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Ribonucleoprotein Complex (sgRNA + Cas9) Mediated *In vitro* Restriction Assay to Predict *In vivo* Editing of the *White* Gene of the Fall Armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)

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Abstract

CRISPR (clustered regularly interspaced short palindromic repeats)-Cas (CRISPR-associated protein) system has emerged as a popular toolkit to edit specific regions of the genome in several taxa. A 20 bp single guide RNA (sgRNA) directs the Cas 9 endonuclease enzyme to a precise location in the genome for double stranded DNA cleavage and further repair, predominantly through Non-homologous End Joining (NHEJ), resulting in addition or deletions. In this regard, the dsDNA cleavage by Cas9 depends mainly on various factors in sgRNA design. Current web-based tools for sgRNA design suggest a wide variety of factors to choose a suitable sgRNA. Therefore, it is important to check the cleaving efficiency of the Cas9 + sgRNA complex called Ribonucleoprotein complex through *in vitro* restriction assay before proceeding with microinjection to achieve *in vivo* restriction of the target gene in an organism. In the present study we have demonstrated the cleaving efficiency of different sgRNAs in restricting the white gene of the Fall armyworm, *Spodoptera frugiperda*.

Keywords : *In vitro* restriction assay, CRISPR/Cas9, Fall Army Worm, Genome editing

The capacity to manipulate biological systems and individuals has great application potential in fundamental research, medicine and biotechnology. Programmable sequence-specific endonucleases that allow precise editing of endogenous genomic loci are now enabling systematic probing of genetic elements and causative genetic variants in a wide range of animals, including some that were previously untraceable genetically. In recent years, various genome editing methods have evolved such as Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and the RNA-guided CRISPR-Cas nuclease system.

Despite the availability of various genome editing tools, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and CRISPR-associated protein 9 (Cas9) system has emerged as a superior method for precise gene mutations (Zong *et al.*, 2017; Soyars *et al.*, 2018 and Dong *et al*., 2020). Initially discovered as an adaptive immunological defense mechanism against DNA invasion in bacteria (Wiedenheft *et al.*, 2012 and Jinek *et al*., 2012), CRISPR/Cas9 has been modified to perform precise genome editing, consisting primarily of two components: a guide RNA (gRNA) and the Cas9 endonuclease. The gRNA is a 20-nucleotide long gene-

specific sequence (Gao & Zhao, 2014) that binds specifically to a target DNA sequence containing the Protospacer Adjacent Motif (PAM), typically having 'NGG' bases at the 5' end.

The PAM region plays a crucial role in Cas9 binding and is situated 3 base pairs downstream from the Cas9 endonuclease cleavage site (Ran *et al.*, 2013). Adjacent to the 3' end of the 20-nucleotide gRNA is an 80-nucleotide long gRNA scaffold sequence that is necessary for Cas9 interaction (Jiang and Doudna, 2017). Once the gRNA-Cas9 complex forms, Cas9 creates a double-strand break precisely 3 base pairs before the PAM region (Jiang *et al.*, 2013). To repair this break, non-homologous end joining (NHEJ) is employed, which is typically error-prone, leading to the insertion or deletion (indel) of genetic material at the cut site. These indel mutations frequently cause frame-shift mutations, disrupting protein translation and compromising gene function.

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) has evolved as a major pest of many crops, including cotton (*Gossypium hirsutum* L.), sorghum (*Sorghum bicolor* L.), maize (*Zea mays* L.) and other pasture grasses (Priyanka & Murali Mohan, 2022). Although chemical management remains as one of the most important techniques for the management of this pest, it has significant draw backs in terms of efficiency, can be time-consuming, and can pose environmental, animal and human threats, as well as side effects (Khadri and Srinivasa, 2018). The rapid evolution of CRISPR/Cas9 gene editing technology has opened up new avenues for scientific pest management. Consequently, from the standpoint of pest management, PgSIT using CRISPR-Cas9-based genome editing has greater potential in establishing area-wide pest control strategies for a diverse spectrum of insects (Kandul *et al.*, 2019). Developing a PgSIT system necessitates the development of sgRNA and Cas9 transgenic lines for males and females, respectively. This necessitates greater biosafety requirements, more time for development and maintenance and the unpredictability of target gene loss of function. As a result, it is critical to verify gene targets in a simple, non-transgenic manner by using a Ribonucleoprotein (RNP) complex (sgRNA: Cas9 protein) injected at the G0 stage of the target species' embryo. Before editing the genes, which are crucial to create the PgSIT system, it is important to validate the marker genes, which have visible phenotypes, in this regard the eye colour gene white was used as a marker gene.

The specificity and efficacy of the CRISPR-Cas9-mediated genome editing is primarily determined by the sequence features of gRNA. Thus, before performing the RNP mediated editing in insects, it is important to validate the efficiency of various sgRNA's in vitro. In this study, we have demonstrated the evaluation of the efficiency of various sgRNA's for the white gene from *S. frugiperda* through in vitro cleavage assay.

Materials and Methods

Cloning and Characterization of *white* gene

Total RNA was isolated from a single adult *S. frugiperda* using the ISOLATE II RNA Mini Kit (BIOLINE, USA) according to the manufacturer's instructions. Nanodrop (Thermo Scientific, USA) was used to evaluate the purity of the RNA and denaturing agarose gel electrophoresis (1.5%) was used to validate its integrity. A total of 1g total RNA was utilized for cDNA synthesis using the Revert Aid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) and was kept at -20 °C until further usage. PCR amplification was carried out using the degenerated primers of white gene Table 1. using the reagents mentioned in Table 2. The PCR conditions

Table 1
Primer and guide details

Primer Name	Sequence
FAW_WhiteF	GGDGTBGAYAARGTWACNTACAC
FAW_WhiteR	GTYTGYARRAASCGVACTTTGAT
g283	CATGAACGACTCATGCTGTG
g528	ACCACGGGGCTGGACAGCTA
g598	AGAAACTGTCAGCATCAGGC

TABLE 2

PCR amplification of white gene

Reagents	Working Concentration	Volume (il)
Nuclease free water	-	8.0
10x Mg+2 free buffer	1x	2.5
25mM MgCl2	2.5mM	2.5
2.5mM dNTPs mix	0.4mM	4.0
Template (cDNA)	1:10 diluted	5.0
Forward Primer	0.2µM	1.0
Reverse Primer	0.2µM	1.0
Taq polymerase	1unit/µl	1.0
Total Volume		25.0

TABLE 3

PCR conditions to amplify white gene

Step	Temperature (°C)	Time	No. of Cycles
Initial Denaturation	95	2 min	1
Denaturation	98	10sec	
Annealing	56	30sec	35
Extension	72	1min 30 sec	
Final Extension	72	10min	1

for the same are listed in the Table 3. The amplified products were then resolved on 1.2 Per cent agarose gel. The PCR product was purified using PCR clean-up kit (Macherey-Nagel, Germany) and further ligated to pTZ57R/T cloning vector (Thermo Scientific, USA). The ligated products were cloned into chemical competent *Escherichia coli* strain DH5 cells. Afterwards, the transformants were detected on an LB plate with ampicillin (100 mg/mL) using blue/white selection (Fig. 1). Plasmids were isolated using Thermo Scientific's Plasmid isolation kit and clones were run on 1.2 per cent agarose gel and validated by differential mobility assay. The cloned plasmids were sequenced in triplicates by Sanger sequencing and the identity of sequences were further confirmed by Nucleotide BLAST database of NCBI (https://www.ncbi.nlm.nih.gov).

Designing Single Guide RNA (sgRNA)

Three guide RNAs (gRNA) were designed (Table 1.) using CRISPOR tool available at https://crispor.tefor.net (Concordet and Haeussler, 2018) also the following principles were followed while designing the guide: The design site was supposed to contain a protospacer adjacent motif (PAM) region with nucleotide sequence NGG; The GC content of sgRNA should be with in the range of 35-50 per cent; Presence of three stem loop structures, RAR, second and third (Fig. 2). Unpaired Seed region of gRNA (i.e., crRNA/spacer). Self-folding potential value of gRNA between 0 and -2.0 kcal/ mole.

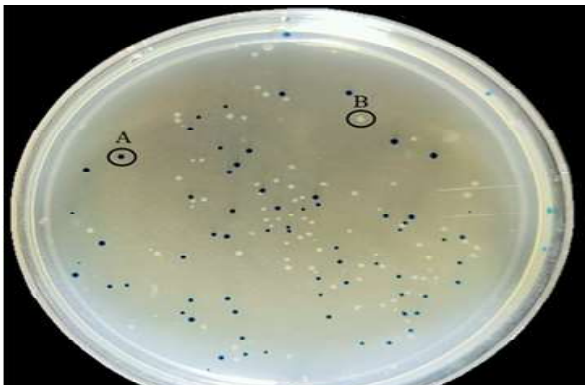

Fig. 1 : Blue-white screening of the E. coli colony to identify the transformants. (A) Blue colour colony indicating the non-transformant. (B) White colour colony indicating the transformant

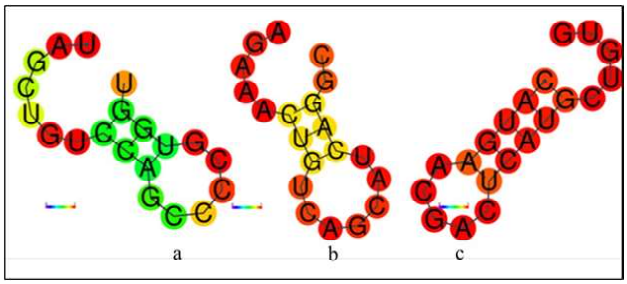

Fig. 2 : Secondary structure of gRNAs a) g528, b) g598, c) g283

***In vitro* Transcription and Synthesis of sgRNA**

Two primers were designed and used to amplify a fragment of sgRNA (114 bp), with the forward primer sgRNA-F containing a T7 polymerase-binding site and the sgRNA target sequence and the reverse primer sgRNA-R containing the remaining sgRNA sequences. The two primers were annealed by PCR to synthesize a template DNA. The total of 50 iL PCR mixture included 10X LA PCR Buffer ll (Mg_2^+free),

2.5 mM MgC l2, 0.40 mM of each dNTP, 100 ng of L. *orbonalis* cDNA and 0.25U of LA Taq DNA Polymerase (TAKARA BIO Inc, USA). The PCR reaction was set as follows: 98 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 20 s, a final extension at 72 °C for 10 min. The purified PCR product was then used as the template for in vitro transcription. In *vitro* transcription of sgRNA was carried out in a total of 50 µL reaction volume, containing 10 µL of 5x transcription buffer, 10 µL of 10 mM NTP mix (2 mM final concentration), 2 µg of purified template, 1.25 µL of RiboLock™ RNase Inhibitor (50 U) and 1.5 µL of T7 RNA polymerase (30 U). The mixture was incubated in a water bath at 37 °C for overnight. The template DNA was removed by incubating with DNase I @ 37 °C for 1hr followed by DNase inactivation by adding 50mM EDTA and incubation @ 65 °C for 5 min. Further the sgRNA was purified by using Monarch® RNA Cleanup Kit following the manufacturer's protocol.

In vitro Restriction Assay

An *in vitro* restriction assay was done to establish that the RNP complex effectively recognizes and cleaves the target site. The TO gene's target sgRNA site-containing PCR product served as the template. The invitro restriction components were all obtained from NEB, United States. In a 20 µL reaction volume, the following components were added: 2 µL of 3.1 NEB uffer, 30 nM of in vitro generated sgRNA cassette and 30 nM of Cas9 enzyme (NEB). The remaining reaction volume is made up to 20 µL by adding ddH2O. The reaction conditions include 30 minutes incubation of the above mixture at 25 °C, which was followed by the addition of 100 ng of purified PCR product and vigorous mixing of the contents. The mixtures were then incubated for 1 hr at 37 °C. Then, the mixture was added with 1 µL of Proteinase K (20 mg/ml) (NEB, USA) and incubated the reaction for 10 min at room temperature. The in vitro restricted products were then analyzed by resolving in 1.5 per cent agarose gel electrophoresis.

Results and Discussion

RNA Isolation and cDNA Synthesis

The integrity of the total RNA isolated from a single adult was confirmed using gel electrophoresis on a 1 per cent agarose gel (Fig. 3). The RNA concentration was measured as 1.7 µg/µl with an A260/280 value of 1.83 using a Nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA). The integrity of the cDNA was confirmed through PCR amplification of the mtCoI internal control gene. The resulting amplicon, with a size of 658 base pairs, was separated via gel electrophoresis on a 1.8 per cent agarose gel (Fig. 4).

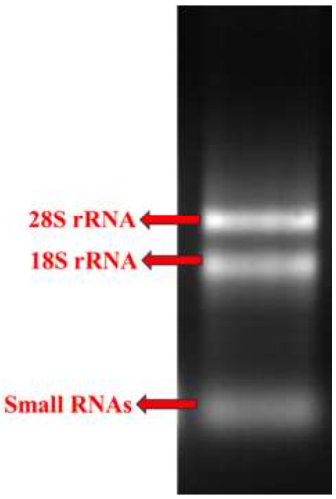

Fig. 3 : Total RNA isolated from adult of *S. frugiperda*

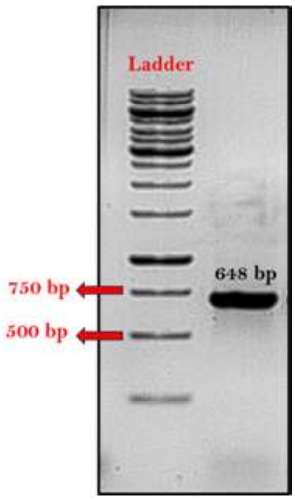

Fig. 4 : PCR amplification of mtCoI internal control gene from *S. frugiperda*

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PCR Amplification of *Spodoptera frugiperda white* Gene

PCR amplification of the *white* gene coding sequence (CDS) using gene-specific primers was confirmed through electrophoresis on a 1 per cent agarose gel, revealing an amplicon with a length of 1290 base pairs (Fig. 5). Subsequently, the gel was used to extract the band and its quantity was determined using a Nano drop spectrophotometer (Nanodrop Lite, Thermo Scientific, USA), resulting in a concentration of 53.4 ng/µl. This eluted product was then employed for the cloning process. The isolated plasmids were assessed *via* electrophoresis on a 1 per cent agarose gel, alongside a reference control DNA plasmid, to observe a shift in band size. In all the clones, the presence of the insert was confirmed, as indicated by the elevated band sizes compared to the reference plasmid (Fig. 6).

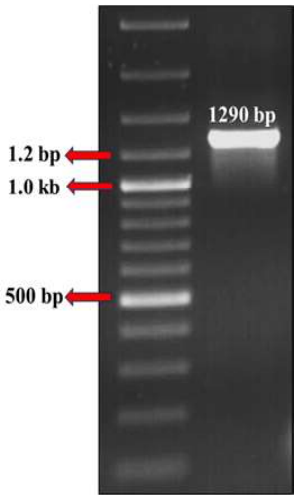

Fig. 5 : PCR amplification of *S. frugiperda* white gene CDS

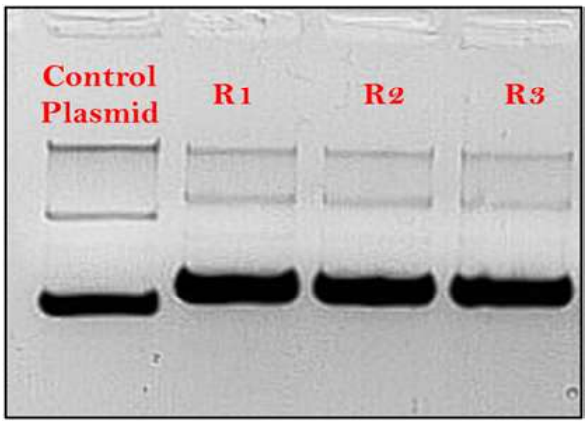

Fig. 6 : white gene clones compared with control plasmid R- Replication

Cloning and *Characterization of White* Gene

The identity of the cloned *white* gene sequence was confirmed by Nucleotide BLAST search in the NCBI database, which showed 97.61 per cent identity with the *S. frugiperda* white gene. Tamura-3-Parameter with a discrete gamma distribution (T92 + G) was identified as the best model for evolutionary tree construction. white sequences were grouped in three distinct evolutionary branches with 100 per cent bootstrap support (Fig. 7). white gene sequences of *S. frugiperda* and *S. litura* were grouped together in a single clade, as both species belong to the same genus. Alignment of the putative amino acid sequence derived from *S. frugiperda* cDNA sequence with other insect white peptide sequences and phylogenetic analysis verified the authenticity of the *S. frugiperda* white sequence.

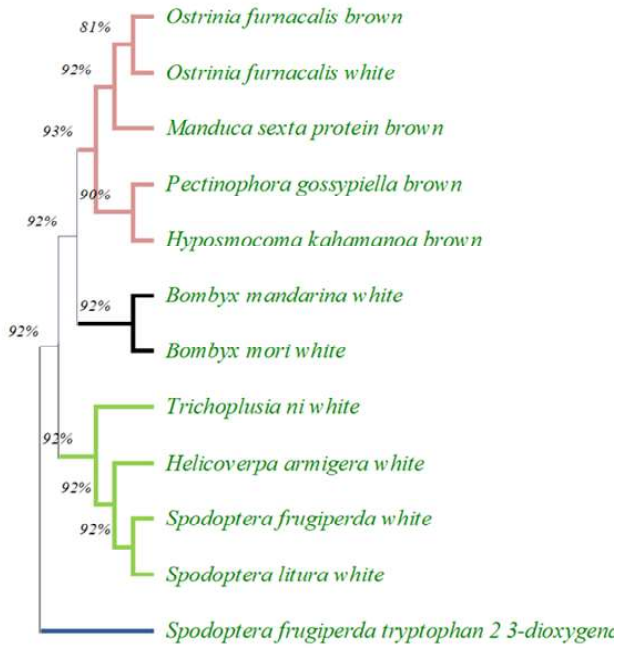

Fig. 7 : Phylogenetic tree of FAW white gene

In vitro Restriction Assay

The in *vitro* restriction was clearly observed in only two guides namely g528 and g598. The restriction of the target sgRNA site-containing PCR product was confirmed by visualizing the restricted fragments at 776 and 514 bp; 706 and 584 bp for g528 and g598 respectively. However, the guide g283 did not

provided the proper restriction however a faint band was noticed at 1022 and 268 bp indicating the inefficient restriction (Fig. 8) Further the free energy (ΔG) of self-folding potential for g283, g528 and g598 was found to be -2.70, -1.80 and -1.40 respectively. The development of secondary structure in side the gRNA portion of the sgRNA can also affect the functionality of the sgRNA. It has been demonstrated that non-functional gRNA has a higher negative value of ΔG for self-folding potential than functional gRNA, indicating its ability to form secondary structure with in itself (Wong *et al*., 2015 and Jensen *et al*., 2017). As a result, in a secondary structure study, a gRNA will have a greater chance of forming secondary structure if it has a more negative ΔG value (Fig. 2). This will make the gRNA less accessible for base pairing with the target sequence. This negatively low self-folding free energy of g283, being less than -2.00, reflected on the efficiency of invitro restriction.

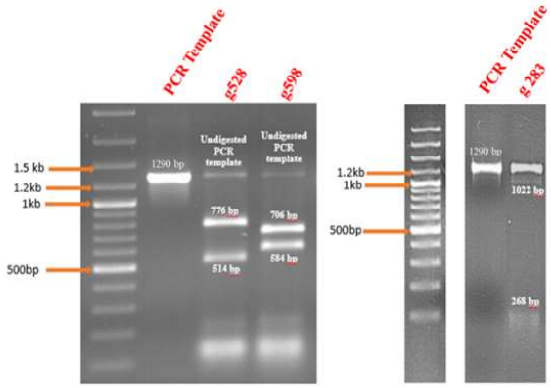

Fig. 8 : In vitro restriction assay of FAW white gene

The CRISPR-Cas system is a widely used toolkit for genome editing in various organisms. Cas9 is directed to a specific genomic region using a small guide RNA (sgRNA). Cas9 efficiency can vary among different sgRNAs, impacting the mutagenesis rate. While existing web-based tools predict multiple candidate sgRNAs, not all of them exhibit the same level of efficiency. To address this, we have demonstrated an in vitro screening method to identify the most suitable sgRNA, ensuring an efficient double-stranded break at the desired genomic target. This method allows for the selection of the optimal sgRNA before introducing genome editing reagents into live cells.

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