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# Cloning of *White* Gene of the Melon Fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera : Tephritidae) and *in vitro* Restriction Analysis of Different Single Guide RNAs (sgRNAs)

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**ABSTRACT**

*Zeugodacus cucurbitae* (Coquillett) is a major pest affecting cucurbit crops. CRISPR/Cas9 mediated mutagenesis of target genes in *Z. cucurbitae* by generating a series of frame-shift mutation will help in changing physiology and behaviour of the insect. *White* gene is an important eye pigmentation gene widely used as marker gene in *Drosophila melanogaster*. PCR and Cloning of *Z. cucurbitae white* gene (2051bp) was performed. Off-target minimized gRNAs were designed by using bioinformatics online software CHOPCHOP by giving *white* coding sequences as input. Efficiency of the designed sgRNA was confirmed by *in vitro* restriction assay.

*Keywords* : CRISPR/Cas9, Guide RNA, *White* gene, *Zeugodacus cucurbitae*

FRUIT flies (Diptera : Tephritidae) are highly invasive and damaging pest species affecting the international trade of fruits and vegetables. More than 4500 species from Tephritidae family flies were described till date, which represents one of the most diverse group of acalyptrate Dipterans from superfamily Tephritoidea (Freidberg, 2006 and David *et al.,* 2016). Direct losses in a large variety of agricultural produce occur due to feeding and development of the maggots in fruits and vegetables (Virgilio *et al.,* 2015).

The melon fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae) is presumed to be from India (Bezzi, 1913), but now this pest has worldwide distribution (Virgilio *et al.,* 2010 and Li *et al.,* 2012) infesting more than 125 host plant species, most of which belong to the family Cucurbitaceae like cucumber (*Cucumis sativus*), bitter gourd (*Momordica charantia*), pumpkins (*Cucurbita moschata*), watermelon (*Citrullus lanatus*), muskmelon (*Cucumis melo*) etc. (White, 2006; Vayssieres *et al.,* 2008). *Z. cucurbitae* adults respond positively to preferred cucurbitaceae host fruit volatiles (Subhash *et al.,* 2018).

Existing management practices for the melon fly are destruction of infested fruits and vegetables, raking of soil, biological control and chemical control. Sterile insect technique (SIT) based area wide management practices helped to eradicate the pest from all the islands of the Okinawa archipelago in Japan (Shimizu *et al.,* 2007). Conventional management practices are associated with many disadvantages related to efficiency, can be time-consuming and come with environmental, animal and human hazards as well as side effects. By considering drawbacks, researchers explored new strategies and recent advancements in genetic control

CRISPR-Cas9 system is considered as a revolutionary technology with high efficiency and precision that can be applied to a wide range of species. A single guide RNA (sgRNA) finds target site in a genome, which can be implemented by scanning for protospacer adjacent motif (PAM) sequences (like 5'-NGG-3'



*The Mysore Journal of Agricultural Sciences*

for SpCas9). The guide RNA (gRNA) domain of the sgRNA determines both the efficacy and specificity of the genome editing activities by Cas9 (Jinek *et al.,* 2012).

Researches on the basis of genetic control of the melon fly, *Z. cucurbitae* are lacking. *White* gene regulating pigmentation in *Z. cucurbitae* will modify the visual behaviour of the target insect, which will help in devising suitable genetic control for the area wide pest management of the same. Optimization of sgRNA design is important for the success of gene editing experiments. The aim of this study is to identify and clone the *white* gene in *Z. cucurbitae* and validate sgRNAs by *in vitro* restriction assay to confirm the efficiency of restriction of the target gene, so that we can proceed for further micro injection studies.

## Material and Methods

### Mass Rearing of the Insect

Stock culture of *Zeugodacus cucurbitae* was maintained on cucumber (*Cucumis sativus* L.) at Division of Basic Sciences, ICAR-IIHR, Bengaluru, India at 25±1 °C, 75±1 per cent relative humidity with 14h:10h L:D photoperiod. The infested cucumber was placed in a container containing thin layer of sieved sand at the bottom for pupation. After pupation, the pupae were collected from sand and transferred to acrylic cages for adult emergence. Grinded sugar along with yeast powder and water was provided as a food source for adults. Insects obtained from this starter culture were used in further experiments.

### *White* Gene Identification and Primer Designing

The pigmentation gene, *white* was selected from the annotated NCBI genome database. Sequence similarity of *white* gene of melon fly with other tephritid species was determined by sequence alignment through ClustalW multiple sequence alignment in BioEdit (version 7.2.6.1) software.

Gene-specific primers were designed manually by using OligoAnalyzer Tool at Integrated DNA Technologies (IDT) site (Table 1). Then the specificity of the primers was confirmed by using the database of NCBI Primer BLAST (https:www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 1
*Zeugodacus cucurbitae white* gene-specific primers

| Primers | Sequences (5'-3') |
|---|---|
| Forward Primer | AAATGGGTCAGGAGGATCAG |
| Reverse Primer | CTCCTCATTTTACTCCTTGCG |

### RNA Isolation and Complementary DNA (cDNA) Synthesis

Male adult flies (5 nos.) were taken from stock culture and kept in a 10 ml falcon tube. Then the falcon tube was kept in a container containing liquid nitrogen for 2 minutes. After that the tube was shaken vigorously to detach different body parts. Then five heads were collected by using fine camel brush and transferred to one 1.5 ml eppendorf tube. Total RNA was extracted by using Trizol Reagent (Sigma Aldrich, USA) according to the manufacturer's protocol. Integrity of RNA was checked on 1 per cent agarose gel and further quantified with Nanodrop spectrophoto meter (Nanodrop Lite, Thermo Scientific, USA).

Complementary DNA (cDNA) was synthesized from the total RNA (2µg) by Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's protocol. cDNA synthesis was confirmed by PCR amplification of *RPL60* internal control gene and confirmed on 2 per cent agarose gel.

### PCR Amplification of *White* Gene and Gel Elution

The cDNA was diluted by using autoclaved milliQ water (1:10::cDNA:Water) and further used as a template to amplify the entire coding region of the *white* gene using gene-specific primers in PCR (Table 1, 2 and 3). Amplicon products were separated to determine size *via* electrophoresis on 1 per cent agarose gel. The desired amplicon band was eluted by using NucleoSpin Extract II kit (Machery Nagel, Germany) by adopting manufacturer's protocol and further used for cloning.

TABLE 2
PCR amplification of white gene

| Chemicals for White Gene Amplification | Working Concentration | Quantities (µl) |
|---|---|---|
| Autoclaved milliQ water | - | 12.6 µl |
| 10X $Mg^{+2}$ free buffer | 1X | 2.5 µl |
| 25mM $MgCl_2$ | 2.5mM | 2.5 µl |
| 2.5mM dNTPs mix | 0.4mM | 4.0 µl |
| Template (cDNA) | 1:10 diluted | 1.0 µl |
| Forward Primer (white gene) 5'-AAATGGGTCAGGAGGATCAG-3' | 0.2 µM | 1.0 µl |
| Reverse Primer (white gene) 5'-CTCCTCATTTTACTCCTTGCG-3' | 0.2 µM | 1.0 µl |
| LA Taq polymerase | 1 unit/ µl | 0.4 µl |
| | Total Volume | 25 µl |

TABLE 3
PCR conditions

| Steps | Temperature | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 95°C | 1 minutes | 1x cycle |
| Final denaturation | 95°C | 10 seconds | |
| Annealing | 56°C | 40 seconds | 35x cycles |
| Extension | 68°C | 2 minutes 10 seconds | |
| Final extension | 68°C | 10 minutes | 1x cycle |
| Store | 4°C | Forever | |

**Ligation of eluted *white* gene amplicon to cloning vector and transformation**

The eluted *white* gene amplicon was ligated into general purpose cloning vector, pTZ57R/T vector (Thermo Scientific, Lithuania) (Table 4). 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 etc. Recombinants were distinguished from non-recombinants by blue-white selection of colony. Blue colony indicates non-recombinant colony, whereas white colony indicates recombinant colony.

TABLE 4
Ligation of *white* gene into cloning vector

| Chemicals Added | Working Concentration | Quantities (µl) |
|---|---|---|
| Autoclaved milliQ water | - | 8.5 µl |
| 5X Ligase buffer | 1X | 4.0 µl |
| *pTZ57R/T* vector | 25 ng | 0.5 µl |
| Template gene (*white*) | 111.8 ng | 6.0 µl |
| T4 DNA ligase | 1 unit/ µl | 1.0 µl |
| | Total Volume | 20µl |

The ligated products were used for transforming *Escherichia coli* DH5-α by standard protocols. The transformed cells were spread on LB agar plates containing X-gal (20 mg/ml), IPTG (100 mM) and ampicillin (100 µg/ml). The plates were then incubated at 37 °C overnight to screen blue and white colonies and all the white colonies (colonies harboring the insert) were inoculated in LB broth containing ampicillin, incubated at 37 °C overnight and stored at 4 °C until further use.

**Plasmid Isolation and Sequencing**

Plasmids were isolated from the overnight culture of the transformed white colonies cultured in LB broth

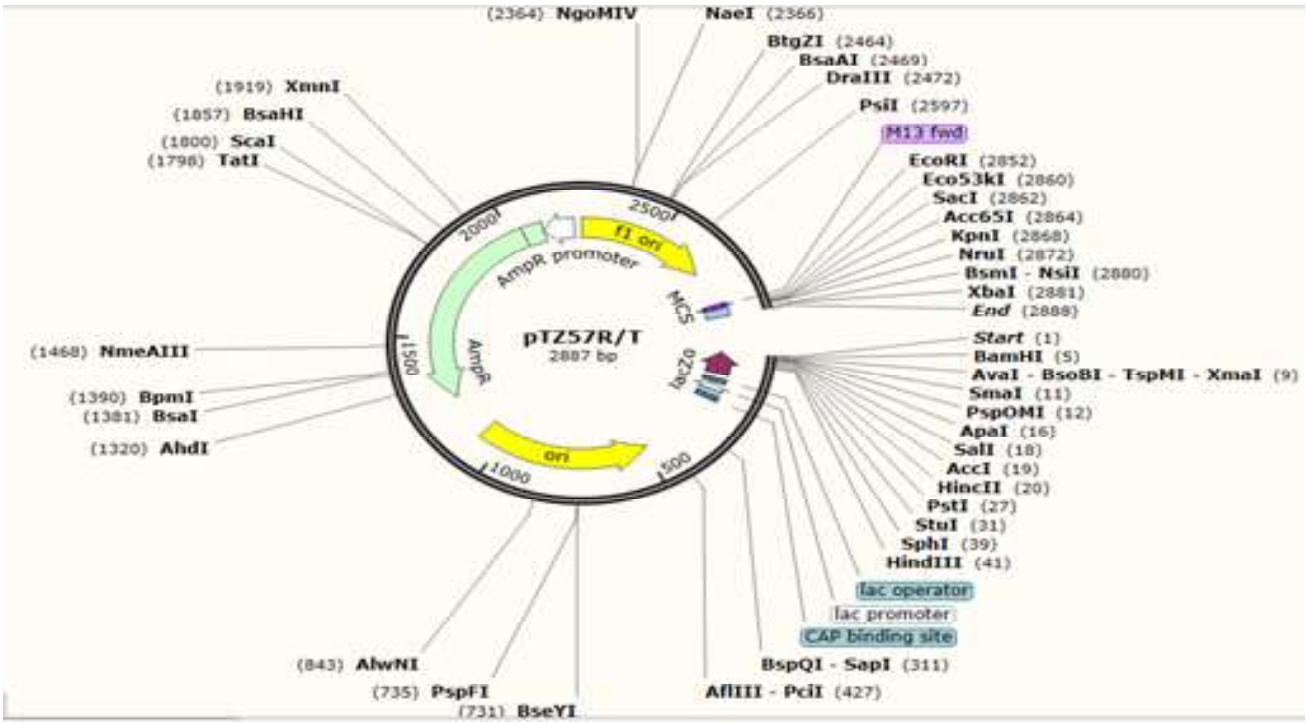


Fig. 1 : pTZ57R/T vector map

using Gene JET™ Plasmid Miniprep Kit (Thermo Scientific, Fermentas, Lithuania) according to manufacturer's protocol and the recombinant plasmid was confirmed in 1 per cent agarose gel electrophoresis with reference plasmid (control DNA1).

Sequencing was carried out in triplicates (three biological replicates and three technical replicates) of the above clones in an automated sequencer (ABI prism ® 3730 XL DNA Analyzer; Medauxin, Bengaluru) using M13 universal primers both in forward and reverse directions.

**Sequence Analysis and Data Interpretation**

Multiple sequence alignments of the *white* gene clone sequences and database *white* gene reference sequences were performed with ClustalW multiple alignment tool and the results were displayed using 'BioEdit (version 7.2.6.1). Percentage query similarity between the *white* gene sequenced clones and database *white* gene reference sequences was noted.

**Identification of Off-Target Minimized gRNA**

CHOPCHOP (version 3) (https://chopchop.cbu.uib.no/) is a web tool for selecting target sites for CRISPR/Cas9, CRISPR/Cpf1, CRISPR/Cas13 or NICKASE/TALEN-directed mutagenesis. It uses different alignment algorithms to predict off-target binding of sgRNAs and TALENs within short search time (Montague *et al*., 2014).

The sequenced *Z. cucurbitae* genome set was imported into the public site of the CHOPCHOP (version 3) tools (Labun *et al*., 2016). The gRNA region was identified from functional domain of the concerned protein by using CHOPCHOP (version 3) tools. Two sgRNA target sites, in exon2 (5'-AGATTATCCGTGGTGAGCGTAGG-3') and exon 7 (5'-ACCGAATGAAGTCGACACATTGG-3') were selected by performing NCBI- BLAST to check off-target effect. Reverse complement of the gRNA was also designed (Table 5).

**Hybridisation of gRNA and Cloning**

The designed gRNA and its reverse complement were hybridized by following manufacturer's protocol (Thermo Scientific sgRNA hybridization kit). Then the hybridized gRNA was ligated to a lab modified linearized IVT cloning vector and incubated at 16 °C

TABLE 5

*Zeugodacus cucurbitae white* gene sgRNA primers and its reverse complement (RC)

| Whsg1 | Whsg7 |
|---|---|
| >Wh sg1- AGATTATCCGTGGTGAGCGTAGG | >Wh sg7- ACCGAATGAAGTCGACACATTGG |
| >Wh sg1 RC- CCTACGCTCACCACGGATAATCT | >Wh sg7 RC- CCAATGTGTCGACTTCATTCGGT |

overnight. Initially, the IVT cloning vector was digested by using endonuclease enzyme (BbsI), the site where sgRNA has to be ligated. Then the ligated product was transformed into *E. coli DH5-α* cell and incubated over night at 37 °C. The recombinant colony was picked for inoculation into fresh LB media and plasmid was isolated from overnight inoculated product. Plasmids were further sequenced in order to identify the insert.

**PCR Amplification of sgRNA Cassette and *in vitro* Transcription**

For *in-vitro* single guide RNA synthesis, sgRNA cassettes (T7 promoter + sgRNA+ scaffold+ terminator) was amplified by using M13 forward and reverse primers. The amplicon size was determined by gel electrophoresis in 1.5 per cent agarose gel.

TABLE 6

Ligation protocol for sgRNA mobilization into IVT vector

| Chemicals added | Working Concentration | Volume (µl) |
|---|---|---|
| Autoclaved MilliQ water | - | 8.5 µl |
| 5X Ligase buffer | 1X | 4.0 µl |
| Linearised IVT vector | 25 ng | 3 µl |
| ds Oligos (Hybridized gRNA) | 2.75 ng | 3.5 µl |
| T4 DNA ligase | 1 unit/ µl | 1.0 µl |
| | Total Volume | 20 µl |

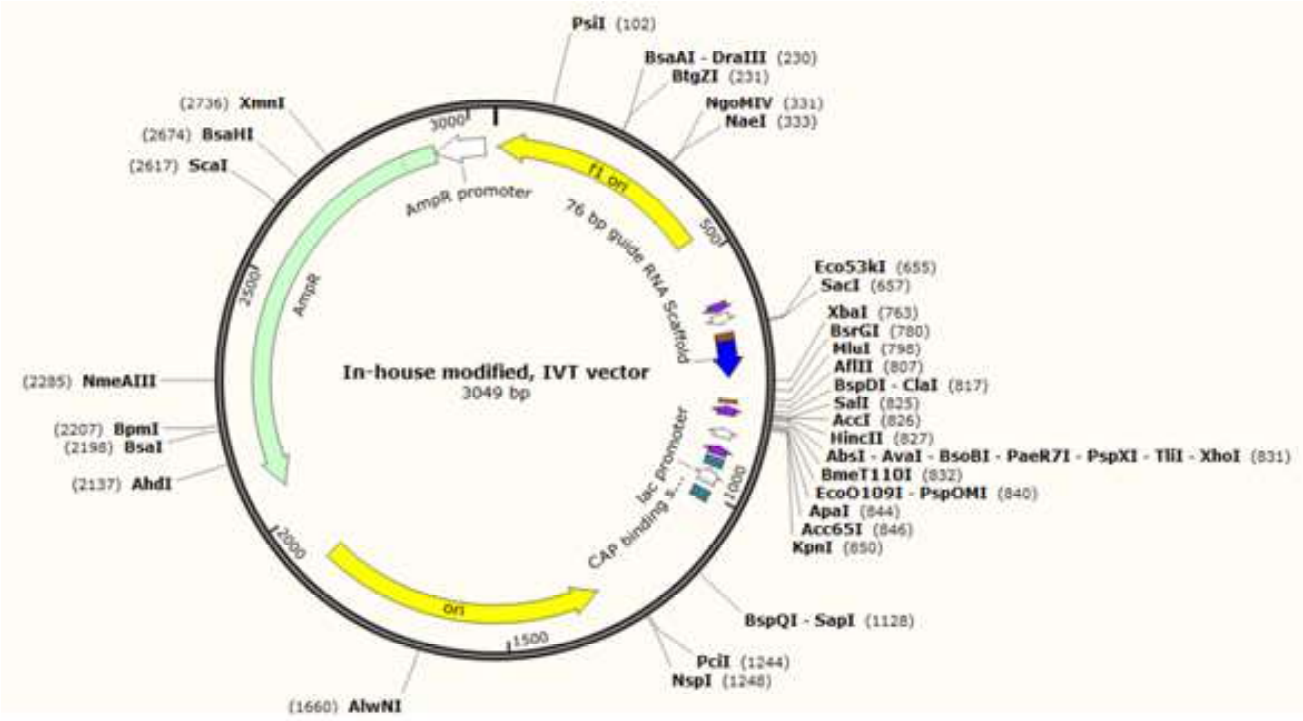


Fig. 2 : IVT vector map

The sgRNAs (24 nucleotides) were synthesized through *in vitro* transcription by using T7 promoter by following the manufactures protocol (NEW ENGLAND Biolab ® Inco) (Table 7). The reaction mixture was incubated 37 °C overnight. Next day the sample was treated with DNase I (4 µl) enzyme and incubated at 37 °C for 1 hour and then the samples were treated with 0.5 M EDTA (5 µl) and incubated at 65 °C for 10 minutes to denature DNase I enzyme. Then the sample was purified by Phenol: Chloroform: Isoamyl alcohol (P:C:I) method to yield good quantity of sgRNAs.

TABLE 7

*In vitro* transcription reaction mixture

| Components | Working Concentration | Volume |
|---|---|---|
| Autoclaved MilliQ water | - | 12.5 µl |
| 5X Transcription Buffer | 1X | 20 µl |
| NTP Mix | 10 mM | 20 µl |
| Template | 5 µg | 40 µl |
| T7 RNA Polymerase | 20 units/ µl | 5 µl |
| Ribolock RNase Inhibitor | 40 units/ µl | 2.5 µl |
| | Total Volume | 100 µl |

The concentration of sgRNAs were quantified using Nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA) and further assayed on 2 per cent agarose gel with RNA ladder that has 100-base band. Concentration of the sgRNAs were estimated by using ImageJ software (v 1.53s) by comparing the intensity of band between ladder and sgRNAs.

### *In vitro* Restriction Assay

*In vitro* restriction of *white* CDS with Cas9 nuclease and synthesized sgRNAs is quintessential to validate the efficiency of sgRNAs. The assay was performed for two sgRNAs (Whsg1, Whsg7) along with a negative control (Table 8). The components of CRISPR/Cas9 system were added in fixed quantities by following manufactures protocol (NEW ENGLAND Biolab ® Inco) with slight modifications (Table 9). Then *in vitro* restriction was confirmed by gel electrophoresis in 2 per cent agarose gel.

TABLE 8

The CRISPR/Cas9 system of two sgRNAs for *in vitro* restriction assay

| Sample No. | Components of CRISPR/ Cas9 System |
|---|---|
| 1. | *White* gene CDS + Cas9 only |
| 2. | *White* gene CDS + Cas9 + Whsg1 |
| 3. | *White* gene CDS + Cas9 + Whsg7 |

TABLE 9

The CRISPR/Cas9 components for *in vitro* restriction assay

| Components | Working Concentration | Volume |
|---|---|---|
| Autoclaved MilliQ water | - | 11.34 µl |
| NEBuffer r3.1 | 1X | 2 µl |
| 150 mM KCl | 5 mM | 0.66 µl |
| sgRNA | 150 ng/ µl | 0.4 µl |
| Cas9 diluent (0.25 µl + 4.75 µl NEBuffer r3.1) | 300 ng/ µl | 0.6 µl |
| | Reaction Volume | 15 µl |
| Pre-incubate for 30 minutes at $25^0$C | | |
| *White* gene CDS | 150 ng/ µl | 5 µl |
| | Total Reaction Volume | 20 µl |
| Incubate for 1 hour at $37^0$C | | |

## RESULTS AND DISCUSSION

### RNA Isolation and cDNA Synthesis

Integrity of total RNA isolated from head region was confirmed by gel electrophoresis in 1 per cent agarose gel (Fig. 3). In nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA), the RNA concentration was 1.3 µg/ µl with A260/280 value of 1.64. cDNA synthesis was confirmed by PCR amplification of *RPL60* internal control gene. The amplicon band size of 119bp was separated by gel electrophoresis on 2 per cent agarose gel (Fig. 4). *RPL60* gene was the most stable reference gene (119bp) found in *Zeugodacus cucurbitae* (Zhang *et al.,* 2018).

### PCR Amplification of *White* Gene and Cloning

PCR amplification of *white* gene CDS with gene-specific primers was confirmed by gel

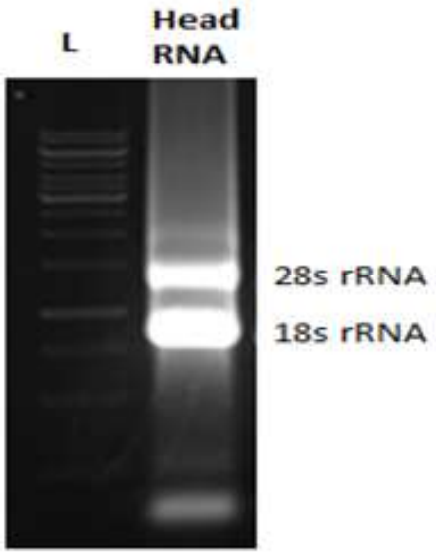


Fig. 3: Total RNA isolated from head region of *Zeugodacus cucurbitae*

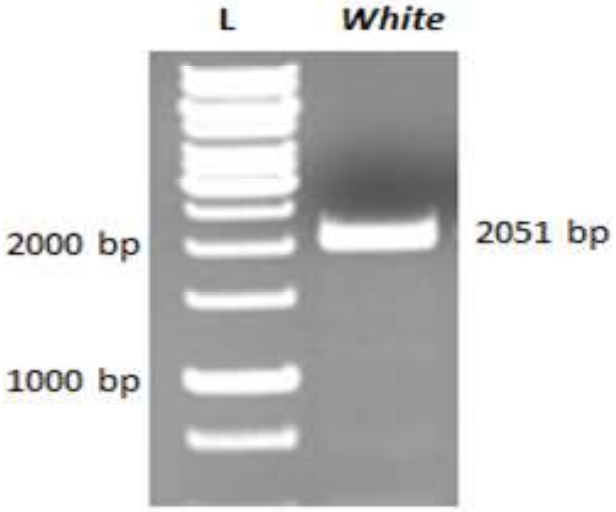


Fig. 5 : PCR amplification of *Zeugodacus cucurbitae white* gene CDS

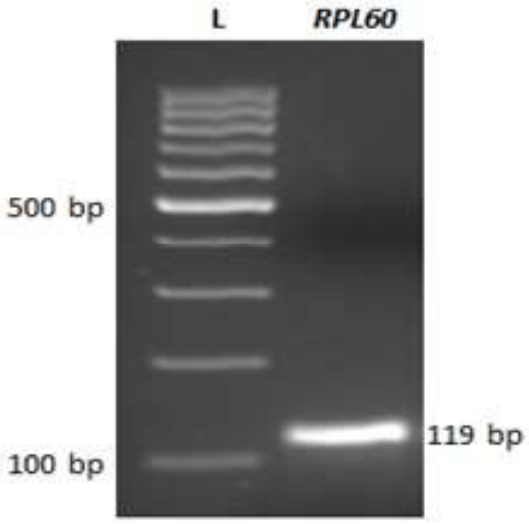


Fig. 4 : PCR amplification of *RPL60* internal control gene

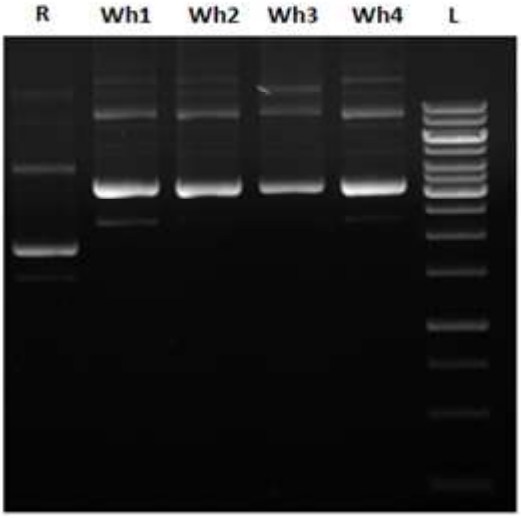


Fig. 6 : *Zeugodacus cucurbitae white* gene clones compared with reference plasmid (R)

electrophoresis on 1 per cent agarose gel with amplicon band size of 2051bp (Fig. 5). Further the band was eluted from the gel and quantified on Nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA). The concentration was 34.5 ng/ µl. This eluted product was further used for cloning. The isolated plasmids were checked by gel electrophoresis on 1 per cent agarose gel along with reference control DNA1 plasmid to observe the raise in band size. In all the clones, insert was observed (all bands were raised compared to reference plasmid) (Fig. 6).

NCBI-BLAST of the cloned sequences showed 98.09 per cent sequence similarity with predicted *Z. cucurbitae* protein white (W) (XM_011189498.2). It showed high sequence similarity with predicted *white* gene sequences of *Bactrocera dorsalis* (XM_011202225.3) (90.44%), *B. tryoni* (XM_040106531.1) (90.20%), *B. latifrons* (XM_018942980.1) (89.61%) and *B. oleae* (XM_036371618.1) (89.51%) (Fig.7). This revealed that the predicted domains are conserved in all the related tephritid fruit flies.

Descriptions | Graphic Summary | Alignments | Taxonomy

**Sequences producing significant alignments** Download Select columns Show 100

select all *76 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

| Description | Scientific Name | Max Score | Total Score | Query Cover | E value | Per. Ident | Acc. Len | Accession |
|---|---|---|---|---|---|---|---|---|
| Zeugodacus cucurbitae isolate CucBan white-eye mRNA, complete cds | Zeugodacus cucurbitae | 3768 | 3768 | 100% | 0.0 | 100.00% | 2040 | OQ632780.1 |
| PREDICTED: Zeugodacus cucurbitae protein white (W), mRNA | Zeugodacus cucurbitae | 3541 | 3541 | 100% | 0.0 | 97.99% | 3253 | XM_011189498.3 |
| PREDICTED: Bactrocera dorsalis protein white (LOC105224216), mRNA | Bactrocera dorsalis | 2710 | 2710 | 100% | 0.0 | 90.64% | 3392 | XM_011202225.4 |
| PREDICTED: Bactrocera neohumeralis protein white (LOC126758950), mRNA | Bactrocera neohumeralis | 2699 | 2699 | 100% | 0.0 | 90.54% | 3326 | XM_050473441.1 |
| PREDICTED: Bactrocera tryoni protein white (LOC120776067), mRNA | Bactrocera tryoni | 2660 | 2660 | 100% | 0.0 | 90.20% | 2040 | XM_040106531.1 |
| Bactrocera dorsalis clone IIHR_BD_WG1 white protein (w) mRNA, complete cds | Bactrocera dorsalis | 2595 | 2595 | 100% | 0.0 | 89.66% | 2037 | MT895645.1 |
| PREDICTED: Bactrocera latifrons protein white (LOC108974879), mRNA | Bactrocera latifrons | 2593 | 2593 | 100% | 0.0 | 89.61% | 2142 | XM_018942980.1 |
| PREDICTED: Bactrocera oleae protein white (LOC106620505), mRNA | Bactrocera oleae | 2582 | 2582 | 100% | 0.0 | 89.51% | 3349 | XM_036371618.1 |
| Ceratitis capitata protein white (W), mRNA | Ceratitis capitata | 2043 | 2043 | 99% | 0.0 | 84.78% | 2250 | NM_001279365.1 |
| C.capitata white gene mRNA | Ceratitis capitata | 2043 | 2043 | 99% | 0.0 | 84.78% | 2252 | X89933.1 |
| PREDICTED: Anastrepha obliqua protein white (LOC129239220), mRNA | Anastrepha obliqua | 1921 | 1921 | 98% | 0.0 | 83.94% | 2987 | XM_054874570.1 |
| PREDICTED: Anastrepha ludens protein white (LOC128858475), transcript variant X4, mR... | Anastrepha ludens | 1905 | 1905 | 98% | 0.0 | 83.81% | 2590 | XM_054094783.1 |

## gRNAs Cloning, PCR Amplification of gRNAs Cassette and *In vitro* Transcription

Designed gRNAs had no potential off-target sites, which were confirmed by NCBI-BLAST. The seed region (final 12 nucleotide of the target sequence within the sgRNA) (Wu, 2014) and protospacer adjacent motif (PAM) sequences were matching perfectly to the target sequence. Studies by Cong *et al.* (2013) revealed that the seed region of sgRNA and PAM sequences play vital role in initiating efficient restriction in the target region. Mismatch in these seed region and absence of PAM site effects in non-recognition of target site. gRNA was hybridized and cloned into IVT cloning vector. Plasmids isolated were confirmed by gel electrophoresis in 1 per cent agarose gel against reference IVT vector (Fig. 8). It confirmed insertion of gRNA into the vector in all the gRNAs. Further the clones were confirmed by sequencing.

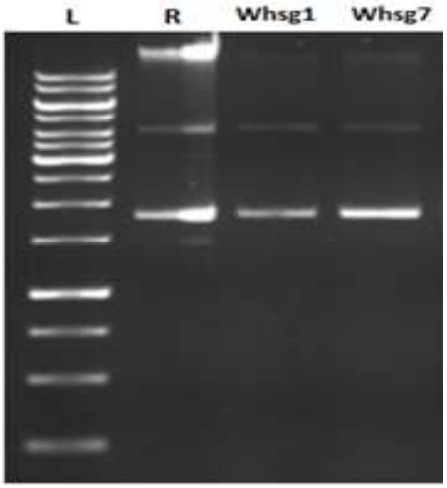


Fig. 8 : *White* gRNA clones compared with reference IVT vector

Guide RNA cassette (T7 promoter + gRNA + scaffold) was PCR amplified with M13 forward and reverse primer. The PCR product was confirmed by gel electrophoresis on 1 per cent agarose gel with amplicon band size of 317bp (Fig. 9). The bands were eluted from the gel and used for *in vitro* transcription. The *in vitro* transcribed sgRNAs were quantified by gel electrophoresis on 2 per cent agarose gel with

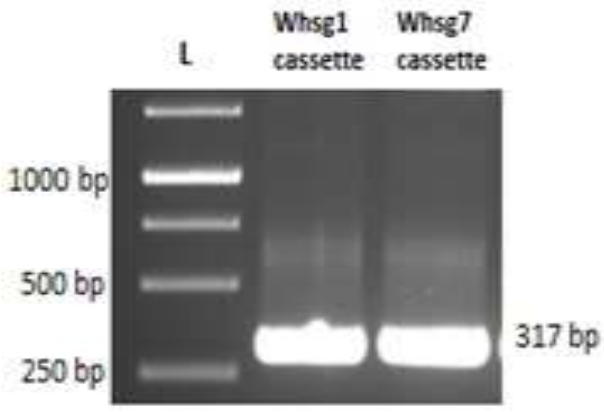


Fig. 9 : PCR amplification of gRNA cassette

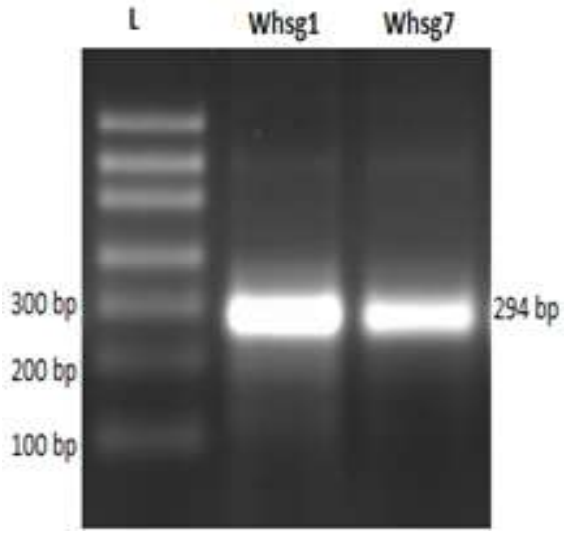


Fig. 10 : Quantification of Wh sgRNA with RNA ladder

RNA ladder (Fig. 10) and processed by using ImageJ software (v1.53s). Concentration of Whsg1 and Whsg7 was 548 ng/ µl and 465 ng/ µl respectively.

### *In vitro* Restriction Assay

*In vitro* restriction assay confirmed the potency of Cas9 protein and sgRNAs to cleave the target site of double stranded DNA. The *in vitro* complex mix was loaded on 1.5 per cent agarose gel. First lane was 1kb ladder, second lane was *white* gene CDS + Cas9, third lane was *white* gene CDS + Cas9 + Whsg1 and fourth lane was *white* gene CDS + Cas9 + Whsg7. Visualisation on agarose gel revealed one solid band of 2051bp in second lane. Third lane showed multiple fragments of bands of size 1890bp and 161bp, which were cut released bands from 2051bp *white* gene CDS band. Fourth lane showed multiple fragments of bands of size 1641bp and 410bp, which were cut released bands from 2051bp *white* gene CDS band (Fig. 11). These sgRNAs were further used for microinjection of ribonucleoprotein (RNP) complex into embryos of *Z. cucurbitae.*

*White* gene was abundantly expressed in the compound eyes of *Bactrocera, Anastrepha* and *Ceratitis.* Mutation of this gene leads to loss of eye pigmentation (Bai *et al.,* 2019 and Sim *et al.,* 2019). Complete loss of *white* gene impairs formation of dimers with Brown and Scarlet proteins, which blocks

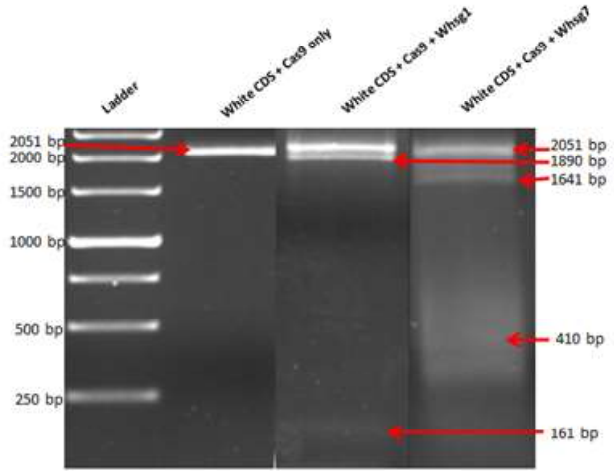


Fig. 11: *In vitro* restriction assay of *white* sgRNAs

the transport of precursor of ommochromes, responsible for eye pigmentations. So, the eyes appear as white as a consequence (Choo *et al.,* 2018).

*White* mutants in *Ceratitis capitata* exhibited unsuccessful mating due to reduced courtship behaviour (Briceno, 2003).

Cloning and sequencing of *white* gene in *Z. cucurbitae* paved way for further characterization and functional analysis. As there is non-availability of published database of *white* gene of *Z. cucurbitae,* functional annotation of this gene following microinjection and phenotypic and molecular validation will enrich the knowledge about its functionality. *In vitro* restriction assay confirmed the restriction efficiency of the designed sgRNAs. Further, it can be proceeded for microinjection of *Z. cucurbitae* embryos. Post mutagenesis studies can be carried out to check physiological and behavioural changes in the flies.

## References

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