CRISPR/Cas9 gene editing in the swallowtail butterfly Papilio xuthus

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Method Article

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Abstract

Butterflies are one of wonderful experimental systems in evolutionary biology, but their such potential has been limited by lack of genetic manipulation technology. CRISPR/Cas9 has emerged as an efficient tool for gene editing especially across a wide spectrum of model organisms[1,2]. This protocol describes the gene editing in the swallowtail butterfly Papilio xuthus \(Figure 1), taking three morphological related gene Abdominal-B, ebony, and frizzled \(Table 1, Figure 2) as examples: including the design of target sites, preparation of sgRNAs and Cas9 mRNA, preparation of butterfly eggs, injection technology, detection of disrupted genes, phenotyping, and validation of gene expression in mutants.

Introduction

Butterflies are famous for their extraordinarily diverse wing patterns, which differ not only among species, but also among populations, sexes, and even seasonal forms[3,4]. Beyond wing pattern, butterflies are also diverse in virtually all aspects of their biology, ranging from behavior and biogeography to cellular biology and biochemistry, with decades of study having placed much of this variation in a well-resolved ecological context[5]. These features make butterflies a promising system to explore the genetics, evolution, and development of morphological diversification and speciation[6,7]. While butterflies have notable strengths in terms of natural variation and ecology, they also suffer critical shortcomings in the quest to characterize the genetic basis of organismal phenotypes, in particular due to a lack of functional genetics methodology. Recently, clustered regulatory interspaced short palindromic repeat \(CRISPR) associated \(Cas) based RNA-guided DNA endonucleases such as the Streptococcus pyogenes Cas9 \(SpCas9) nuclease \(CRISPR/Cas9) has emerged as an efficient tool for gene editing across a wide spectrum of organisms[1,2], including insects such as fruitfly Drosophila melanogaster[8,9] and silkworm Bombyx mori[10,11]. Here, following the dissection of high quality reference genomes for the two highly heterozygous and closely related butterflies P. xuthus and P. machaon, we develop an efficient CRISPR/Cas9 gene editing method that results in obvious phenotypes with three genes, Abdominal-B, ebony, and frizzled in P. xuthus \(Figure 3, Tables 2-4).

Reagents

Plasmids: Cas9 expression vector pTD1-T7-Cas910 \(gifted by Huang Yongping's Lab, Shanghai Institute of Biological Science), PMD18-T7-gRNA \(our lab), PMD18-T and PMD19-T \(Takara, Japan) Animal Tissue Direct PCR kit \(FOREGENE, China) Gentra Puregene Blood Kit \(Qiagen, Germany) TIANgel Maxi Purification Kit \(Tiangen, China) QIAquick Gel Purification Kit \(Qiagen, Germany) Qiagen PCR purification kit\(Qiagen, Germany) QIAprep Spin Miniprep Kit\(Qiagen, Germany) MAXIscript T7 Kit \(Life technology, USA) mMESSAGE mMACHINE T7 ULTRA kit \(Life Technologies, USA) Q5 high-quality DNA polymerase \(NEB, USA) T4 PNK \(NEB, USA) T4 Ligase \(NEB, USA) DraI \(NEB, USA) BsaI \(NEB, USA) NotI \(NEB, USA) DNase I \(NEB, USA) ExTaq polymerase \(Takara, Japan) Kanamycin T7 endonuclease I \(T7EI) \(NEB, USA) 0.05 M NaOH 20X TBS \(1L): 1 M Tris, 3 M NaCl, 4 g KCl 1X PBS \(pH7) \(IL): 8.5 g NaCL, 3.5 g Na2HPO4.12H2O, 0.25g NaH2PO4.2H2O 1X TBS-T \(1L): 50 ml 20XTBS, 1ml Tween20 Bovine albumin \(0218054991) \(MPbio, USA) Antibodies: anti-ABD-B \(1A2E9) \(DSHB) 12; HRP-Goat Anti-Mouse IgG \(SA00001-1) \(Proteintech); β-actin antibody \(66009-1-Ig) \(Proteintech) SupersignalTM West Pico Chemiluminescent Substrate \(Thermo scientific) Trizol\(Invitrogen) cDNA using the RevertAid H Minus First Strand cDNA Synthesis kit \(Thermo, USA) SYBR Premix Ex Taq \(Takara, Japan) Polyvinylidene difluoride \(PVDF) membranes \(Millipore, Bedford, MA, USA). RIPA Buffer \(Sigma)

Equipment

Thermal cycler Water bath \(37°C incubator) Centrifuge Vortex NanoDrop Spectrophotometer 2000c \(Thermo, USA) TransferMan NK2 and FemtoJet microinjection system \(Eppendorf, Germany) Nikon SMZ800 Stereoscopic microscope \(Nikon, Japan) Glass tube needles \(Φ1.0**0.8**100 mm) \(Zhengtian, China) Glue gel Slide Soft paintbrush Model for arranging eggs \(8*8) \(our Lab) Shaking table QUANTSTUDIOTM 12K FLEX \(Applied Biosystems)

Procedure

Design of sgRNA target sequence SgRNA target sites were designed by seeking sequences corresponding to N20NGG on exon regions of the sense or antisense strand of the DNA by ZiFit Targeter program13. Then we BLAST \(Basic Local Alignment Search Tool) these candidate target sequences against the P. xuthus genome to eliminate those with potential off-target sites using strict criteria, where the candidate editable site is defined only when the seed region \(12 nucleotides \(nt) to protospacer adjacent motif \(PAM) NGG) is unique1. From candidate editable sites, we selected those with the first two bases of GG, GA or AG for sgRNA synthesis. **SgRNA preparation** SgRNA can be produced by plasmid-based or PCR-based strategies. We list the protocol of two methods here, but based on our experiences, we recommend PCR-based strategy. For plasmid-based method, please go 1. For PCR-based method, please go 3. 1. _Construction of empty gRNA plasmid._ 1) Order the double-strand oligonucleotides including sequences of T7 promoter \(underlined with framed denoting the transcription start site), restriction endonuclease BsaI \(Bold) and DraI \(Bold and italic) sites, and guide RNA \(underlined and italic) as following: TAATACGACTCACTATAGGAGAGACCGAGAGAGGGTCTCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGT(2) Ligate the above double-strand oligonucleotides into PMD18-T. ddH2O 3 μl PMD18-T Simple vector 1 μl Double-strand oligonucleotides 1 μl Solution I 5 μl 10 μl Incubate at 16°C for overnight. 3) Transformation and plate on Kanamycin \(Kan) + plate \(50 mg/ml). 4) Confirm correct insertion of double-strand oligonucleotides by sequencing using M13-47 primer. 5) Mini-prep PMD18-T7-gRNA plasmid using QIAprep Spin Miniprep Kit. 2. _Construction of sgRNA expression plasmid_ 1) Order oligos \(oligo1 and oligo2, produced by ZiFit Targeter program) 2) Aneal and phosphorylate oligos ddH2O 6.5 μl Oligo1 \(100 μM) 1 μl Oligo2 \(100 μM) 1 μl 10X T4 Ligation Buffer 1 μl T4 PNK 0.5 μl 10 μl 37 °C \(no hot-cap) 30 min 95 °C 5 min 95 °C, -1.3°C/cycle 45 s × 60 cycles 4 °C hold 3) Preparation of PMD18-T7-gRNA plasmid ddH2O * μl PMD18-T7-gRNA plasmid 4 μg 10X CutSMART Buffer 5 μl BsaI 1 μl 50 μl \(*Calculate based on PMD18-T7-gRNA plasmid concentration) Incubate at 37°C for overnight. Purify the digestion product using TIANgel Maxi Purification Kit. 4) Ligation of

annealed oligos with BsaI-linearized PMD18-T7-sgRNA Dilute annealed oligos with 100 folds Diluted annealed oligos 1 μl Linearized PMD18-T7-sgRNA \(40 ng/μl) 3 μl 10 X T4 buffer 1 μl T4 Ligase 0.5 μl 10 μl Incubate at 16°C for 6 h. 5) Transformation and plate on Kan+ plate \(50 mg/ml). 6) Confirm correct insertion of sgRNA oligos by sequencing using M13-47 primer. 7) Mini-prep PMD18-T7-sgRNA plasmid using QIAprep Spin Miniprep Kit. Please go 4\(1). 3. _SgRNA template preparation using PCR-based methods_ 1) Order a unique oligonucleotide encoding T7 polymerase binding site and the sgRNA target sequence N20 \(CRISPRF: GAAATTAATACGACTCACTATAN20GTTTTAGAGCTAGAAATAGC) and common oligonucleotide encoding the remaining sgRNA sequence \(sgRNAR: AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCT TATTTTAACTTGCTATTTCTAGCTCTAAAAC). 2) Primer self amplification of CRISPRF and sgRNAR ddH2O 67 μl 5X Q5 Buffer 20 μl 10 mM dNTPs 2 μl 10 μM CRISPRF 5 μl 10 μM sgRNAR 5 μl Q5 high-quality DNA polymerase 1 μl 100 μl 98 °C 30 s 98 °C 10 s 60 °C 30 s × 35 cycles 72 °C 15 s 72 °C 10 min Purify with Qiagen PCR purification kit, and elute in 30 μl EB. Please go 4 \(2). 4. _Transcription of sgRNAs in vitro_ \(Ensure that reagents, tubes and tips are RNase-free and that the work is done in a ribonuclease-free environment). 1) Preparation of sgRNA plasmids RNase-free water * μl 10X CutSMART Buffer 5 μl sgRNA plasmid 10 μg DraI 1 μl 50 μl \(*Calculate based on sgRNA plasmid concentration) Incubate at 37°C for 6 h. Check if plasmids are digested completely by loading 2μl in 1% gel electrophoresis. Purify digested product using QIAquick PCR Purification Kit and elute with 10 μl RNase-free water. 2) In vitro transcription of sgRNA using MAXIscript T7 Kit \(Life technology, USA) \(1) Thaw the frozen reagents, and then keep 4 nucleotides and enzyme on ice but keep 10X Reaction Buffer at room temperature. \(2) Assembly transcription reaction at room temperature and incubate Nuclease-free water * DNA template **** 10X Reaction Buffer 2 μl 10 mM ATP 1 μl 10 mM CTP 1 μl 10 mM GTP 1 μl 10 mM UTP 1 μl Enzyme Mix 2 μl 20 μl \(==**==Calculate based on DNA template concentration; ==***== 1 μg linearized PMD18-T7-sgRNA plasmid or 600 ng purified self-amplification PCR-product) Mix thoroughly and incubate at 37°C for 4-6 h. \(3) Add1 μl TURBO DNase, mix well, and incubate at 37°C for 15 min. \(4) Add RNase-free water to the DNase I-treated transcription reaction up to 50 μl. \(5) Add 5 μl 5 M Ammonium Acetate and vortex to mix. \(6) Add 3 volumes 100% ethanol. \(7) Chill the solution at −20°C for 30 min or longer. \(8) Spin for >15 min at maximum speed in a 4°C centrifuge. \(9) Carefully discard the supernatant, and wash the pellet once with cold 70% ethanol. \(10) Elute in 10 μl RNase-free water, and determine sgRNA\(s) concentration by NanoDrop 2000. We recommend a concentration of more than 1 μg/μl for later use. \(11) Store sgRNA \(s) in 5 μl aliquots at −70°C. **Cas9 mRNA preparation** \(Ensure that reagents, tubes and tips are RNase-free and that the work is done in a ribonuclease-free environment) 1. _Preparation of pTD1-T7-Cas9_. RNase-free water * μl 10XCutsmart 5 μl pTD1-T7-Cas9 plasmid 10 μg NotI-HF 1 μl 50 μl \(*Calculate based on plasmid concentration) Incubate at 37°C for 6 h. Check if plasmids are digested completely by loading 2μl in 1% gel electrophoresis. Purify digested product using QIAquick Gel Purification Kit and elute with 10 μl RNase-free water. 2. _In vitro transcription of Cas9 mRNA using mMESSAGE mMACHINE T7 Kit_ \(Life technology, USA) 1) Dissolve the reagents. Thaw the frozen reagents, and then keep the ribonucleotides T7 \(2X NTP/CAP) and enzyme mix on ice but keep 10X T7 Reaction Buffer at room temperature. 2) Assemble transcription reaction at room temperature and incubate Nuclease-free water * μl Linearized plasmid template 1 μg 2X NTP/CAP 10 μl 10X T7 Reaction Buffer 2 μl Enzyme Mix 2 μl 20 μl \(*Calculate based on Linearized plasmid template concentration) Mix thoroughly and incubate at 37°C for 4-6 h. 3) Add 1 μl TURBO DNase, and mix well, incubate at 37°C for 15 min. 4) Add 30 μl Nuclease-free water and 30 μl Lithium Chloride \(LiCl) Precipitation Solution, mix thoroughly, and chill at -20°C for more than 1-2 h. 5) Centrifuge at 4°C for 15 min at maximum speed to pellet the RNA. 6) Carefully remove the supernatant. Wash the pellet once with ~1 ml 70% ethanol, and re-centrifuge to maximize removal of unincorporated nucleotides. 7) Carefully remove the 70% ethanol, and re-suspend the RNA in Nuclease free water. 8) Check by gel electrophoresis to verify the integrity of Cas9 mRNA, and determine the Cas9 mRNA concentration by NanoDrop 2000. We recommend a concentration of more than 2 μg/μl for later use. 9) Store frozen at −20°C or −70°C. **Preparation of microinjection mixture** \(sgRNAs and Cas9 mRNA) 1.Thaw aliquots of the Cas9 mRNA and sgRNAs on ice. 2. Dilute the Cas9 mRNA and sgRNAs with RNase-free water to a concentration that is designed for microinjection in a final volume of 10 μl. 3.Pipette the mixture up and down several times. 4.Centrifuge at 4°C for 1 min at maximum speed. **Egg preparation** 1. Adult and hand pair Pupae were reared in plastic baskets at room temperature or under the conditions of 26 °C, 75% relative humidity and 18h/6h light/darkness. Emerged adults were crossed via hand-pairing[14,15]. 2. Egg laying, collecting and preparing Mated females were transferred into net room with host plants for oviposition. Eggs were collected from the host plant leafs following 15-60 min oviposition bouts, dipped into clear water and then aligned on the microscope slide mounted into a model designed by our lab using soft paintbrush, fixed by glue. **Microinjection and egg hatching** 1. _Microinjection_ 2 nl mixture of sgRNA\(s) and Cas9-encoding mRNA with the different combination of final concentration was injected through the chorion into each egg under a stereoscopic microscope \(Nikon SMZ800), using an TransferMan NK2 and FemtoJet microinjection system. 2. _Hatching_ Injected eggs contained in the petri dishes were placed in an incubator at 25°C and 70% relative humidity. When embryos are close to hatch, host leaves \(e.g a horticulturally rutaceous plant \(Zanthxylum piperitum) are placed into the dishes for newly hatched larva feeding. **Breeding and Phenotyping** 1. _Breeding_ The leaves with newly hatched larvae are carefully transferred into large petri dishes. All larva were fed on host leaf collectively or individually in glass containers. Container is cleaned and leaves of host plant are renewed every day. Pupae are transferred into plastic baskets to eclose. 2. _Phenotyping_ For developed but unhatched eggs, which are morphologically black, we dissect larvae from eggs and observe their morphological traits using stereoscopic microscope. Larvae of the newly hatched and the second instar were carefully checked using stereoscopic microscope and pictured using microscopic digital camera if there are morphological changes compared with wild type. From third instar larvae on, morphologic changes in different developmental stages are observed using stereoscopic microscope or by naked eyes. In the cases of the three genes, we mainly observe abdomen habitus for Abd-B; for ebony, we mainly pay attention to the color of larvae and adults; for frizzled, we mainly observe the cuticular structures. **Genotyping** 1. _Sampling, genomic DNA extraction, and target loci amplification_ Sampling strategies are determined based on the anticipated phenotypes of target genes and the developmental situation of injected individuals. Genomic DNA extraction and PCR for unhatched larva dissected from developed eggs are carried out using Animal Tissue Direct PCR kit \(FOREGENE, China) following manual instruction; genomic DNA of hatched larva, prepupa, pupa and adult are extracted using Gentra Puregene Blood Kit \(Qiagen, Germany), and their target sites are amplified using exTaq polymerase. Amplified target region \(300-800 bp) are purified with Qiagen PCR purification kit, and eluted in 30μl Nuclease-free water. 2. _T7 endonuclease I mutation detection assays_ Purified or unpurified PCR products of target loci were for T7 endonuclease I \(T7EI) mutation detection assays as previously described 16. It is noted that T7E1 might cause false positives if the target DNA sequences carry allelic polymorphism, AT-rich region, or sequence differences due to PCR errors. 3. _Cloning and Sequencing_ T7EI positive and/or morphologically mutated individuals were further confirmed by Sanger sequencing 12 TA clones. **Validation of expression of disrupted gene** Expression changes \(mRNA level or at protein level) of disrupted gene in mutant and wild type can provide direct clues to gene function. 1. _Quantitative reverse transcription-PCR \(qRT-PCR)_ 1) Design and order primers for target gene and internal control gene. Note: The gene for cytoplasmis actin gene A3 that is expressed constitutively in the cell was used as an internal standard to estimate the relative expression of mRNA in the current study. 2)

Total RNA was extracted using the guanidinium thiocyanate-phenol-chloroform extraction method \(Trizol, Invitrogen) according to manufacturer's protocol. Note: For the individuals of small body such as eggs, the first instar larvae etc., at least five individuals are needed as one biological sample for extraction of total RNA. Three biological replicates were carried out for both mutants and wild types. 3) Reverse transcribe mRNA into cDNA using the RevertAid H Minus First Strand cDNA Synthesis kit \(Thermo) according to instructions. 4) Quantitative RT-PCR was performed using QUANTSTUDIOTM 12K FLEX \(Applied Biosystems) with the SYBR Premix Ex Taq \(Takara), with four experiment duplicates for each sample. ddH2O 7 μl 2X SYBR® Premix Ex Taq \(Tli RNaseH Plus) 10 μl 10μM PCR Forward Primer 0.5 μl 10μM PCR Reverse Primer 0.5 μl 100 ng DNA template 2 μl 20 μl 95 °C 20 s 95 °C 15 s 95 °C, -1.6°C/s 58 °C 20 s × 40 cycles 5) Data analysis The relative expression of target gene in mutants and in wild type was analyzed by 2-ΔΔCT method. The differences in expression of Abd-B gene between mutants and wild type were compared by SPSS16.0 statistics software \(SPSS Inc., Chicago, USA) using Independent_Sample t Test. A p value <0.05 was considered significant. 2. _Western blot analysis_: Abd-B protein as an example 1) Antibody preparation a. Retrieve antibody to the protein of target gene ortholog in other insects \(e.g. fruitlfy) in the Developmental Studies Hybridoma Bank \(DSHB) and, if yes, order. E.g. \(1A2E9) \(DSHB). b. Select internal control gene \(e.g. β-actin) and order its antibody. c. Order the second antibody. 2) Protein samples were prepared in RIPA buffer \(Sigma) from mutants and wild-types. For small body such as eggs, the first instar larvae, about 10 individuals are needed as one biological sample for extraction of protein. 3) All protein samples from 10 individuals were run on 10% SDS-polyacylamide gels. 4) Transferred to the polyvinylidene difluoride \(PVDF) membranes. 5) Blocking: incubate membranes in 10 ml 5% bovine albumin for 1 h in shaking table. 6) Detect target gene product a. Dilute the 1st antibody of target gene protein in bovine albumin. Dilute rate can be determined by pilot experiment. Antibody of Abd-B gene product \(1A2E9) \(DSHB) developed from fruitfly12 is diluted in bovine albumin with a ratio of 1: 50. b. Add 10 ml 1st antibody, and incubate 2 h in shaking table, recover the 1st antibody solution for later use. Wash the membranes 3 times with 1XTBS-T, each 5 minutes. c. Dilute the secondary antibody Horseradishperoxidase-coupled anti-mouse IgG \(SA00001-1) \(Proteintech) with a ratio of 1: 1000. d. Add 10 ml the 2nd antibody, and incubate 2 h in shaking table, recover the 2nd antibody solution for later use. wash the membranes 3 times with 1XTBS-T, each for 5 min. e. Detect chemiluminescence with SupersignalTM West Pico Chemiluminescent Substrate \(Thermo scientific) according to the manufacturer's instructions. 7) _Striping and blocking_ a. Wash the membranes 3 times with 1XTBS-T, each 5 min. b. Wash the membranes 3 times with 0.05M NaOH, each 30 min. c. Wash the membranes 3 times with 1X PBS \(pH7.0), each 5 min. d. Wash the membranes 3 times with 1XTBS-T, each 5 min. e. Blocking. Add 10 ml 5% bovine albumin and incubate 1 h in shaking table, and then discard bovine albumin. 8) Detect internal gene β-actin product a. Dilute 1st antibody of internal protein in bovine albumin with a ratio of 1: 500. b. Add 10 ml β-actin 1st antibody, incubate 2 h shaking table, recovery the β-actin 1st antibody solution for later use. Wash the membranes 3 times 5 minutes each with 1XTBS-T. c. Dilute the 2nd antibody HRP-coupled anti-mouse IgG with a ratio of 1: 1000. d. Add 10 ml the 2nd antibody, and incubate 2 h in shaking table, recover the 2nd antibody solution for later use. wash the membranes 3 times with 1XTBS-T, each for 5 min. e. Detect chemiluminescence with SupersignalTM West Pico Chemiluminescent Substrate according to the manufacturer's instructions.

Timing

4-5 days for the construction of sgRNA expression plasmid, or 1-2 days for sgRNA\(s) preparation using PCR-based methods. 1 day for the in vitro transcription and preparation of sgRNA\(s) and Cas9 mRNA. 1 day for collection and preparation of eggs and their microinjection. 3-5 days for eggs hatching. 40-60 days for breeding, phenotyping and genotyping.

Troubleshooting

1. Low yield of sgRNAs. We recommend to use PCR product as template, and to make the double in vitro transcriptions for precipitating sgRNA\(s). 2. Low yield of Cas9 mRNA. We recommend to improve the quality of the lineanized plasmid DNA template and to make the double in vitro transcriptions for precipitating Cas9 mRNA. 3. Low mutated rate of genotype and phenotype. 1) To ensure the high quality of sgRNA\(s) and Cas9 mRNA. 2) To co-microinject sgRNA\(s) of two adjacent target sites separated from 50 to 200 bp if such target sites are available in target genes. 3) To shorten the period from the beginning of egg laying to finishing of microinjection within 2 h by: a. shortening egg laying bout in case of high fertile females. b. Simplifying the protocol by omitting disinfection treatment. 4) To increase the concentration of microinjection mixture with appropriate ratio \(2: 3 to 1:1) of sgRNA\(s) and Cas9 mRNA

Anticipated Results

1. High concentration and appropriate ratio of sgRNA\(s) and Cas9 mRNA are critical for phenotype mutation \(Table 2-3). 2.Two sgRNA \(s) are better than one \(Table 2). 3. Phenotype mutants shows the low expression of disrupted target genes \(Figure 3).

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Figures

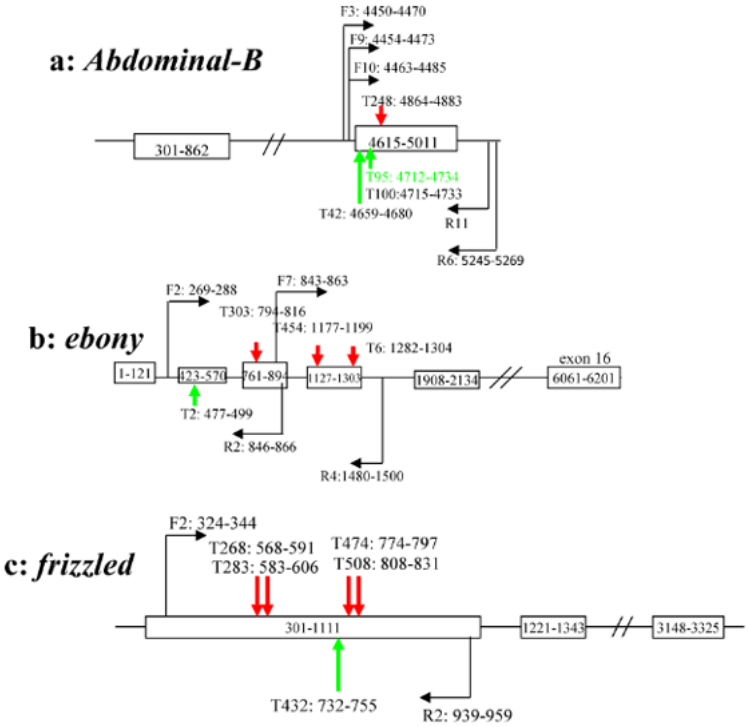

Figure 1

Figure 2 Schematic diagram of the sgRNA-targeting sites and primers used for amplifying target sites of different genes in P. xuthus. a, Px_03961_Abd-B; b, Px_01073_e (ebony); c, Px_15230_fz (frizzled). sgRNA: single guide RNA. Boxes represent exons. Lines represent intron or untranslated regions (UTR) of 5' or 3'; //: long intron; T: Cas9 targets with the number after colon showing the location; red arrow: targets in plus strand; green arrows: targets in minus strand. F: forward primer with the number after colon showing the location; R: reverse primer with the number after semicolon showing the location. In (a) (Px_03961_Abd-B) and (c) (Px_15230_fz), the fragments including 300 bp of 5' UTR, gene region and 300 bp of 3' UTR. In (b) (Px_01073_e), the fragment includes only gene region from the start codon ATG to the stop codon TAA

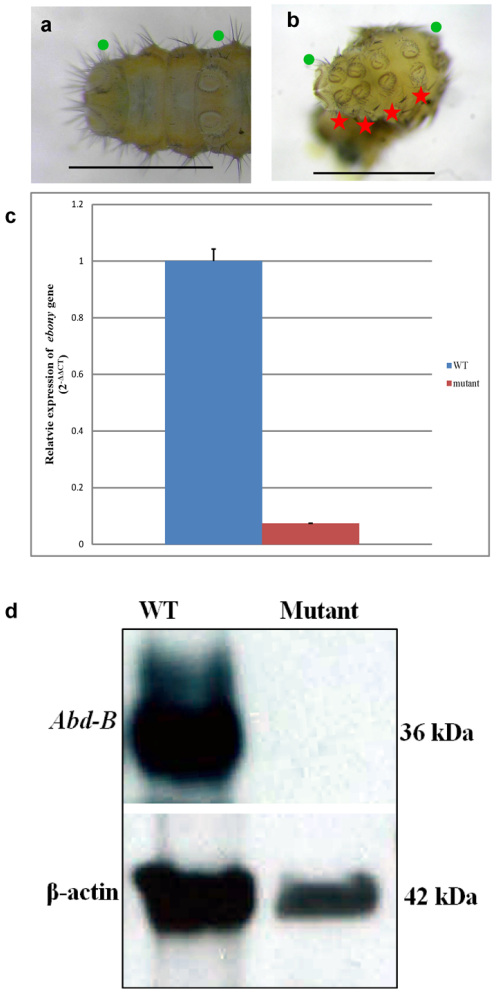

Figure 2

Figure 3 Abd-B mutants, expression of Abd-B gene in mutants (a) and in wild-type (WT) (b) detected with quantitative reverse transcription-PCR (RT-PCR) (c) and western blotting (d). a, Mutant with prolegs on segment Abdominal segment 7(A7) - A10; b, wild type without prolegs on segment A7-A10. c, Significant reduction of expression of Abd-B gene in P. xuthus mutants compared to WT individuals examined by RT-PCR (t-test, P=0.026). The maximum from the wild type was set as 1. Bars, means ± SD (n=3). x-axis: mutants and WT individuals; y-axis; the values of the relative expression (2-ΔΔCT) of Abd-B. d, The relative expression of Abd-B gene in 10 mutants and 10 WT individuals of P. xuthus examined by western blot using the fruitfly Abd-B antibody (1A2E9) (DSHB) and β-actin antibody (66009-1-Ig) (Proteintech) used as loading control.

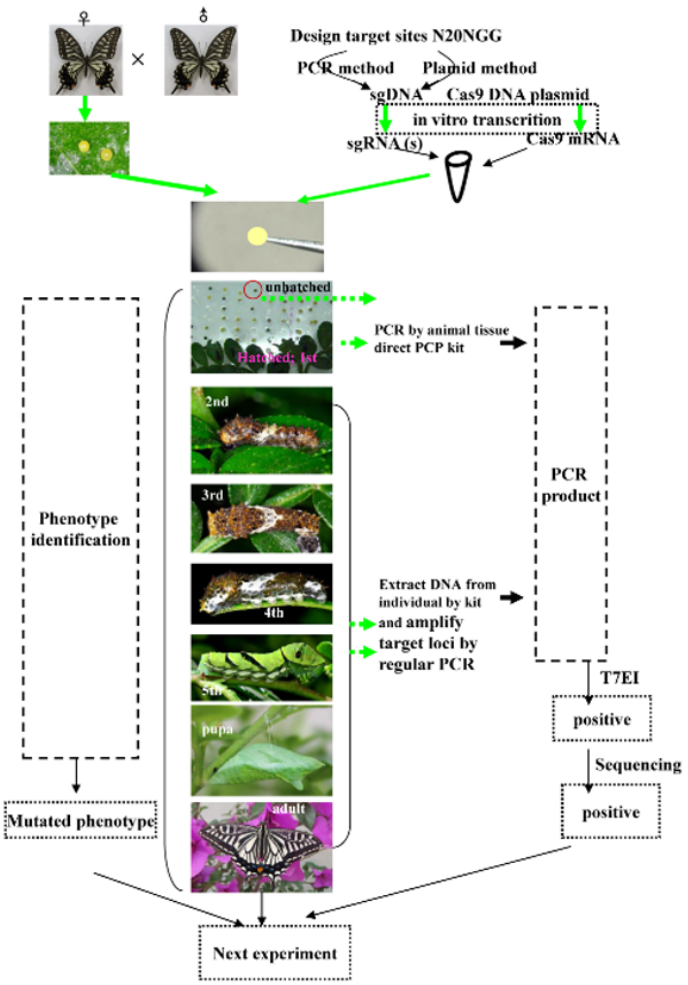

Figure 3

Figure 1 Flowchart of CRISPR/Cas9 technology in butterfly. 1st, 2nd, 3rd, 4th, 5th: different instar larvae; T7EI: T7 endonuclease I mutation detection assays.

Supplementary Files

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