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Pheno-molecular confirmation and protein structure analysis of Wild Vs CRISPR white mutant *Bactrocera dorsalis*

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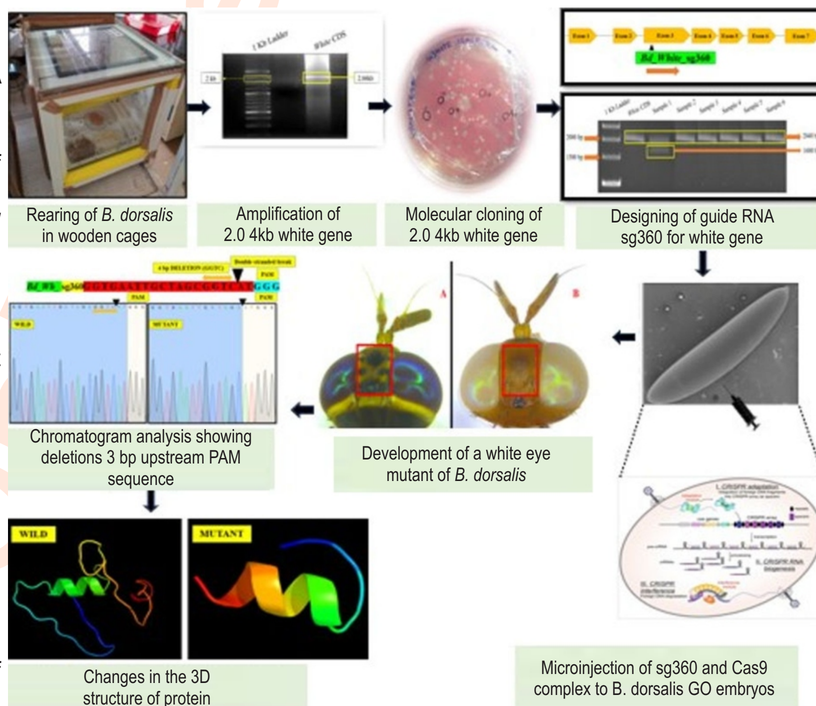
Abstract

Aim: This investigation aimed for embryonic G0 microinjection of ribonucleoprotein (RNP) complex (sg360+Cas9) for restriction of white melanin synthesising gene in Bactrocera dorsalis followed byphenotypic and molecular confirmation and 2D and 3D protein structure comparison of wild Vs CRISPR white mutant.

Methodology: White gene responsible for melanin pigmentation was cloned followed by synthesis and in vitro restriction analysis of designed sgRNA (sg360). Microinjection was targeted in G0 embryosfollowed by phenotypic characterisation and molecular validation. Comparative analysis of amino acid sequences, 2D and 3D structure of wild Vs CRISPR white mutant was done using ClustalW and Phyre2 respectively.

Results: Microinjected RNP complex restricted white gene leading to generation of white eyed mutants. Amino acid alignments showed significant frame shift (valine substituted for tryptophan) and introduction of stop codons in the mutant hindering white protein production. Predicted 2D protein structure showed more α-helix in the mutant (72%) compared to the wild (22%). Similarly, β-sheets were nullified and major deletions in the3D protein structure were observedin mutant.

Interpretation: The study validated variation in amino acids alignments and 2D and 3D protein structure and conformation, leading to production of non-functional white protein in *B. dorsalis*.



Key words: *Bactrocera dorsalis*, CRISPR/Cas9, Microinjection, White protein

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Introduction

The most significant class of horticultural crop pests are tephritid fruit flies that inflict significant financial losses (De Meyer *et al.,* 2015). Their effects include severe losses in export markets as a result of quarantine limitations apart from direct yield losses due to fruit infestation (Tanga, 2015). There are 440 species in the genus Bactrocera (Liu *et al.,* 2019). They are especially invasive due of their characteristics as polyphagia, high fertility and greater mobility (Ekesi *et al.,* 2007). One of the major biosecurity concerns is the damage potential and corresponding yield losses caused by the pest B. dorsalis (Hendel) (Clarke *et al.,* 2005). The pest is highly significant in terms of its impact on horticultural ecosystem throughout South east Asia and the Pacific. The earliest known documentation of the pest occurred in 1912 in Kaohsiung, Taiwan, China (Wan *et al.,* 2012). Verghese *et al.* (2002) proposed that India, with its 200 species, accounts for 5% of the global fruit fly species. *B. dorsalis* has potential to inflict significant harm on over 250 varieties of vegetables and fruits that are cultivated for commercial purposes (Cheng *et al.,* 2014; Meng *et al.,* 2019; Zhu *et al.,* 2022). The pest *B. dorsalis* undergoes 3-5 generations annually under tropical climatic conditions (Chen and Ye, 2007).

Genetic control technologies have been advanced throughuse of genome editing tools such as ZFNs (Zinc finger nucleases), TALENs (Transcription activator-like effector nucleases), and RNAi (RNA interference). Nevertheless, an optimal and effective gene modification technology should possess rapid and efficient capabilities, while ensuring absence of any off-target mutations (Gaj *et al.,* 2013). Precision guided sterile insect techniques (pgSIT) is an enhanced version of the traditional sterile insect technique (SIT), in which CRISPR technology is employed to specifically target crucial genes. This method can be employed to manage significant agricultural crop pests and disease-transmitting vectors (Kandul *et al.,* 2019). The CRISPR/Cas9 system was initially identified as a prokaryotic adaptive immunity system. The mechanism involves the combination of mature CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) to form a single guide RNA (sgRNA). This sgRNA guides the Cas9 endonuclease to the target site by recognising a specific sequence called a protospacer-adjacent motif (PAM) (Deltcheva *et al.,* 2011).

Upon binding, Cas9 generates a double-stranded break in the DNA, which activates the cellular DNA repair system. This repair can occur through two pathways: the error-prone nonhomologous end-joining (NHEJ) pathway and the error-free homology-directed repair (HDR) pathway (Moon *et al.,* 2022). Insects rely on body colouration for both physiological and ecological functions. The white gene facilitates production of a component of an ATP-binding cassette (ABC) transporter. The white protein is incorporated to the pigment cells of compound eyes, ocelli, malpighian tubules and testis. The white protein also facilitates transportation of diverse biological substances, including second messengers, neurotransmitters, bioamines, and metabolic intermediates (Sullivan *et al.,* 1979). Thus, this investigation was aimed at restricting the function of white gene in *B. dorsalis* utilising CRISPR/Cas9 as a genome editing tool. By leveraging CRISPR/Cas9, a precise and efficient tool for targeted gene editing, the research sought to specifically induce mutations in the white gene locus, thereby examining the potential of genetic manipulation to influence pest phenotypes. The white gene is involved in regulating eye pigmentation, and its disruption is often used as a marker for genetic manipulation in insects. This disruption was further aimed to be validated by phenotypic and genotypic characterisation of mutant flies. Protein level transformations were assayed to verify the efficacy and reproducibility of mutations in *B. dorsalis.* The targeted disruption of this gene could affect various physiological processes in *B. dorsalis,* such as visual capabilities, behaviour or reproductive fitness, which may contribute to novel pest control strategies. Ultimately, this approach aims to explore the potential of gene editing for reducing pest populations through genetic alterations, offering an alternative to traditional chemical control methods in pest management.

Materials and Methods

Insect maintenance: *B. dorsalis* laboratory population has been established in ICAR- IIHR, Hesaraghatta, Bengaluru (13.13°N, 77.49°E). Since mango is a costly and seasonal host, ripe banana fruits (cv Ney Poovan, Yelakki) were used for larval feeding and as oviposition substrate. By evaluating morphological characteristics and DNA-barcoding of the mitochondrial COI gene, the identity of the insect was verified. B. dorsalis was cultured in cuboidal chambers measuring 30 x 30 x 30 cm, bound by a wire mesh on oneside, glass on the threesides, a cloth which has an opening to handle flies on the other one side; and a wooden base. The culture room environment was maintained at 14 hour light-to-dark photoperiod and controlled temperature and humidity levels (25±1°C and 75±1%). Yeast and sugar hydrolysate powder in the ratio of 1:1 was given in a cap for adult flies to feed, along with cotton which had been soaked in a nutrient mixture containing distilled water and A to Z multivitamin syrup in proper proportions (Rajan *et al.,* 2023).

Molecular cloning of *B. dorsalis* white gene: Five adults from the *B. dorsalis* laboratory population were used to extract the total RNA, using "RNAiso Plus (Takara Bio Inc.) as per manufacturers' instructions. Using NanoDrop (Thermo Fisher Scientific), the RNA's quality was assessed, and 1.5% agarose gel electrophoresis was carried out to confirm the RNA's integrity. One (1µg) of extracted total RNA was converted into single-stranded cDNA by utilizing the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Using the PCR, the overall coding region" was amplified using white gene-specific primers (Forward primer: GTTGTACGCAAGGAAACGG, Reverse primer: CTCTTGTCATCTGCCAGTTC). The cycling conditions included two minutes of initial denaturation at 95°C, 40 sec of annealing at 55°C, 2 min of extension at 68°C, and 10 min of final extension at 68°C. The PCR amplicon which hadbeen cloned to the *pTZ57R/T* cloning vector (Thermo Fisher Scientific)

was gel-purified by utilizing the Nucleo Spin Extract II kit (Machery Nagel). By utilizing M13 universal forward as well as reverse primers, isolated plasmids "from transformed E. coli DH5-α colonies were subjected to Sanger sequencing (ABI prism® 3730 XL DNA Analyzer; Medauxin) to verify gene identity. Using 1000 bootstrap repetitions, ClustalW multiple alignments of the attained white gene sequences with database reference gene sequence was carried out in BioEdit (version 7.2.6.1).

Preparation of sgRNA, sgRNA hybridization and PCR amplification of sgRNA cassette: The sgRNAs were designed for the white gene using the online design tool of CRISPR, CHOPCHOP V3 (Montague *et al.,* 2014). The gRNA region was identified from functional domain of the concerned protein. Off target minimized guide RNA sequence, in exon 3 (5'-GGTGAATTGCTAGCGGTCATGGG -3') was selected for white gene by performing NCBI-BLAST to check off-target effect. Reverse complement of the gRNA was also designed. The gRNA and its reverse complement were hybridised (Thermo Scientific sgRNA hybridization kit). Forthe *in-vitro* single guide RNA synthesis,white gene specific primers were used for the first PCR reaction and hybridization of the oligonucleotide of primers of sgRNAs was done. Vector, *pBSK* was digested by using restriction endonuclease enzyme, Hind III and then the sgRNAs were ligated to the restriction site. Cloning and sequencing was done to confirm the ligated sgRNAs.

***In-vitro* transcription of sgRNA:** The amplified product was eluted from gel by utilizing the NucleoSpin Extract II kit (Machery Nagel) following the protocol of the manufacturer. Two micrograms were utilized as template for an *in-vitro* transcription reaction, which was then treated with DNase1 (Thermo Fisher Scientific) and then incubated at 37°C for one hour after being treated with 5X transcription buffer, T7 RNA polymerase (30U), 10mM NTP mix, and RiboLock RNase inhibitor (50U) in a 150 µl total reaction volume. The resultant *in-vitro* transcribed sgRNA was hybridised, purified and quantified utilizing Nano Drop (Thermo Fisher Scientific) and NEB (New England Bio Lab)'s Monarch RNA Clean Up Kit.

***In-vitro* restriction assay:** To evaluate restriction effectiveness of the designed sgRNAs, an *in-vitro* restriction test was performed. NEB r3.1 buffer (10X), white gene-specific CDS, 30 nM of EnGen Spy Cas9 NLS enzyme (New England Bio Lab), 30 nM of *in-vitro* transcribed sgRNAs (sg360), and 5 mM of KCl were used to set up a reaction mixture in a 20 µl reaction volume. After assembling the reaction mixture without the white CDS on ice, it was incubated for a span of 30 min at 25°C in a water bath. The combined mixture was incubated for 1hr at 25°C in a water bath after adding white CDS. The identical mixture compositionminus the sgRNAs was used to set up the negative control. The entire reaction mixture which was loaded onto a 1.5 percent agarose gel, after a one-hour incubation period. Different-sized digested products were produced by the reaction mixture containing the white gene-specific CDS, depending on the sgRNA cut site position in the white CDS. Using specifically designed sgRNAs, the digested size of the band had been compared to the anticipated size of the band of digestion from the Cas9 cut site to the ends of the white CDS. The negative control showed no signs of digestion.

Microinjection: The injection was performed in the middle region of the egg, or slightly towards the posterior pole, using a total of 4-6 pulses. Precautions were taken to avoid leakage of cytoplasm from the egg. To avoid drying out and enhance hatching rate, olive oil was applied to the eggs. The entire experiment was conducted within one hour of egg laying, at an average temperature of 25ºC.The G0 adults so obtained were used for phenotypic and genotypic examination.

Phenotypic analysis: In the CRISPR/Cas9 genome editing program,phenotype is the main confirmation of a successful alteration. The flies that emerged were examined under a stereo binocular microscope for the purpose of studying its physical characteristics, and images were captured using a Leica microscope. The flies exhibiting mutant phenotype were utilised for the extraction of genomic DNA.

Molecular validation: Genomic DNA was isolated from wild and white-eyed mutants. Specific primers were designed to amplify the region of genomic DNA (using similar PCR conditions as mentioned earlier) where CRISPR edit was expected from both wild and mutant flies. The difference in genomic sequences of this specific region in mutant flies as compared to the wildtype would confirm the occurrence of a successful edit using CRISPR. Cloning and sequencing of the edited region of the pigmentation gene, white was done. Cloned sequences were aligned using nucleotide alignment tool, Bio Edit along with the white gene of control fly. The alignment sequences were analysed for knock out or indel mutations.

Amino acid alignments and protein structure prediction: Further, the sequences were trimmed and subjected to amino acid alignments using Clustal Omega (V1.2.4.) to predict the difference in amino acid sequences of the mutant fly as compared to the wildtype. Furthermore, the 2D structure prediction was performed to ascertain the anticipated conformation, protein structure, and patterns of helices and sheets within the protein. This prediction was created usingPhyre2 program. The 3D protein structure prediction was also done subsequently. A comparison was made between the wild and mutant fly protein structures to analyse the differences.

Results and Discussion

NCBI BLAST showed 98.66 per cent similarity of the cloned sequences with predicted *B. dorsalis* protein white (LOC105224216) mRNA. It also 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

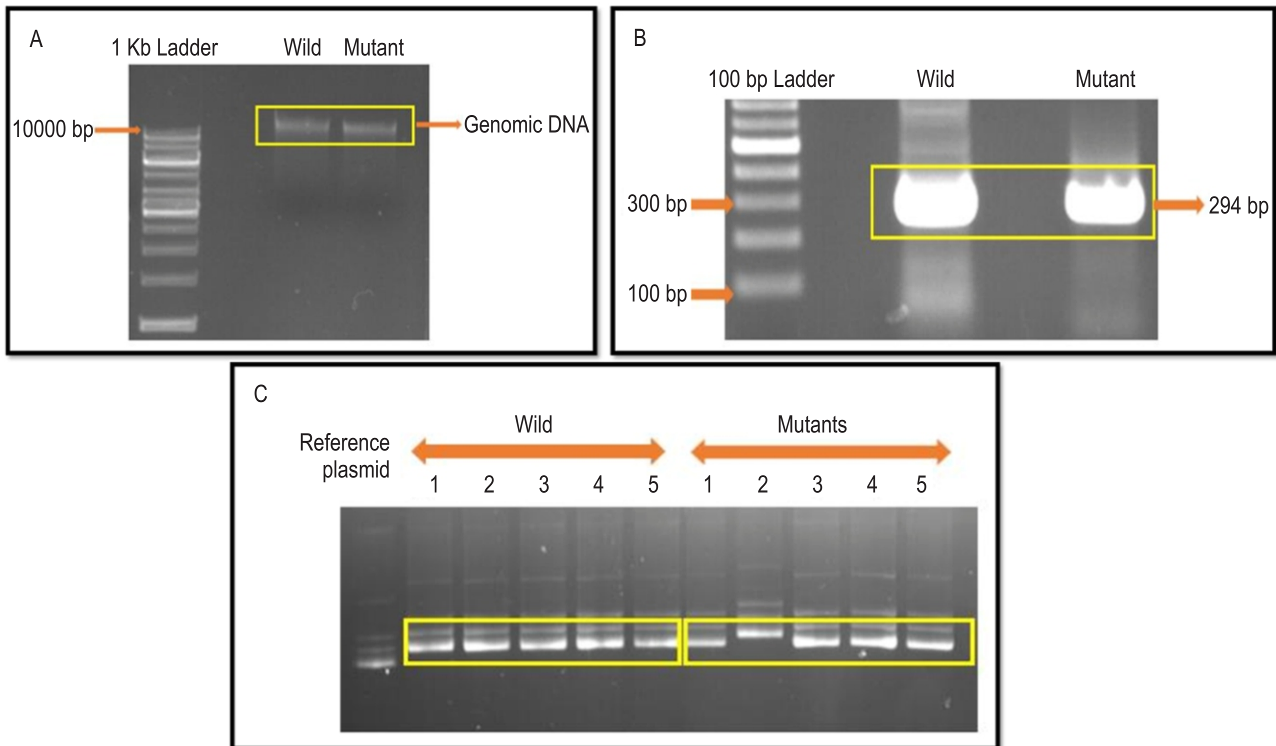

Fig. 1: (A) Genomic DNA isolated from wild and white-eyed mutant flies; (B) PCR amplification of specific region where CRISPR edit was expected from genomic DNA; (C) B. dorsalis wild and mutant clones for CRISPR edit specific region sent for sequencing.

related tephritid fruit flies. The *in-vitro* digestion of the gene was performed following the protocol of New England Biolab® Inco. In thefirst experiment, a single guide RNA (sg360) was taken which was used to test six samples with varied components of CRISPR/ Cas9 system. The samples were: white CDS+sg 360+Cas9, white CDS+Cas9+ no sg360,Cas9+sg360+ no white CDS, white CDS + sg360+ no cas9, white CDS + no Cas9 + no sg360 and sg360+no cas9+no white CDS. Sample one (white CDS +sg360+Cas9) gave significant results where Cas9 enzyme digested the white gene DNA. The digested strand of white gene DNA was 1680 bp. The other samples did not show any significant results because the components required for *in-vitro* digestion were altered (Fig.1).

CRISPR/Cas9 based genome editing has led to efficient alteration of genomic sequences within the protein coding region of white gene in *B. dorsalis.* This in turn led to many morphological and physiological changes within the organism. The mutant flies showed a white-eyed phenotype as compared to metallic green eye colour of the wild flies. The contrasting differences in the eye colour is attributed to the loss of melanin pigments in the mutant owing to deletion of bases and frameshift mutations introducing stop codon within the white gene sequence or production of non-functional white protein in the organism. The wild as well as the mutant fly were picturised (Fig. 2). The enzyme arylalkyl amine N-acetyl transferase (aaNAT) is responsible for converting dopamine into N-acetyl dopamine, which is a significant component of colourless or translucent cuticles (Bai *et al.,* 2019). The white gene expresses a protein belonging to the ATP-binding cassette (ABC) transporter superfamily. This protein forms a heterodimer with either Brown or Scarlet proteins to facilitate transport of the eye pigment precursors guanine or tryptophan, respectively (Dermauw and Leeuwen, 2014).

The presence of tryptophan, which is a precursor of serotonin, and guanine, which is a precursor of biopterin, a co-factor involved in serotonin and dopamine synthesis, indicates that the white protein is connected to levels and distribution of the neurotransmitters serotonin, dopamine and histamine in the brain (Borycz *et al.,* 2008). Furthermore, white blood cells facilitate transportation of secondary messengers and several small compounds (Sullivan *et al.,* 1979; Evans *et al.,* 2008). Disruption of white gene resulted in manifestation of white-eye colour and variations in pigmentation in the head and thoracic region. The mutant fly exhibited a reduction in head and thorax pigmentation relative to the wild fly. White-eye phenotypes were seen in *B. dorsalis* (Bai *et al.,* 2019) and *B. tryoni* (Choo *et al.,* 2018) as a result of CRISPR/Cas9 driven alterations which affected eye colour. The white-eyed mutants also exhibited hazy and fading mutant head spots, which were less well-defined and delimited compared to the prominent head spots of the wild phenotypic. Bai *et al.* (2019) saw similar phenotypic effects when they used

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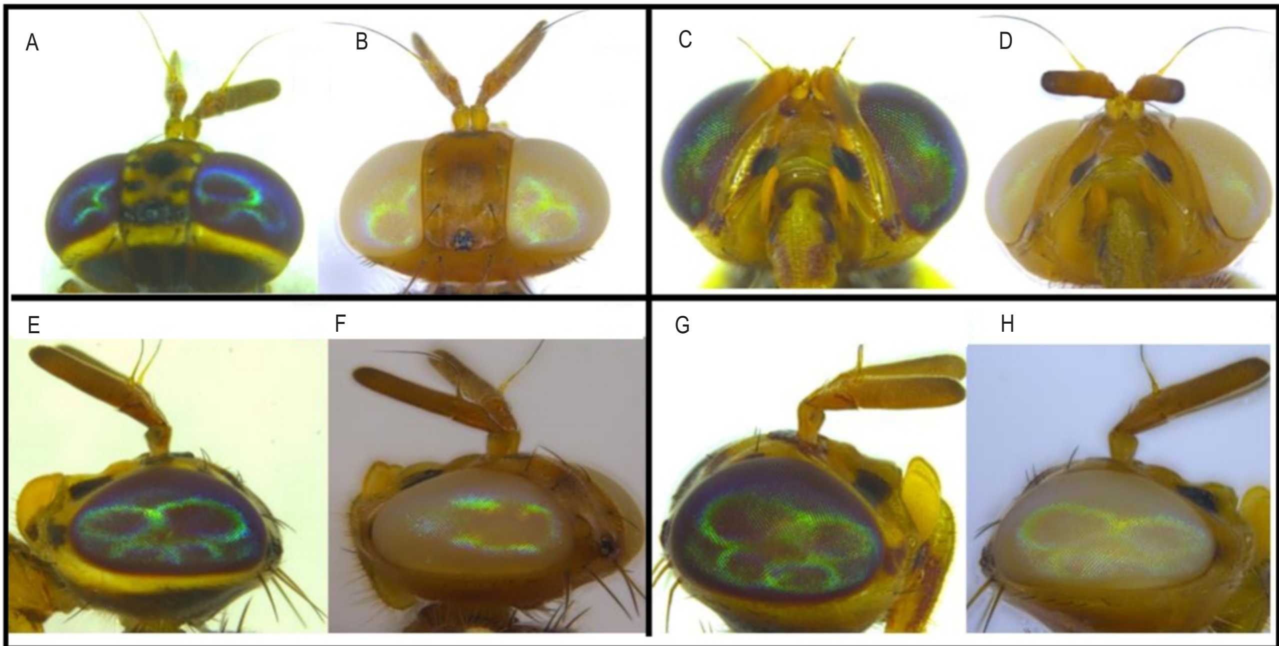

Fig. 2: Comparative view of eye phenotype of G_0 *B. dorsalis* (A) Dorsal view of wild; (B) Dorsal view of white mutant; (C) Ventral view of wild; (D) Ventral view of white mutant; (E) Left lateral view of wild; (F) Left lateral view of white mutant; (G) Right lateral view of wild; (H) Right lateral view of white mutant).

CRISPR/Cas9 to knock down the white protein in *B. dorsalis*.

Upon alignment of cloned sequences in Bio Edit software, it was observed that out of the total four mutant sequences, three mutant colonies (1, 4 and 5) showed a double stranded break and four base pairs (GGTC) deletion, 2 base pairs upstream of PAM sequence towards 5' end. Also, it was observed that in these clones PAM sequence was intact.Mutant colony 3, showed nearly 14 base pairs deletion. The PAM sequence was also lost in this mutant (Fig. 3A). Thus, it was hypothesised that different types of edits might have occurred in two different alleles of the gene. But, in TA cloning each single colony represent only a single allele of the gene cloned. Thus, it was concluded that the type of edit was different in the two alleles cloned. This may be a possible reason for having 2 different types of edit with variable number of deletions in and around the PAM sequence. Further, though this colony showed up a more efficient edit, but was not considered reliable because the results were not obtained in replicates. Analysis of chromatogram of the wild and mutant fly also revealed that there was a double stranded break and deletion of 4 base pairs, 2 base pairs upstream PAM sequence. Change in the chromatogram images of mutant fly with respect to the wild fly also provides satisfactory evidence of occurrence of edit. The above results taken together suggest that sg360 bounded to Cas9 complex could generate expected deletion of bases (base deletion around PAM sequence) at the target site. Thus, this study underlines the practical utility CRISPR/Cas9 based genome editing for genetic population control of serious agricultural pest as *B. dorsalis.* Further, the sequences were trimmed and subjected to amino acid alignments using Clustal Omega. The trimmed sequences markedan edit (region of 4 bp deletion) nearly 36 bp after the starting point of translation. This means that there was a change in the amino acid sequence and frame shift after 12 amino acids from the point of initiation of translation in the trimmed sequences, because, codons are triplets. Similar results were obtained in the alignment of amino acid sequences where substitution of valine for tryptophan was noticed (Fig. 3B).

Due to a change in amino acid sequences 12 amino acids away from the initial point of translation and frame shift, there was introduction of many stop codons (marked by *) within the amino acid sequence of the mutant organism. This could be a reason that justifies the termination of translation process. Consequently, it can be hypothesised that there was no production of functional white protein in the mutant organism leading to loss of function of white gene in the organism and development of edited phenotype. Thus, protein analysis confirmed that the differences in the amino acid sequences of mutant fly and the wild fly led to failure of metallic blue- green eye phenotype in the wild and production of white (colourless) eye phenotype in mutant as the protein may have been truncated or non-functional. This research thus cites a perfect example for CRISPR based knock out of gene function in *B. dorsalis.*

Moreover, the protein's 2D structure was utilised to ascertain the arrangement of helices and sheets within the

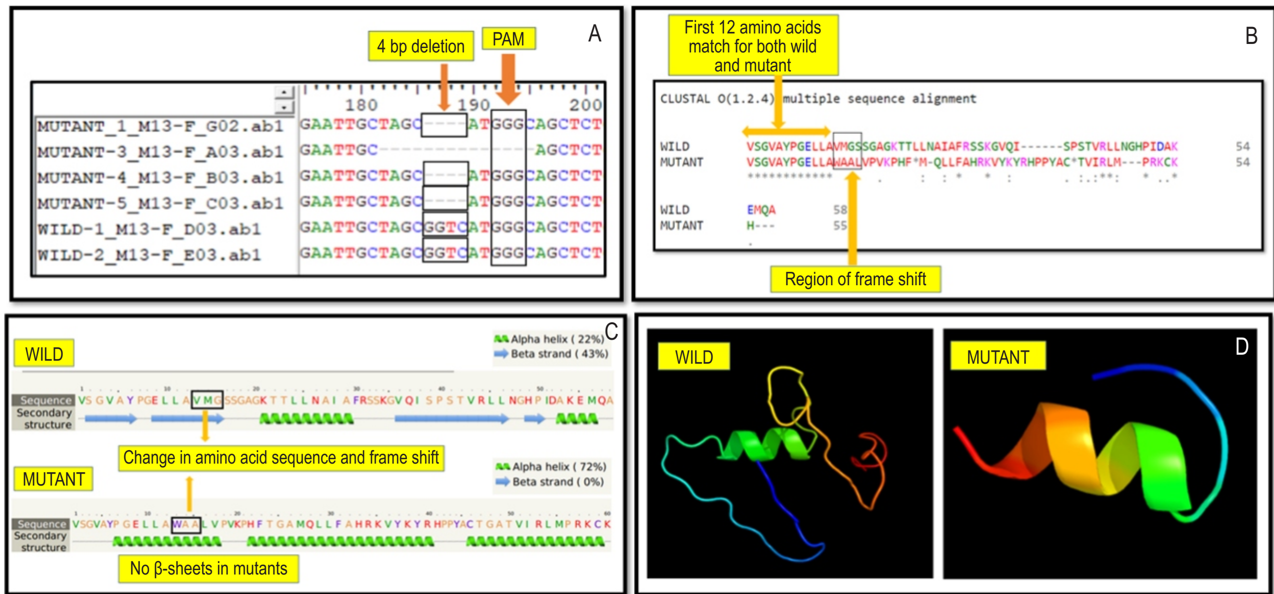

Fig. 3: (A) Genotypic analysis of mutant sequences; (B) Amino acid alignments showing frame shit mutations at the site of edit; V (valine) is substituted for (tryptophan); (C) Predicted 2D structure of the protein using Phyre2; (D) 3D structures of wild and mutant protein predicted by Phyre2.

protein. Significant alterations were observed in the arrangement and composition of the mutant protein compared to the wild-type protein. The mutant protein exhibited a higher proportion of α-helix (72%) compared to the wild type (22%). Also, the proportion of β-sheets varied significantly amongst the mutant and wild proteins. While in the wild, the β-sheets were around 43 per cent, in mutant due to frame shift it was completely nullified (Fig. 3C). These secondary (α-helix and β-sheets) structures play a vital role in protein folding and determines the number of caves and pockets in the protein which in turn determine its functional efficiency (Hidayati *et al.,* 2021). Similar variations were also observed in the 3D structures of protein predicted by Phyre 2 software (Fig. 3D). The proteins exhibit variances in shape and conformation as a result of disparities in the overall number of helices and sheets. All these variations in the protein structures could be attributed to alteration in protein function leading to production of non-functional protein or no protein at all.

The CRISPR/Cas9 mediated approach has proven to be a powerful tool for genetic control of insect pest such asmango fruit fly, *B. dorsalis*. From our analyses based on comparison of phenotype and genotype of mutants and wild, it is evident that experiment on demonstration of CRISPR/Cas9 based genome editing was successful. Thus, the technology has proved itself to be a potential genome editing tool for management of key agricultural pests in thelong run and provide an opportunity for sustainable control of an aggressive pest suchas *B. dorsalis.* Further, studies relating to the assessment of the mutant individuals with respect to biology and life span, egg laying behaviour, ovipositional preferences, fecundity, damage potential, restriction of vision and ability to recognise host and mate must be emphasised. Subsequent experiments in this area must focus on the development of progeny with similar heritable mutations. Consequently, CRISPR/Cas9 technology should be exploited for the development of gene drives, which can spread genetic modifications through insect populations to control serious agricultural pests. They must be aimed to sterilize male insects, reduce fertility or lifespan, or make pests more susceptible to insecticides, ultimately reducing pest populations offering sustainable alternative to chemical pesticides.

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