The Pharma Innovation Journal 2023; 12(9): 2541-2545

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(9): 2541-2545

www.thepharmajournal.com
Received: 02-05-2023
Accepted: 03-06-2023

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Cloning of eye pigmentation gene white from the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Tephritidae: Diptera) and CRISPR/Cas 9 based *in vitro* restriction assay of different sgRNAs

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Abstract
Bactrocera dorsalis (Hendel) (Diptera: Tephritidae) is a destructive pest causing tremendous economic losses. The present study was, aimed at validation of genetic targets for genetic biological control of this notorious pest *via* CRISPR/Cas 9 mediated genome editing. Gene white (2040 bp) crucial for melanin pigmentation and cuticle sclerotization was cloned. Further, off-target minimised guide RNA for white (sg 360) was synthesised *in vitro.* The designed gRNAs were used for restriction assay to check the cleavage efficiency of sgRNAs.

Keywords: *Bactrocera dorsalis*, CRISPR/Cas 9, single guide RNAs, White gene

Introduction
Bactrocera dorsalis (Hendel), is a notorious pest species that shows extreme polyphagia and causes financial depreciation of higher scale [1]. The area under colonisation of this pest has escalated in few decades, owing to the encroaching nature of this pest. It was first recorded in Taiwan in 1912, [2] further spreading to Asia-Pacific regions [1]. It causes fruit yield losses and also results in rejection of exports due to quarantine regulation.
Globally, around 5,000 species across 500 genera of Tephritidae including key pest species such as *Anastrepha ludens* (Loew), *B. tryoni* (Froggatt), *Ceratitis capitata* (Weidemann), *Dacus ciliates* (Fabricius) and *Rhagoletis pomonella* (Walsh) [3, 4] are reported. With their common traits of polyphagia, high fecundity, and superior mobility, they are particularly invasive [5]. Genus Bactrocera consists of at least 75 species, with the pest *B. dorsalis* (Hendel) being a major biosecurity concern [1].
Apart from causing economic damage, the pest also projects a higher level of resistance to insecticides, necessitating the importance of sustainable pest management of the pest species [6]. The pest species is thought to be India's single biggest crop destroyer [7]. During the months of April and May the flies attack semi ripe and mature fruits. Also susceptible to this pest attack are other fruits such as guava, citrus, plum, peach, sapota and loquat etc. In India, the loss in fruit yield ranges from 1 to 31 per cent with a mean of 16 per cent [8].
Many conventional methods of pest management such as cultural, physical, mechanical, biological and chemical control are available, however, these options had some disadvantages related to efficiency, time-consuming, mammal hazardous and residual effects. To overcome these drawbacks, scientists engaged themselves in exploring the potential of genetic pest management of *Bactrocera dorsalis*.
It was found that CRISPR/Cas 9 genome editing technology emerged as a revolution in genome editing because it is capable of inducing and creating specific variations in specific genomic locations [9]. The revolution at CRISPR shows no signs of slowing down. The technology consists of various components such as, first RNA structures, a CRISPR RNA (crRNA) and second, (tracr RNA) trans-activating structures. Combining these two RNA structures forms the single guide RNA (sgRNA) [10].
The CRISPR-Cas9 system operates through a ribonucleoprotein complex where the Cas9 enzyme target specific binding site of target DNA by interacting with homologous single guide RNA (sgRNA) and the excision lobe cuts the DNA [11]. One can easily find spacer and repeats at CRISPR locus. The stages in the CRISPR defence mechanism include: (i) adaptation of the CRISPR via the incorporation of short sequences of the invaders as spacers; (ii) expression of

CRISPRs to form the pre-crRNAs and further processing to form crRNAs; and (iii) interference and disruption of target DNA by the crRNA guides. It is also being exploited as a tool for the potential control of pest insects and vector-borne diseases. The idea that CRISPR based systems could be used for genome editing in eukaryotes was initially conceived by Doudna and Charpentier in 2012 [12].

White gene is known to encode a protein that is a member of ATP-binding cassette (ABC) transporter superfamily that heterodimerises with other essential proteins to transport the eye pigment precursor guanine or tryptophan. It also influences many behavioural and physiological pathways in insects. Thus, mutation in this gene will result in development of colourless compound eyes in the mutant fly.

Thus, the primary objective of this investigation is to locate and clone the white gene in *B. dorsalis* followed by validation of sgRNAs by *in vitro* restriction assay to verify the effectiveness of restriction of the target gene, and open the door for additional microinjection experiments.

Materials and Methods

The investigation on was planned at the Division of Basic Sciences, Indian Institute of Horticultural Research (IIHR), Bengaluru, Karnataka.

Rearing of mango fruit fly

Under laboratory conditions, *B. dorsalis* culture was maintained at 27±1ºC and 75±1 % relative humidity with 14h: 10h photoperiod in wooden rearing cages (size 30 x 30 x 30 cm^3). Banana was supplied as a substrate for oviposition and as larval food. The prepupal wandering larvae were transferred for pupation to rectangular plastic boxes (25 x 10 cm), containing sand. The pupae were collected through manual sieving of the soil. The pupae collected for adult emergence were transferred to a plastic cylindrical rearing cage (14 cm height and 11 cm diameter). Sugar, yeast extract, multivitamin syrup and water were provided to the adult flies as a food source.

Identification of White gene and primer designing

The genome database of the annotated NCBI was used to choose the pigmentation gene white. With the aid of Clustal W multiple sequence alignment in the Bio Edit (version 7.2.6.1) programme, the sequence similarity of the white gene with other insect species was ascertained. At the Integrated DNA Technologies (IDT) site, gene-specific primers (Table 1) were manually constructed using the Oligo Analyzer Tool.

Table 1: *B. dorsalis* white gene-specific primers

Primers	Sequences (5'-3')
Forward Primer	ATGGGTCAGGAGGATCA
Reverse Primer	TTACTCTTTGCGTCTA

RNA Isolation, Complementary DNA (cDNA) Synthesis and PCR Amplification of *β-tubulin* gene

Total RNA of mango fruit fly (seven-day old pupa) was isolated by following the TRIzol-T reagent (guanidinium thiocyanate and acid phenol) methodology. The RNA purity was checked by Nanodrop (Thermo Scientific, USA) and the integrity of RNA isolated from seven-day old pupae was checked using denaturing gel electrophoresis in 1% agarose gel and visualised under ethidium bromide (0.5 µg/ml). Following this, cDNA was formed by following the manufacturer's protocol - Revert Aid First Strand cDNA Synthesis Kit #K1622, Thermo Fisher Scientific. Amplification of *β-tubulin* gene suggested that cDNA has been properly synthesised.

PCR amplification of white gene of *B. dorsalis*

Amplification of white CDS (Table 2) of *B. dorsalis* was done using specially designed primers *Bd* white forward (5'ATGGGTCAGGAGGATCA3') and *Bd*_white reverse (5' TTACTCTTTGCGTCTA3') under optimum conditions (Table 3). The amplified products were resolved on 1% agarose gel and stained with ethidium bromide (10 g/ml) (UVP, USA) before being observed in a gel documentation system. The Nucleo Spin Extract I kit (Machery Nagel, Germany) was used to elute the amplicon band, which was subsequently put to use for cloning.

Table 2: The protocol for white gene amplification in PCR

Sl. No.	Chemicals for White Gene Amplification	Working Concentration	Quantities (µl)
1.	Autoclaved milli Q water	-	10.6 µl
2.	10 X Mg+2 free buffer	1 X	2.5 µl
3.	25 mM MgCl2	2.5 mM	2.5 µl
4.	2.5 mM dNTPs mix	0.4 mM	4.0 µl
5.	Template (cDNA)	1:10 diluted	2.5 µl
6.	Forward Primer (white gene)	0.2 µM	1.25 µl
7.	Reverse Primer (white gene)	0.2 µM	1.25 µl
8.	LA Taq polymerase	1 unit/ µl	0.4 µl
Total Volume			25 µl

Table 3: PCR conditions for white gene

Sl. No.	Steps	Temperature	Time	Cycles
1.	Initial denaturation	95 °C	2 minutes	1x cycle
2.	Final denaturation	95 °C	10 seconds	35x cycles
3.	Annealing	55 °C	40 seconds	
4.	Extension	68 °C	2 minutes	
5.	Final extension	68 °C	10 minutes	1x cycle
6.	Store	4 °C	Forever	

Cloning and Sequencing of white gene

Ligation of the gel eluted products to *pTZ57R/T* cloning vector (Figure 1) was done as per manufacturer's protocol. The main features of the vector are the blue and white colony selection, the presence of ampicillin resistance marker gene and the integrated sequence of M13 primers for easy sequencing. Recombinants were distinguished from non-recombinants by blue-white selection of colony.

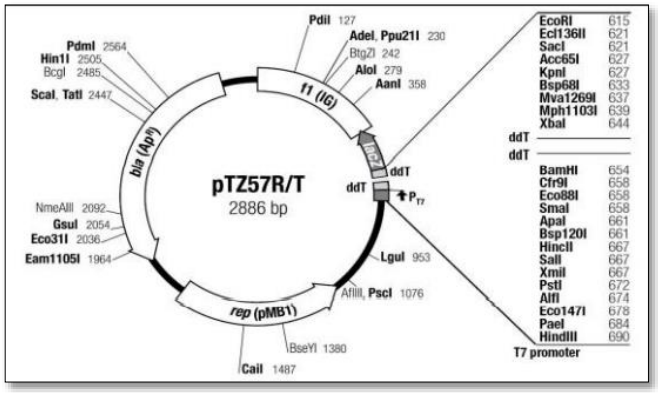

Fig 1: Cloning vector, *pTZ57R/T* vector map

After transformation, the cells were diffused in LB agar plates containing a suitable antibiotic, preferably ampicillin. This was followed by incubation at 37 °C overnight. Following this the transformed white colonies were picked up using a sterilised toothpick and inoculated into LB broth containing ampicillin and incubated in shaker incubator at 37 °C overnight at 180 rpm.

Plasmid isolation from the positive clones was then carried out via the Gene JETTM Plasmid Miniprep Kit (Thermo Scientific, Fermentas, Lithuania) in accordance with the directions provided by the manufacturer. On 1% agarose gel electrophoresis, the reference plasmid control DNA1 and the recombinant plasmids were compared.

The aforementioned clones were sequenced in triplicates using M13 universal primers in both orientations using an automated sequencer at Medauxin, Bengaluru.

Sequence analysis and data interpretation

The ClustalW was found appropriate to perform multiple sequence alignments of the sequences. The sequenced clones' percentage of query similarity to the database reference genome sequence was reported.

Designing of off-target minimised guide RNA (gRNA)

The efficacy of the CRISPR/Cas9 based genome editing depends upon the specificity of single guide RNA. The sgRNAs were designed from the sgRNA sites of the white gene using the online design tool of CRISPR and the software of PAM sequences called CHOPCHOP V3. Off target minimized guide RNA sequence (Figure2), targeted in exon3 (5'-GGTGAATTGCTAGCGGTCAT-3') was selected for white gene by performing NCBI-BLAST to check off-target effect.

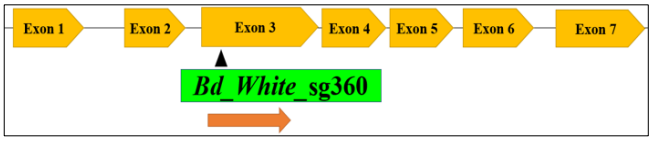

Fig 2: Schematic representation of position of single guide RNA binding to *Bactrocera dorsalis* genome for restriction of white gene. (The yellow-coloured boxes represent the exon sequences, the black line connecting two exons represent the intron sequence and the orange arrows indicates the direction of restriction)

sgRNA hybridization and PCR amplification of sgRNA cassette

The designed gRNA and its reverse complement were hybridised following manufactures protocol (Thermo Scientific sgRNA hybridization kit). The hybridised gRNA was then attached to an IVT cloning vector linearized in the lab, and incubated at 16 °C overnight. The IVT cloning vector was initially digested using the endonuclease (HindIII) at the sitein which sgRNA must be ligated. Following this, the ligated mixture was employed to transform *E. coli* DH5α cell and incubated for the following day at 37 °C. The recombinant colony was selected and inoculated into new LB media, and the overnight inoculated product was further processed to isolate the plasmid. Plasmids underwent additional sequencing in order to locate the insert.

***Invitro* transcription of sgRNA**

The sgRNAs (20 to 25 nucleotides) were synthesised through *invitro* transcription by following the manufactures protocol (NEW ENGLAND Biolab® Inco).

***In vitro* Restriction assay**

The procedure of *in vitro* digestion (Table 4) was done in an effective manner employing the guidelines provided by the manufacturer (NEW ENGLAND Biolab® Inco). It was performed to test the efficiency of single guide RNA, sg360 for white CDS restriction. The samples were 1. White CDS+ sg 360+Cas9, 2. White CDS+Cas9+ no sg360, 3. Cas 9+ sg 360+ no White CDS, 4. White CDS + sg360+ no cas 9, 5. White CDS + no Cas9 + no sg360 and 6. Sg 360+no cas9+no White CDS.

Table 4: The CRISPR/Cas9 components for *invitro* restriction assay for white gene

Sl. No.	Components	Volume (µl)
1.	Nuclease-free water	14.25
2.	10X NE Bufferr3.1	2.0
3.	sgRNA	0.5
4.	Cas9 diluent (0.25 µl + 4.75 µl NEBuffer r3.1)	0.25
	Reaction volume	17.0
	Pre-incubate for 10 minutes at 25 ºC	
5.	Substrate DNA	1.0
6.	Nuclease-free water	2.0
	Total volume	20.0

Results and Discussion

RNA Isolation, Complementary DNA (cDNA) Synthesis and PCR Amplification of *β-tubulin* gene

Integrity of RNA extracted from seven-day old pupae was confirmed by gel electrophoresis in 1% agarose gel. The RNA concentration was 2118 ng/µl with an A260/280 value of 2.0 in nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA). Gel documentation photography has confirmed three intact bands of RNA (28S, 18S, 5.8S) for the above sample (Figure3), cDNA formation was verified by PCR amplification of the *β-tubulin* gene.

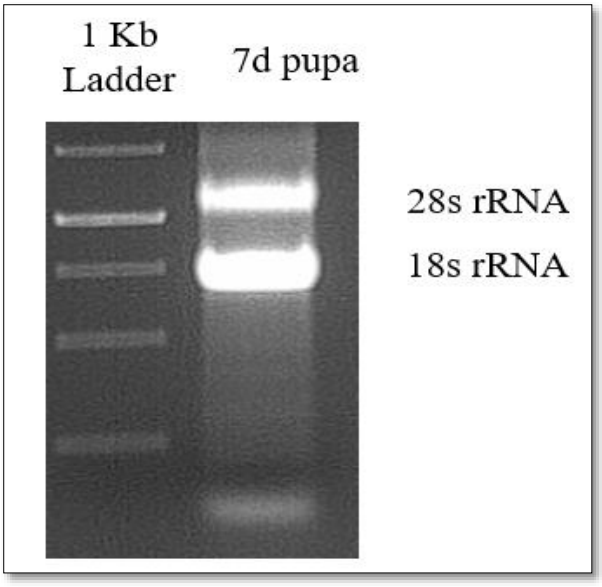

Fig 3: RNA isolated from *B. dorsalis* seven-day old pupae on 1% agarose gel

PCR Amplification of White Gene and Cloning

1% agarose gel electrophoresis with an amplicon band size of

2040 bp (Figure 4) verified that the white gene CDS had been amplified by PCR using gene-specific primers. Nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA) quantified the band after it had been eluted from the gel. The concentration was observed to be 65.6 ng/µl. This was followed by ligation and blue-white colony selection. The sequenced data signified that the cloned sequences (Figure 5) were white gene of *B. dorsalis*, when the sequences were compared in nucleotide BLAST.

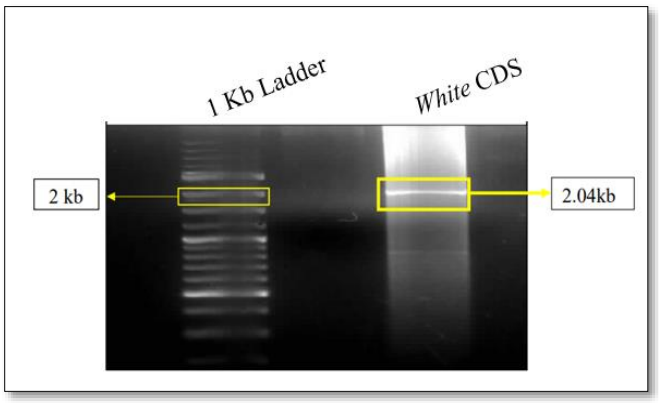

Fig 4: PCR amplification of white CDS visualised on 1% agarose gel

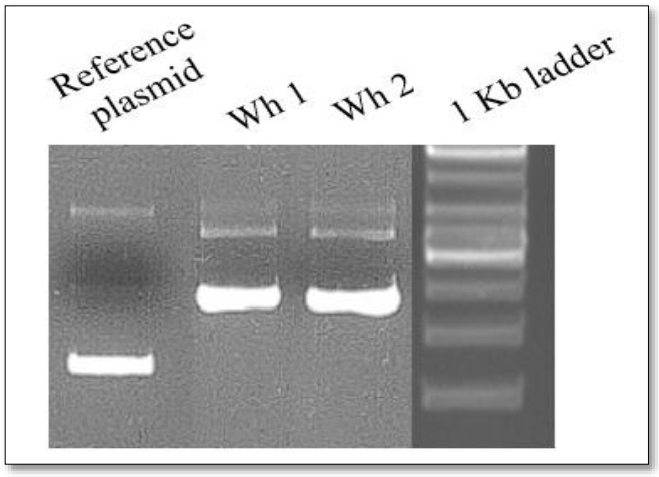

Fig 5: *B. dorsalis* white gene clones compared with reference plasmid F

NCBI BLAST showed 98.66 per cent similarity of the cloned sequences with the predicted *B. dorsalis* protein white (LOC105224216) mRNA (Figure 6). It showed high sequence similarity with predicted white gene sequences of *B. tryoni* (XM_040106531.1) (95.53%), *B. latifrons* (XM_018942980.1)(95.97%)and*B. oleae* (XM_036371618.1) (94.08%). This revealed that the predicted domains are conserved in all the related tephritid fruit flies.

Fig 6: NCBI-BLAST results for *B. dorsalis* cloned white CDS

gRNA Cloning, PCR Amplification of gRNA Cassette and *In Vitro* Transcription

The specific sgRNA sites were obtained and identified based on the PAM site and those sequences were sent for synthesis for getting primers for amplification of the particular sgRNA for respective sites. Cloning and sequencing was done to confirm the ligated sgRNAs. By using sgRNA cassette specific primers, the sgRNAs cassettes were amplified and amplicon size was determined to be 150 bpin 1.2% agarose gel electrophoresis (Figure 7). The *invitro* transcribed sgRNAs were quantified by gel electrophoresis on 2% agarose gel. The concentration of sg360 was found to be 1749.4 ng/µl.

***In vitro* restriction assay**

The ability of the Cas9 protein and sgRNAs to cleave the target site identified by PAM was denoted using restriction experiment *in vitro*. The 1.5% agarose gel was loaded with the *in vitro* complex mixture.

Sample one (White CDS +sg 360+Cas 9) gave significant results where Cas9 enzymedigested the white gene DNA. When the digested strand was compared with a hyper ladder of 1kb, it was observed that, the digested strand of white gene DNA was 1680 bp (Figure 8). The other samples didn't show any significant results because the components required for *invitro* digestion were altered according to the need of experiment to check the activity of CRISPR/Cas9 system in presence and absence of sgRNA.

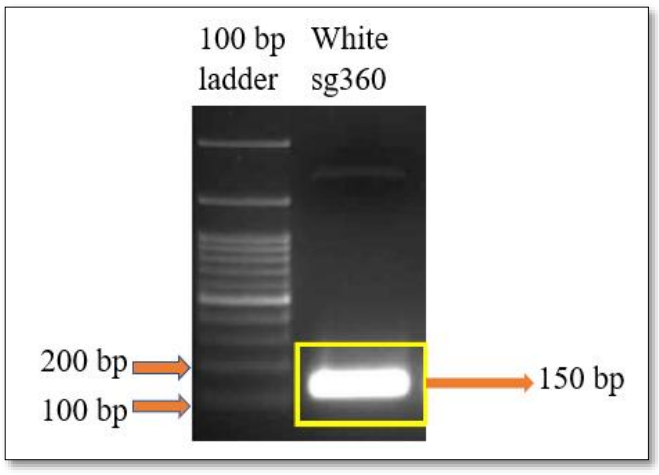

Fig 7: PCR amplification of gRNA cassette for white gene

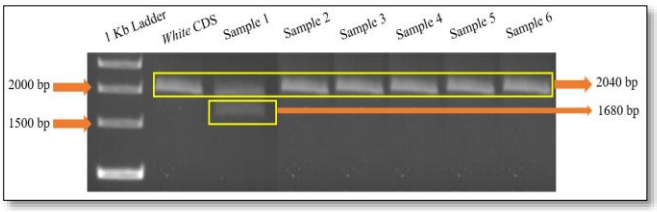

Fig 8: *In vitro* restriction assay of white sg RNA. The samples are, 1. White CDS+ sg 360+Cas 9, 2. White CDS+ Cas 9+ no sg 360, 3. Cas 9+sg 360+ no White CDS, 4. White CDS + sg 360+ no cas 9, 5. White CDS + no Cas 9 + no sg 360 and 6. Sg 360+no cas 9+no White CDS.

The above results on CRISPR/Cas9 based *invitro* digestion showed that, the single guide RNAs which we designed are compatible with the Cas9 enzyme and ready for editing of white gene of mango fruit fly, *B. dorsalis.* Thus, these sgRNAs were further used for microinjection of ribonucleoprotein (RNP) complex into embryos of *B. dorsalis*.

Thus, mutation of the white gene leads to loss of eye pigmentation in *B. dorsalis* and may also lead to change in behavioural and physiological pathways, which might lead to genetic management of the pest.

Further functional study can be made possible by the cloning and characterisation of the white gene in *B. dorsalis*. *In vitro* restriction assay confirmed the restriction efficiency of the designed sgRNAs. Further, it can be proceeded for microinjection of embryos. Post mutagenesis study can be carried out to check physiological and behavioural changes in the flies.

Acknowledgement

This endeavour was aided by Department of Agricultural Entomology, UAS, GKVK, Bengaluru and Indian Institute of Horticultural Research (ICAR-IIHR), Bengaluru. I sincerely express my gratitude towards the reviewers for their needful help in ameliorating the quality of this paper. The authors declare that there is no conflict of interest with the contents of this article.

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