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CRISPR/Cas9 mediated knockout of the *abdominal-A* homeotic gene in the global pest, diamondback moth (*Plutella xylostella*)

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ABSTRACT

The diamondback moth, *Plutella xylostella* (L.), is a worldwide agricultural pest that has developed resistance to multiple classes of insecticides. Genetics-based approaches show promise as alternative pest management approaches but require functional studies to identify suitable gene targets. Here we use the CRISPR/Cas9 system to target a gene, *abdominal-A*, which has an important role in determining the identity and functionality of abdominal segments. We report that *P. xylostella abdominal-A* (*Pxabd-A*) has two structurally-similar splice isoforms (A and B) that differ only in the length of exon II, with 15 additional nucleotides in isoform A. *Pxabd-A* transcripts were detected in all developmental stages, and particularly in pupae and adults. CRISPR/Cas9-based mutagenesis of *Pxabd-A* exon I produced 91% chimeric mutants following injection of 448 eggs. Phenotypes with abnormal prolegs and malformed segments were visible in hatched larvae and unhatched embryos, and various defects were inherited by the next generation (G_1). Genotyping of mutants demonstrated several mutations at the *Pxabd-A* genomic locus. The results indicate that a series of insertions and deletions were induced in the *Pxabd-A* locus, not only in G_0 survivors but also in G_1 individuals, and this provides a foundation for genome editing. Our study demonstrates the utility of the CRISPR/Cas9 system for targeting genes in an agricultural pest and therefore provides a foundation the development of novel pest management tools.

1. Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.), is one of the most destructive and cosmopolitan pests of cruciferous crops. It attacks many economically important food crops such as oilseed rape and cabbage, and the annual total cost of damage and management worldwide is estimated at USD4-5 billion (Furlong et al., 2013; Zalucki et al., 2012). *Plutella xylostella* has developed resistance to all major classes of pesticides, including dichlorodiphenyl-trichloroethane (DTT) and *Bacillus thuringiensis* (Bt) (Angkersmit, 1953; Johnson, 1953; Tabashnik et al., 1990), making it difficult to control and demanding the development of novel management strategies. Genetics-based technologies such as *piggyBac*-mediated transgenesis, support the ability to develop population-suppression DBM strains by release of insects carrying a dominant lethal trait (RIDL) (Martins et al., 2012). Although *piggyBac* is a versatile transposon element for engineering insects, its random integration, relatively low transforming frequency, possible instability of integrated sequences, and limited carrying capacity hamper its wide application in pest control trials (Fraser Jr,

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CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein) is a newly emerged genome-editing tool with advantages over the earlier, time- and labor-consuming techniques: zinc finger nucleases (ZFN) and transcription activator-like effecter nucleases (TALENs) (Miller et al., 2007, 2011; Porteus and Baltimore, 2003; Sander et al., 2011; Wood et al., 2011). This novel methodology needs only the Cas9 nuclease coupled with a single guide RNA (sgRNA) to cleave a specific sequence. Since the first demonstration of engineering the type II CRISPR-Cas9 system to function in eukaryotes (Cong et al., 2013), the technique has been used to edit genomes of numerous species including human (cells), mice, nematodes (*Caenorhabditis elegans*) and insects (*Drosophila melanogaster*), and for substantially improving the ease of genome editing and studies of genome regulation (Cho et al., 2013; Friedland et al., 2013; Gilbert et al., 2013; Gratz et al., 2013; Li et al., 2013; Qi et al., 2013).

The *abdominal-A* (*abd-A*) gene, belonging to the homeotic gene (Hox) family, is a member of *Drosophila* bithorax complex, which is required for segmental identity during embryogenesis (Sánchez-Herrero et al., 1984). These genes encode transcription factors that modulate segment development by interacting with a large number of downstream target genes (Pavlopoulos and Akam, 2011). Gene products of *abd-A* are involved in many biological processes during early embryogenesis of *Drosophila* and other insects. These include the differentiation of the anterior body and rear somite axis, cardiac tube organogenesis, heart cell fate in the dorsal vessel, genesis of the nervous system and fat body, gonad formation and development, midgut formation and muscle patterning (Cumberledge et al., 1992; Foronda et al., 2006; Lovato et al., 2002; Marchetti et al., 2003; Mathies et al., 1994; Michelson, 1994; Perrin et al., 2004; Ponzielli et al., 2002). Products of *abd-A* also play a role in larva-to-pupa metamorphosis in the silkworm, *Bombyx mori*, and abdominal pigmentation of adult *D. melanogaster* (Deng et al., 2012; Rogers et al., 2014). RNAi of *abd-A* in silkworms results in complete or partial absence of ventral appendages (prolegs and legs) from the third to sixth abdominal segments in late-stage embryos, indicating its importance in the normal development of these segments (Pan et al., 2009).

We cloned the *P. xylostella* ortholog, designated *Pxabd-A*, profiled levels of expression across different life stages and sexes, and applied the CRISPR/Cas9 system to generate loss-of-function individuals and visible defect phenotypes. Severe abdominal morphological defects and significant lethality resulted from disruption of the gene. Our results demonstrate the possibility of further gene function studies based on genome editing and developing novel approaches for genetic control of this globally important pest insect.

2. Materials and methods

2.1. Experimental DBM strain

The experimental DBM strain (Fuzhou-S) was derived from insecticide-susceptible insects collected from a cabbage (*Brassica oleracea* var. *capitata*) crop in Fuzhou (26.08°N, 119.28°E) in 2004 and later used for genome sequencing (You et al., 2013). Larvae were reared on potted radish seedlings (*Raphanus sativus* L.) at 25 ± 1 °C, 65 ± 5%RH and L:D = 16:8 h, in a greenhouse without exposure to insecticides.

2.2. Cloning of Pxabd-A

Total RNA was isolated from five *P. xylostella* 3rd instar larvae using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized with Hiscript™ Reverse Transcriptase (Vazyme Biotech) by using 500 ng total RNA. The *Pxabd-A* cDNA was amplified with a pair of primers (F: 5′- ATG AGT TCC AAG TTC ATC ATC G - 3′; R: 5′- TTA CGT GGG CAC CTT GTT GA - 3′) corresponding to the predicted coding sequence of *Pxabd-A* (gene ID in DBM genome: Px004264) in the *P. xylostella* genome (http://iae.fafu.edu.cn/DBM/index.php). PCR was carried out with KOD-plus polymerase (TOYOBO, Japan) under the following conditions: 98 °C for 2 min, and then 30 cycles at 98 °C for 30 s, 55 °C for 30 s, 68 °C for 70 s, and a final elongation step at 68 °C for 10 min. PCR products were isolated on a 1% agarose gel stained with ethidium bromide. The target band was extracted using the Omega gel extraction kit (Omega) and cloned into PJET1.2 vector (Thermo scientific) for sequencing.

2.3. Alignment and phylogenetic analysis of Pxabd-A

Multiple alignments were conducted using the MUSCLE algorithm based on the amino acid sequences encode by *abd-A* orthologs from 12 species (*P. xylostella*; *B. mori*, *Bombus terrestris*, *Apis mellifera*, *Tribolium castaneum*, *Anopheles gambiae*, *Drosophila melanogaster*, *Acyrthosiphon pisum*, *Myrmica rubra*, *Strigamia maritima*, *Hymenolepis microstoma* and *Echinococcus granulosus*) available in GenBank. The phylogenetic tree was constructed using the maximum likelihood method with a bootstrap value of 1,000 in MEGA5.1.

2.4. Expression profiling of Pxabd-A

The quantitative reverse transcription (qRT)-PCR-based expression profiling of *Pxabd-A* was performed using cDNA samples of eggs, 1st and 2nd instar larvae, male and female 3rd/4th instar larvae, prepupae, pupae, and adults. qRT-PCR was performed using transcript-specific primers (*Pxabd-A*-qRT-F: 5′- GAA GGA GAT CAA CGA GCA GG -3′, *Pxabd-A*-qRT-R: 5′- GTG GGC ACC TTG ACT TT -3′; isoform A-qRT-F: 5′- CCA TCA CTG ATT TTC CAT TTC CAG -3′, isoform A-qRT-R: 5′- CGT CAG GTA GTG GTT GAA GTG GAA -3′) for *Pxabd-A* and isoform A, respectively. SYBR Select Master Mix for CFX kit (Life Technologies) was used to conduct qRT-PCR under the conditions: 50 °C for 2 min, then 95 °C for 2 min, and 40 cycles at 95 °C for 15 s, 60 °C for 30 s. The *P. xylostella* ribosomal protein gene S64 was used as the reference (F: 5′- CAA TCA GGC CAA TTT ACC GC - 3′; R: 5′- CTG GGT TTA CGC AGT TAC G - 3′).

2.5. In vitro transcription of Cas9 and sgRNA

A 23 base-pair (bp) sgRNA targeting site located at nucleotides 163–185 bp (5′- GGA CTG AGT GCA GCG GCT CTA GG - 3′) was selected in Exon I of *Pxabd-A*. The control sgRNA (5′- GGC GAG GGC GAT GCC ACC TA -3′) was used to target the exogenous gene encoding the EGFP protein, and the targeted efficiency of EGFP-sgRNA verified in a DBM embryo cell line (established in our laboratory). The sgRNA was generated from a ready-to-use 500 bp linearized vector by annealing oligonucleotide duplexes encoding the 20 bp target sequence upstream of protospacer adjacent motif (PAM), with the sgRNA expressed under control of the T7 promoter. The sgRNA template was subcloned into the PJET1.2 vector (Fermentas, Ontario, CA) and sequenced to verify the structure. sgRNA was synthesized *in vitro* using the MAXIcript T7 kit (Ambion). The PTD1-T7-Cas9 vector (ViewSolid Biotech, Beijing, China) was linearized with the *Not*I restriction enzyme (Fermentas, US), and the Cas9 mRNA synthesized *in vitro* using the mMESSAGE mMACHINE T7 Kit (Ambion).