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Cloning and *In Vitro* Restriction Analysis of the Sex Peptide Receptor Gene in Fall Armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae)

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Abstract

The polyphagous pest *Spodoptera frugiperda* (J. E. Smith) damage a number of important crops. The fertility and the reproductive rate of *S. frugiperda* can be altered by the CRISPR/Cas9 mediated mutagenesis of target genes, which produces a cascade of frame-shift mutations. The gene mediating reproductive behaviour in adult moths by suppressing the female receptivity in a variety of lepidopteran pests is the sex peptide receptor gene. PCR amplification and cloning of the *S. frugiperda* sex peptide receptor gene (1039 bp) was carried out. Utilizing CRISPOR, an online bioinformatics tool, SFSPR exons were used as input to create 'On target maximised and off target minimised sgRNA (20 bp)'. The *in vitro* restriction assay using designed sgRNA resulted in band size of 790 bp which is a release from the 1039 bp CDS which further verified the designed sgRNA's efficiency.

Keywords : *Spodoptera frugiperda*, CRISPR/Cas9, Single guide RNA, SPR gene, *in vitro* restriction

Spodoptera frugiperda (J.E. Smith, 1797) is a serious lepidopteran pest belonging to the Noctuidae family. As a larva it prefers to feed on leaves and young shoots, particularly buds and develops into a chewer of plant tissue (He *et al*., 2020). Due to its feeding habits and polyphagous nature, it is a devastating pest of crops in the Western Countries. Additionally, it prefers a wide range of host plants and possesses a great ability for adaptation and dissemination (Casmuz *et al*., 2010; Montezano *et al*., 2018 and Paredes-Sanchez *et al*., 2021).

S. frugiperda is regarded as a persistent pest in the Americas due to its behaviour in field and it has recently spread to Africa, India (Sharanabasappa *et al*., 2018) and China. Year-round prevalence of *S. frugiperda*, resulting in economic loss of major food crops, which encourages the inappropriate use of chemical pesticides (in terms of the type of pesticide used, increases in recommended application doses, number of applications per season/year, time and rate of application), which has resulted in the development of insecticide resistance and unfavourable impact on the environment and people (Bolzan *et al*., 2019 and Lira *et al*., 2020). Owing to the negative consequences of conventional chemical management strategies on the environment, animals and people, scientists investigated the novel approaches in genetic regulation of target pests.

The CRISPR/Cas9 system is the ground-breaking tool with exceptional accuracy and efficiency that can be exploited to effectively manage polyphagous pest like *S. frugiperda*. Studies on the genetic regulation of the fall armyworm, *S. frugiperda* are scanty. In this regard, the *sex peptide receptor* (*SPR*) gene which

regulates the reproductive behaviour in adult moths can be a good candidate gene for the genetic control of *S. frugiperda*. Through employing CRISPR/Cas9 mediated genome editing of *SPR*, it is possible to alter the target insect's reproduction rate, which further aid in the development of appropriate genetic control for the target pest.

Effective sgRNA optimization is critical to the accomplishment of gene editing. The goal of the present work is to identify the *SPR* gene in *S. frugiperda* and validate the 'Off target minimized sgRNA' using an *in vitro* digestion assay to confirm the effectiveness of restriction of the target gene and the ribo nucleo protein (RNP) complex can be further proceeded for microinjection in the embryos of *S. frugiperda*.

Material and Methods

Mass Culturing of the *Spodoptera frugiperda*

The early instar larvae of *Spodoptera frugiperda* were reared individually on young maize leaves and the later instar were maintained on chickpea based diet. The insect culture was maintained under the controlled rearing environment of 25 ± 1 °C, 65 ± 5 per cent relative humidity with 14h:10h (L:D) photo period at the Division of Basic Sciences, ICAR-IIHR, Bengaluru, India. Adults were released in mating cages (30 x 30 x 30 cm), with glass on top and mesh netting covering three sides supplemented with a 10 per cent honey solution as immediate energy source upon emergence (Anu *et al*., 2024). Further experiments were conducted using insects that were acquired from this starting culture.

Identification of *Sex Peptide Receptor* (*SPR*) Gene and Designing Gene Specific Primers

The NCBI genome database was referred to retrieve the information about the *SPR* gene. Using ClustalW in BioEdit software (version 7.7.1), the multiple sequence alignment and the sequence similarity of the *SPR* gene of the *Spodoptera frugiperda* (*SFSPR*) with other insect species was discovered.

Total RNA Isolation and Complementary DNA (cDNA) Synthesis

The abdomen of the single adult female moth was dissected to isolate the total RNA. Using TRIsure™ reagent (BIOLINE), the complete RNA was isolated according to the manufacturer's instructions. On 1.5 per cent agarose gel, the RNA integrity was examined and it was then quantified using a Nano drop (Thermo Scientific, USA). Following the manufacturer's protocol, cDNA was synthesized from 2µg of total RNA using the Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA). Internal control gene mtCOI, was used to confirm the cDNA synthesis and was verified on 1 per cent agarose gel.

PCR Amplification of *SFSPR* Gene

The complementary DNA was diluted using autoclaved milliQ water in the ratio 1:10 and then utilized as a PCR template to amplify the complete coding region (CDS) of the *SFSPR* gene using the gene-specific primers (Table 1) and optimized PCR conditions (Table 2 and 3). PCR amplicon was resolved on 1 per cent agarose gel and purified using a Favorgen Biotech Corp GEL/PCR purification micro kit.

Table 1

Gene specific primers for *Spodoptera frugiperda* Sex Peptide Receptors gene

Primers	Sequences (5'-3')
SFSPR Forward Primer	GACATCACAGATGACATAA
SFSPR Reverse Primer	GTACTAGATACATAGACAGAG
SFSPRsg	GAAATTAATACGACTCACTATAGGGAACGTTGTACTAATGGCTA gttttagagctagaaatagc
CRISPR reverse	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTA GCCTTATTTTAACTTgctatttctagctctaaaac

Table 2

PCR components for amplification of *SFSPR* gene

Reagents	Volume	Final Concentration
10X LA PCR buffer (Mg^{+2} free)	2.5 µl	1X
dNTPs mix (2.5 mM)	4.0 µl	0.4mM
$MgCl_2$ (25 mM)	2.5 µl	2.5mM
Template (cDNA)	1.0 µl	100ng
SFSPR Forward Primer	1.0 µl	0.2 µM
SFSPR Reverse Primer	1.0 µl	0.2 µM
TaKaRa Taq	0.2 µl	1 unit/ µl
Sterile PCR water	12.8 µl	-
Total Volume	25 µl	

Ligation of *SFSPR* gene

The general-purpose cloning vector pTZ57R/T (Thermo Scientific, Lithuania) was ligated with the eluted *SFSPR* gene amplicon. The total reaction volume of 20 µl includes 1X Ligase buffer, pTZ57R/T vector, T4 DNA ligase and *SFSPR* gene (Table 4). The vector's primary characteristics are the selection of blue and white colonies, the integration of M13 primers for sequencing and the presence of an ampicillin resistance marker gene. White colonies denote recombinant colonies, while blue colonies indicate non-recombinant colonies. The blue-white selection of the colony allowed recombinants to be identified from non-recombinants (Pradhan *et al*., 2023).

Table 4

Ligation of *SFSPR* gene into pTZ57R/T cloning vector

Reagents	Volume	Final Concentration
MilliQ water	9.0 µl	-
5X Ligase buffer	3.0 µl	1X
pTZ57R/T vector	1.0 µl	55 ng
SFSPR gene template	1.0 µl	158 ng
T4 DNA ligase	1.0 µl	1 unit/ µl
Total Volume	15 µl	

Table 3

PCR conditions for *SFSPR* gene amplification

Steps	Temperature (°C)	Duration	Cycles
Initial Denaturation	95	2 minutes	1
Denaturation	98	10 seconds	35
Annealing	56	10 seconds	
Extension	68	45 seconds	
Final extension	68	10 minutes	1
Store	4	∞	

Cloning and Transformation

A chemically competent strain of *Escherichia coli*, DH5α, was used to clone the ligated products. The transformed *E. coli* cells were spread on Luria Bertani (LB) agar plates supplemented with IPTG (100 mM), X-gal (20 mg/ml) and ampicillin (100 µg/ml). Following an overnight incubation at 37 °C, the plates were screened for blue and white colonies. All of the positive colonies (white colonies) - those that harboured the insert - were then inoculated with ampicillin containing LB broth and incubated at 200 rpm for 37 °C.

Isolation of Plasmid and Sequencing

Using the Plasmid isolation kit (Thermo Fisher Scientific) and the manufacturer's instructions, plasmids were harvested from the transformed white colonies cultured in Luria Bertani (LB) broth. The recombinant plasmids were verified on 1 per cent agarose gel electrophoresis with control plasmid. Using M13 universal primers, sequencing of the aforementioned clones was done for three biological replicates using Sanger sequencing.

Analysis of Sequencing Outcomes and Interpretation of Data

In order to compare the *S. frugiperda SPR* sequence with other homologous sequences retrieved from the NCBI database, alignment was carried out using the default parameters of ClustalW (Version 7.7.1) in BioEdit software. The translation tool on SnapGene 7.2.0 was used to infer the target genes' amino acid sequence. Furthermore, MEGA 11 (Version 11.0.13)

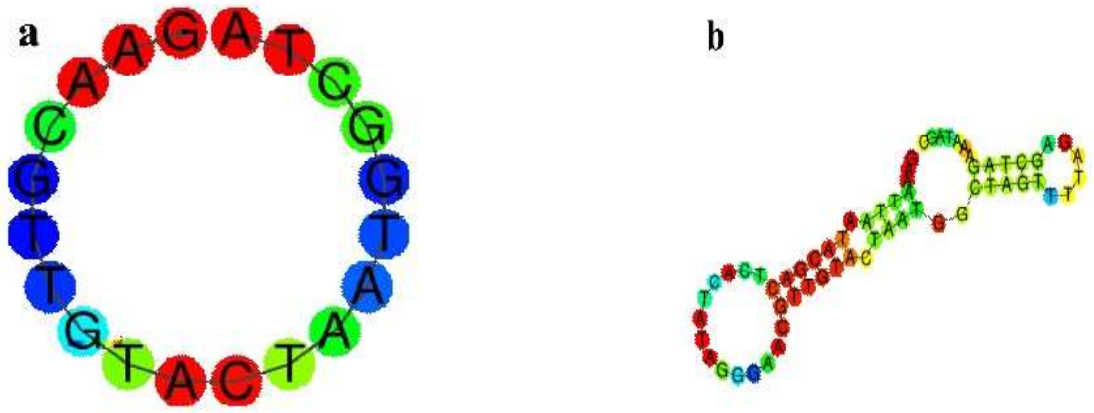

Fig. 1 : Structure of sgRNA for *SFSPR* gene (a) SFSPRsg (b) SFSPRSg casette

was used to create a phylogenetic tree using the greatest likelihood approach. To guarantee accuracy and resilience, the tree construction process underwent 1000 bootstrap replications and thorough gap deletion.

Identification of Single Guide RNA (sgRNA)

The exon sequence of *SFSPR* gene was submitted to the CRISPOR tool (http://crispor.tefor.net/) (Hwang *et al*., 2013) and using the criteria: 5' - GGN - 18nts NGG - 3', the off-target minimized and on-target maximized sgRNA was created. The sgRNA target site was found in exon 2 (5'- GAACGTTGT ACTAATGGCTA-3') (Fig. 1). Additionally, a sgRNA reverse complement was created (Bhargava *et al*., 2024). RNA fold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNA WebSuite/RNAfold.cgi) was used to further verify the secondary structure of the chosen sgRNA.

***In vitro* Digestion Assay**

To confirm the effectiveness of sgRNAs, *SFSPR* CDS must be restricted *in vitro* using SpCas9 and sgRNA (SFSPRsg). For this experiment, all of the reagents were procured from New England Bio Lab. The reaction mixture consisted of 30nM of *SFSPR* template, 100nM of EnGen Spy Cas9 NLS enzyme, 1× NEB r3.1 buffer and an *in vitro* produced SFSPRsg cassette (Table 5). Following a 30-minute incubation period at 25°C, the *in vitro* digested products was verified on 1.5 per cent agarose gel electrophoresis.

Table 5

Components for *in vitro* digestion assay

Reagents	Volume	Final Concentration
MilliQ water	62 µl	-
5X Transcription buffer	30 µl	1X
NTP Mix	30 µl	10 mM
SFSPRsg template	20 µl	5 µg
RNase Inhibitor	3.0 µl	40 unit/ µl
T7 RNA Polymerase	5.0 µl	20 unit/ µl
Total Volume	150 µl	

Results and Discussion

Total RNA Isolation and Complementary DNA (cDNA) Synthesis

Agarose gel electrophoresis (1.5%) was used to verify the integrity of the total RNA extracted from the female abdomen (Fig. 2), where the RNA content was verified using Nanodrop (Thermo Scientific, USA) and concentration was found to be 2348 ng/µl with the A260/280 value 2.01. By amplifying the mtCOI internal control gene *via.,* PCR, the cDNA was confirmed.

PCR Amplification of *SFSPR* Gene

By using gene-specific primers, the *SFSPR* gene CDS was amplified in polymerase chain reaction (PCR) using a thermo cycler (ABI Applied Biosystems), the results were visualized using 1 per

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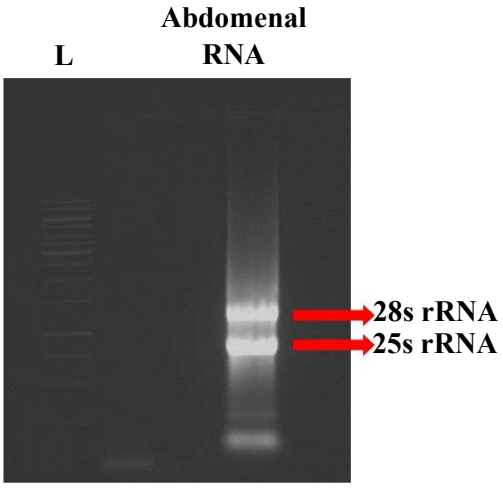

Fig. 2 : Total RNA isolation from abdomen of female *Spodoptera frugiperda*

cent agarose gel, which showed amplified band size of 1039 bp (Fig. 3). The band was excised and eluted from the gel and quantified using a Nanodrop (Thermo Scientific, USA), the concentration recorded was 158 ng/µl with the A260/280 value 1.82. The eluted product was subsequently utilized for cloning.

Cloning and Transformation

Cloning was done using gel eluted *SFSPR* gene product. Following the Blue-white screening the positive colonies were processed to harvest the recombinant plasmid. The isolated plasmids were analyzed on 1 per cent agarose gel alongside control reference plasmid to observe any shift in band size to higher size. All clones showed the presence of the insert, as evidenced by the higher band sizes compared to the control reference plasmid (Fig. 4).

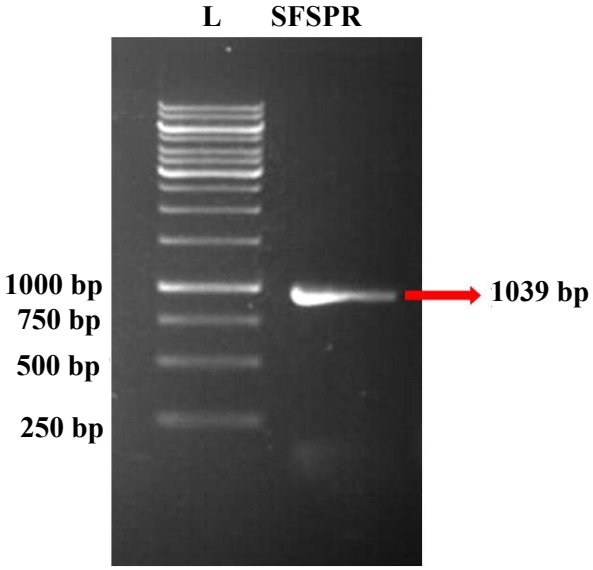

Fig. 3 : PCR amplification of *Spodoptera frugiperda SPR* gene

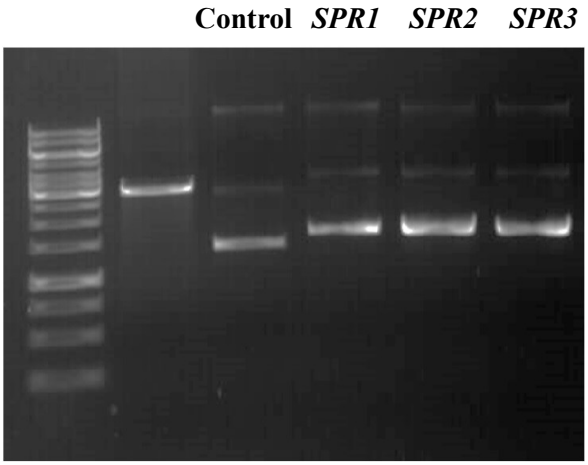

Fig. 4 : Control plasmid compared with *Spodoptera frugiperda SFSPR* gene clones

The cloned sequences were run through to BLAST at the NCBI, it was found that there was 100.00 per cent sequence similarity with the predicted *SPR* gene sequence in *Spodoptera frugiperda*, accession number XM_050699791.1. Additionally, it demonstrated a high degree of sequence similarity with 100 per cent query coverage for the anticipated *SFSPR* gene sequences of *Spodoptera litura* (XM_022973010) (91.22%), *Helicoverpa armigera* (XM_064042429) (82.05%) and *H. zea* (XM_047181648) (81.66%) (Fig. 5). This demonstrated that the expected domains are shared by all related lepidoptera.

Homology Modelling of SFSPR Protein

Using SWISS MODEL (http://swissmodel.expasy.org/), a three-dimensional (3D) model of the *S. frugiperda* SFSPR protein was produced. With the help of the UCSF Chimera software version 1.7, which can be accessed at https://www.cgl.ucSf.edu/chimera/ , the 3D protein structure was further examined (Anu *et al*., 2024). This allowed for a detailed analysis of the structural characteristics and interactions within

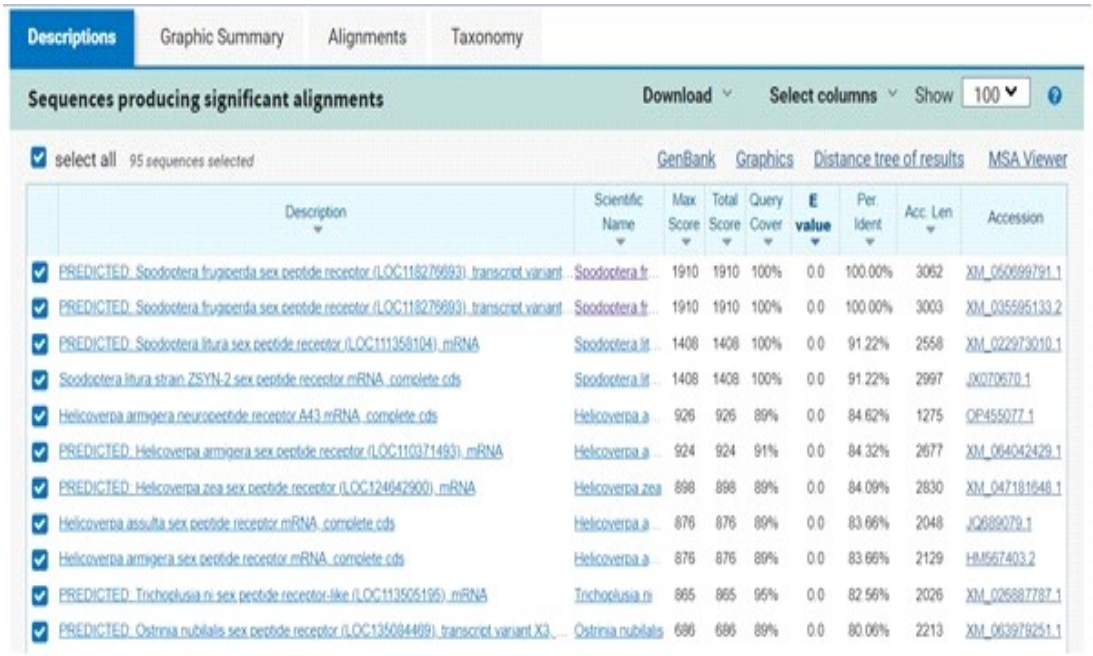

Fig. 5 : Sequence similarity of the *SPR* gene among related lepidoptera

the protein. The SFSPRsg location is indicated in lime color in the homology model (Fig. 6).

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

The sgRNA cassette (T7 promoter + SFSPRsg + scaffold) was amplified in PCR thermo cycler using complementary SFSPRsg scaffold sequence. Thermo Fisher Scientific's MEGAscript™ T7 Transcription Kit was utilizsed to execute *in vitro* sgRNA transcription in accordance with the manufacturer's instructions (Table 6). Following *in vitro* transcription, the sgRNA was purified appropriately. The concentration of *in vitro* transcribed sgRNA, SFSPRsg was 1876 ng/µl.

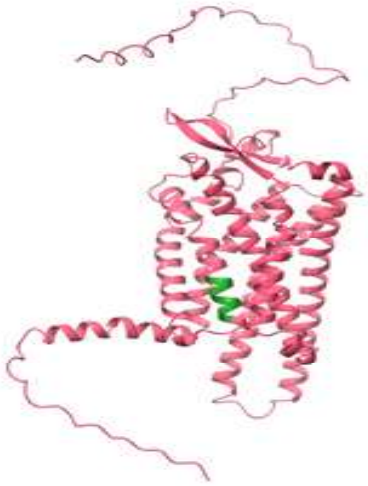

Fig. 6 : Three dimensional protein structure of *S. frugiperda* SPR. The lime color in the protein structure represents the SFSPRsg sequence

Table 6

***In vitro* transcription reaction components for sgRNA synthesis**

Reagents	Volume	Final Concentration
MilliQ water	62 µl	-
5X Transcription buffer	30 µl	1X
NTP Mix	30 µl	10 mM
SFSPRsg template	20 µl	5 µg
Ribolock RNase Inhibitor	3.0 µl	40 unit/ µl
T7 RNA Polymerase	5.0 µl	20 unit/ µl
Total Volume	150 µl	

In vitro Restriction Assay

An *in vitro* restriction experiment was used to confirm that the Cas9 protein and sgRNA could cleave the double stranded DNA in the target location and the results were visualized on a 2 per cent

agarose gel. The first lane had one kb ladder, then an *SFSPR* gene CDS and the third lane has *SFSPR* gene CDS + Cas9 + SFSPRsg. In the second lane of the agarose gel visualization, there was only one distinct, 1039 bp firm band (*SFSPR* gene CDS). The band fragments, measuring 1039 bp and 790 bp (Fig. 7), were cut bands released from the 1039 bp.

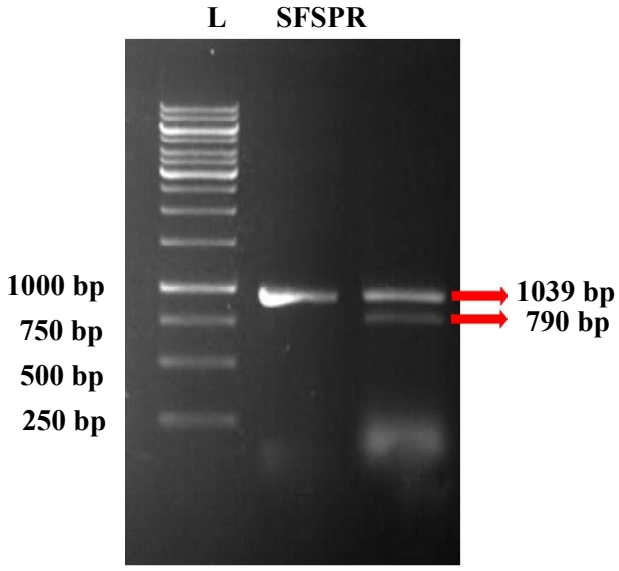

Fig. 7 : *In vitro* restriction assay of SFSPRsg

The *SFSPR* gene was expressed in adults of *Spodoptera litura* (Li *et al*., 2014) and *S. frugiperda.* The silencing of *SPR* gene in *S. litura* mediates changes in the female post-mating behavior which resulted in failed response of female towards male secretion and the mutant female continue to show virgin behaviors thus affecting the reproduction rate with very few number of eggs (Li *et al*., 2014) without affecting the male competitiveness. Similarly, in *Bactrocera dorsalis* silencing of *SPR* gene resulted in reduction in the egg laying capacity of the mutant females and greatly impacted the eclosion rate of their offspring (Zheng *et al*., 2015).

The current study characterized the *SPR* gene in *S. frugiperda* through cloning and sequencing and leads to unique avenue for further functional analysis in the adults. The sgRNAs' efficacy for restricting the target gene was validated by an *in vitro* restriction experiment. Due to its evolutionary conservation among lepidopterans, SFSPR can be a promising candidate target for the biorational/ genetic pest management of related lepidopteran pests. Furthermore, microinjection, edit characterization and mating studies can be used to understand its functionality and behaviour in mutant adult.

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