or wildtype individuals by simple microscope screening. Hence, rapid and automated phenotypic screening based on sophisticated image analysis (which has been demonstrated for the identification of mosquito genotypes (Crawford et al., 2020; Koskinioti et al., 2021)) are required if such a benefit is to be realized in a larger lab population or field setting.

Pigmentation variation, due to divergence of ebony sequences among different D. melanogaster populations, may be involved in adaptation to various geographic environments (Pool and Aquadro, 2007). However, it remains unclear whether Pxebony expression also contributes to DBM adaptation to diverse environments, which helped DBM to become a cosmopolitan pest. In addition to melanin synthesis, evidence supporting other biological roles of ebony has been found in the model insect D. melanogaster. For example, ebony might regulate cuticular hydrocarbon (CHC) composition, where the lack of ebony biased the formation of long chain instead of short chain CHCs, correlated with pigmentation changes (Massey et al., 2019). Additionally, Ebony was detected in the visual and nervous system, participating in the regulation of fly vision, rhythm activity as well as mating behaviour (Suh and Jackson, 2007; Takahashi, 2013; Ziegler et al., 2013). This demonstrates that ebony is a pleiotropic gene, at least in Drosophila, harbouring multiple biological functions, which require further studies to be investigated in DBM.
Experimental procedures

Insect rearing

All CRISPR-treated mutants were generated from the Vero Beach wildtype strain and reared under the same conditions (relative temperature = 25 °C, relative humidity = 50%, and light:dark cycle = 16 h: 8 h) unless specified. Larvae were fed with beet armyworm artificial diet (Frontier Biosciences, Germantown, MD, USA) while adults with 10% sugar solution (Xu et al., 2020).

Phylogenetic analysis

For identifying the putative ebony homologue in DBM, B. mori Ebony protein sequence (accession number: BAH11147.1) was used to BLAST against the P. xylostella PacBio1 genome databases (http://lepbase.org/). Ebony protein sequences of other insect species, including A. aegypti, Anopheles gambiae, D. melanogaster, Apis mellifera, Tribolium castaneum, Leptinotarsa decemlineata, S. litura, Papilio xuthus and Papilio machaon, were also downloaded from NCBI databases by applying B. mori Ebony as the BLAST query. Evolutionary relationships were further analysed with Mega 7. To be specific, the sequence alignment was completed with ClustalW, and a phylogenetic tree was created using the Maximum Likelihood Method with 1000 bootstraps.

Germline gene editing with CRISPR/Cas9

To generate null mutants of Pxebony, CRISPR/Cas9-based gene editing was applied as described in our previous report (Xu et al., 2020). Specifically, a 516 bp fragment was amplified from wildtype genomic DNA with forward primer LA4977 (5'-GAGTGCCTATCTCAGCAGC-3') and reverse primer LA4979 (5'-TCGGTACCAGGACCAACCGTTC-3') using the Q5 High-Fidelity DNA Polymerase (NEB, Hitchin, UK). The amplified product was Sanger sequenced, and searched for possible sgRNA targets with an online tool CHOPCHOP (https://chopchop.cbu.uib.no/) based on the N20NGG rule. DNA templates used for in vitro transcription of two sgRNAs were independently amplified with forward primers (ebony-sgRNA1-F: 5'-GAATAAATGAGCTACTA TAGAGGAGGAGGAGGCACCAGTCAAGTTTATAGAGCTACTAGAA-3'; ebony-sgRNA2-F: 5'-GAATTAATACGACTCTATAGAGGAGGAGGAGGCACCAGTCAAGTTTATAGAGCTACTAGAA-3') and reverse primer LA137 (5'-AACGGACCGACTTCTAGCTTCTCTCAAAACTGTTCTCTTCTGACTTTCTCTAAAC-3'). PCR products were purified using agarose electrophoresis and Monarch DNA Gel Extraction Kit (NEB) and then transcribed using MEGAscript T7 (Thermo Fisher, Waltham, MA, USA). The RNA products were subsequently purified with MEGAclear cleanup Kit (Life Technologies, Rockville, MD, USA). For preparing injection mixture, 150 ng/μl of each sgRNA was mixed prior to being complexed with 300 ng/μl Cas9 protein at 37 °C for 20 min. Given concentrations are at final volume of mix.

Eggs collected within 30 min post oviposition were injected with the sgRNAs/Cas9 mixture and then maintained in Petri dishes containing wet cotton balls for maintaining humidity. Hatched larvae were reared with artificial diet until pupation. Surviving pupae were sexed and set up in five deli pots for crossing, with 10 G0 females and 10 G0 males in each pot. Eclosed adults were allowed to randomly mate and produce G1s, of which adults with distinguishable dark pigmentation were pair-crossed to generate Pxebony knockout strains. Since G1 knockouts exhibited the same phenotype, one of these strains was taken forward for further analysis, and named as Pxebony hereafter.

Identification of mutations

Both G0 and G1 adults were screened and photographed with a camera-integrated Leica E24 HD stereo microscope (Leica Biosystems, Milton-Keynes, UK). Genomic DNA of obviously darker pigmented moths was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Additionally, the 516 bp fragment containing both sgRNA target sites was amplified and cloned using the pJET1.2 Cloning Kit (Thermo Fisher). Positive amplicons were sent for Sanger sequencing to confirm mutations.

Fitness analysis of Pxebony mutants

To investigate potential fitness effects caused by Pxebony knock-out, the Pxebony null mutant strain and their wildtype counterparts were assessed for hatchability (proportion of egg hatching), larval survival rate, time to pupation and pupal eclosion rate.

Initially, 40 pupae of each strain (wildtype and Pxebony) were separately set up in cross pots and allowed to mate freely once eclosed. Two days after mating, parafilm sheets coated with cabbage extract were put in each pot for 1 h oviposition after which time the parafilm sheet was discarded and replaced with a fresh sheet. This step aimed to remove partially developed embryos carried in female adults and ensure that eggs collected on the replacement sheet were at a very similar developmental stage (i.e., freshly fertilized). The new parafilm sheets were placed in cross pots for 30 min to collect fresh eggs, which were then kept in Petri dishes to allow them to hatch, and the hatch rates were subsequently recorded. Additionally, 76 neonate larvae of the same line hatched on the same day were moved from Petri dishes to a deli pot as one biological replicate, and reared with artificial diet till pupation. The total developmental period from neonate larvae to pupae was recorded as larval longevity, and the number of successfully pupated individuals was also counted for analysing larval survival rate. All the experiments above were conducted with three biological replicates. In addition, to investigate eclosion ability, 15 pupae were pooled as one biological replicate and five replicates in total were carried out, after which the number of successfully emerged adults were counted.

Data collected in this section were presented with mean±SEM. The t-test analysis was conducted with GraphPad Prism 8.3.1 software to compare fitness differences, which were sorted into '***' (P < 0.005), '****' (P < 0.001) and ‘n.s.’ (not significant).

In conclusion, our work showed the possibility of using Pxebony as an accessible target in constructing DBM genetic control systems, as well as proving the material for further studies on lepidopteran epidemics formation, circadian rhythms and mating behaviours.

Acknowledgements

This work was supported by European Union H2020 Grant nEUROSTRESSPEP (634361). XX was additionally
supported by the CSC Scholarship from the Chinese Government, and THS was supported by a UK Biotechnology and Biological Sciences Research Council (BBSRC) Impact Acceleration Account grant (BB/S506680/1). LA was supported by core funding from the BBSRC to The Pirbright Institute (BBS/E/00007033, BBS/E/I/00007038 and BBS/E/I/00007039).

Data availability statement
The data that support the findings of this study are available on request from the corresponding author upon reasonable request.

References
Bi, H., Xu, J., He, L., Zhang, Y., Li, K. and Huang, Y. (2019) CRISPR/Cas9-mediated ebony knockout results in puparium melanin in Spodoptera litura. *Insect Science*, 26, 1011–1019.

Crawford, J.E., Clarke, D.W., Criswell, V., Desnoyer, M., Cornell, D., Deegan, B. et al. (2020) Efficient production of male Wolbachia-infected Aedes aegypti mosquitoes enables large-scale suppression of wild populations. *Nature Biotechnology*, 38, 482–492.

Furlong, M.J., Wright, D.J. and Dosdall, L.M. (2013) Diamondback moth ecology and management: problems, progress, and prospects. *Annual Review of Entomology*, 58, 517–541.

Futahashi, R., Sato, J., Meng, Y., Okamoto, S., Daimon, T., Yamamoto, K. et al. (2008) yellow and ebony are the responsible genes for the larval color mutants of the silkworm Bombyx mori. *Genetics*, 180, 1995–2005.

Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M., Bier, E. et al. (2015) Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. *Proceedings of the National Academy of Sciences of the United States of America*, 112, E6736–E6743.

Koskinioti, P., Augustinos, A.A., Carvalho, D.O., Misbah-ul-Haq, M., Pillwax, G., de la Fuente, L.D. et al. (2021) Genetic sexing strains for the population suppression of the mosquito vector Aedes aegypti. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 376, 20190808.

Kyrou, K., Hammond, A.M., Galizi, R., Kranjc, N., Burt, A., Beaghton, A.K. et al. (2018) A CRISPR–Cas9 gene drive targeting doublesex causes complete population suppression in caged Anopheles gambiae mosquitoes. *Nature Biotechnology*, 36, 1062–1066.

Li, M., Bui, M., Yang, T., Bowman, C.S., White, B.J. and Akbari, O. S. (2017) Germline Cas9 expression yields highly efficient genome engineering in a major worldwide disease vector, Aedes aegypti. *Proceedings of the National Academy of Sciences of the United States of America*, 114, E10540–E10549.

López Del Amo, V., Bishop, A.L., CHM, S., Bennett, J.B., Feng, X., Marshall, J.M. et al. (2020) A transcomplementing gene drive provides a flexible platform for laboratory investigation and potential field deployment. *Nature Communications*, 11, 352.

Madeira, F., Park, Y.M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N. et al. (2019) The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Research*, 47, W636–W641.

Massey, J.H., Akiyama, N., Bien, T., Dreisewerd, K., Wittkopp, P. J., Yew, J.Y. et al. (2019) Pleiotropic effects of ebony and tan on pigmentation and cuticular hydrocarbon composition in Drosophila melanogaster. *Frontiers in Physiology*, 10, 518.

Osanai-Futahashi, M., Ohde, T., Hirata, J., Uchino, K., Futahashi, R., Tamura, T. et al. (2012) A visible dominant marker for insect transgenesis. *Nature Communications*, 3, 1295.

Pool, J.E. and Aquadro, C.F. (2007) The genetic basis of adaptive pigment variation in Drosophila melanogaster. *Molecular Ecology*, 16, 2844–2851.

Scott, M.J., Gould, F., Lorenzen, M., Grubbs, N., Edwards, O. and O’Brochta, D. (2017) Agricultural production: assessment of the potential use of Cas9-mediated gene drive systems for agricultural pest control. *Journal of Responsible Innovation*, 5, S98–S120.

Suh, J. and Jackson, F.R. (2007) *Drosophila* Ebony activity is required in glia for the circadian regulation of locomotor activity. *Neuron*, 55, 435–447.

Takahashi, A. (2013) Pigmentation and behavior: potential association through pleiotropic genes in *Drosophila*. *Genes & Genetic Systems*, 88, 165–174.

True, J.R. (2003) Insect melanism: the molecules matter. *Trends in Ecology & Evolution*, 18, 640–647.

Wang, Y., Huang, Y., Xu, X., Liu, Z., Li, J., Zhan, X. et al. (2020) CRISPR/Cas9-based functional analysis of yellow gene in the diamondback moth, Plutella xylostella. *Insect Science*. https://onlinelibrary.wiley.com/doi/10.1111/1744-7917.12870.

Wittkopp, P.J. and Beldade, P. (2009) Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Seminars in Cell and Developmental Biology*, 20, 65–71.

Wittkopp, P.J., True, J.R. and Carroll, S.B. (2002) Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the development and evolution of pigment patterns. *Development*, 129, 1849–1858.

Wittkopp, P.J., Carroll, S.B. and Kopp, A. (2003a) Evolution in black and white: genetic control of pigment patterns in *Drosophila*. *Trends in Genetics*, 19, 495–504.

Wittkopp, P.J., Williams, B.L., Selegue, J.E. and Carroll, S.B. (2003b) *Drosophila* pigmentation evolution: divergent genotypes underlying convergent phenotypes. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 1808–1813.

Xu, X., Harvey-Samuel, T., Yang, J., Alphey, L. and You, M. (2020) Ommochrome pathway genes kynurenine 3-hydroxylase and cardinal participate in eye pigmentation in *Plutella xylostella*. *BMC Molecular and Cell Biology*, 21, 63.

You, M., Yue, Z., He, W., Yang, X., Yang, G., Xie, M. et al. (2013) A heterozygous moth genome provides insights into herbivory and detoxification. *Nature Genetics*, 45, 220–225.

You, M., Ke, F., You, S., Wu, Z., Liu, Q., He, W. et al. (2020) Variation among 532 genomes unveils the origin and evolutionary history of a global insect herbivore. *Nature Communications*, 11, 2321.

© 2021 The Authors. *Insect Molecular Biology* published by John Wiley & Sons Ltd on behalf of Royal Entomological Society., 30, 615–623.
Zhang, L., Martin, A., Perry, M.W., van der Burg, K.R.L., Matsuoka, Y., Monteiro, A. et al. (2017) Genetic basis of melanin pigmentation in butterfly wings. Genetics, 205, 1537–1550.

Ziegler, A.B., Brüsselbach, F. and Hovemann, B.T. (2013) Activity and coexpression of Drosophila black with ebony in fly optic lobes reveals putative cooperative tasks in vision that evade electroretinographic detection. Journal of Comparative Neurology, 521, 1207–1224.

Supporting Information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Protein sequences alignment of the wildtype and G1 Pxeony knockouts. An online tool EMBL-EBI (Madeira et al., 2019) was used to visualize the deduced protein sequences. WT indicates wildtype allele while others represent mutant alleles with different deletions.