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In vitro evaluation of sgRNA-Cas9 RNPs for knocking out essential pigmentation genes in *Bemisia tabaci*

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Abstract

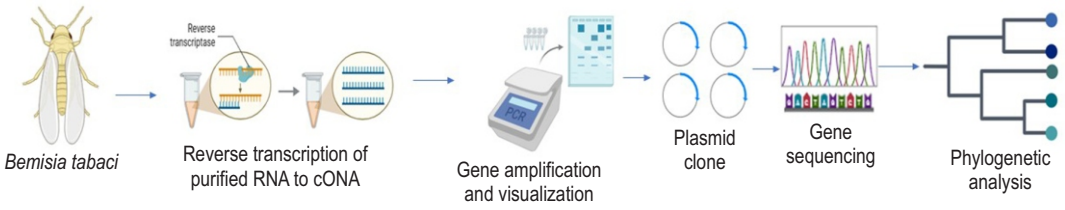
Aim: This study aimed to design and screen highly effective sgRNAs for RNP-based genome editing in *Bemisia tabaci* Asia II 1and to validate the efficiency and specificity of the synthesized RNP complexes through *in-vitro* assays, thereby enabling precise and targeted gene editing.

Methodology: Amplicons of two essential pigmentation-related genes were used to evaluate the efficiency of the designed sgRNA-Cas9 complex through an *in-vitro* cleavage assay, enabling the identification of optimal RNP combinations for *in-vivo* targeted gene editing.

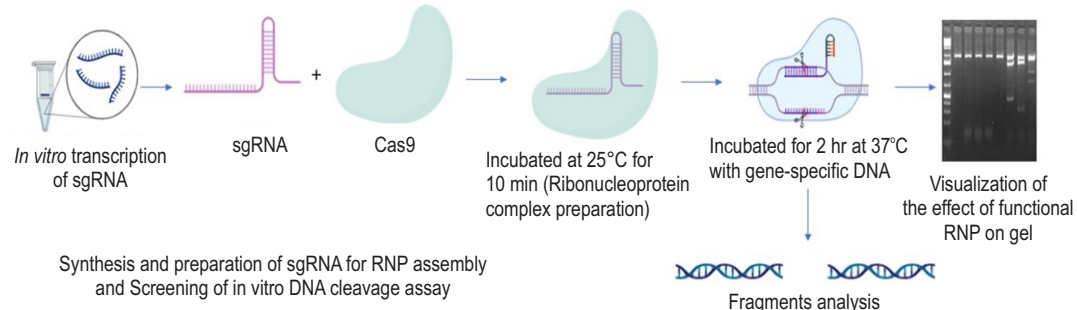
Results: The *LAC2* and *w* genes of *B. tabaci* were successfully characterized, and submitted to NCBI (accession numbers PV012220 and PV012221). *In-vitro* cleavage assays confirmed efficient and precise cutting of target gene amplicons by specific Cas9-sgRNA RNP complexes at expected positions.

Interpretation: Phylogenetic analysis revealed that the *LAC2* and *w* genes were highly conserved in *B. tabaci*. *In-vitro* cleavage with sgRNA-Cas9 RNP complexes provides a rapid and effective pre-validation of CRISPR-Cas functionality in insects. This approach eliminates the need for preliminary cell line testing before *in-vivo* application.

Key words: Gene editing, *LAC2*, Ribonucleoprotein complex, w gene, Whitefly



Gene Amplification, sequencing, and phylogenetic analysis



Synthesis and preparation of sgRNA for RNP assembly and Screening of in vitro DNA cleavage assay

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Introduction

Whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), is a major polyphagous sucking pest that poses a severe threat to global agriculture. It causes direct damage by feeding on plant sap and promoting the growth of sooty mould, which interferes with photosynthesis. Additionally, *B. tabaci* serves as a vector for over 400 plant viruses and is a key vector for spreading begomoviruses (Wang *et al.,* 2016, 2020; Ghosh and Ghanim, 2021). *B. tabaci*-transmitted begomoviruses cause 20-100% yield losses in crops (Varma *et al.,* 1992; Souz *et al.,* 2018). Managing *B. tabaci* and its transmitted viruses is challenging, as traditional methods, such as insecticides and transgenic crops, pose environmental, health, and public acceptance concerns. There are no transgenic crops available towards resistance to *B. tabaci*. Recent approaches, such as CRISPR-Cas9, offer innovative pest management strategies through precise genetic modifications, enabling targeted alterations in *B. tabaci* traits.

This powerful tool holds significant promise for controlling a wide range of insect pests. CRISPR-Cas9 technology plays a vital role in elucidating gene functions in *B. tabaci,* thereby contributing to the development of sustainable pest management strategies. Among the commonly targeted loci in CRISPR-Cas9-mediated gene editing for non-model insects are the white gene (*w*) and laccase 2 (*LAC2*). The w encodes an ABC transporter essential for eye pigmentation (Mackenzie *et al.,* 1999; Figon and Casas, 2019), and its loss-of-function results in visible pigment loss, facilitating straightforward mutant screening (Ge *et al.,* 2016). It has been widely targeted across various insect orders (Summers *et al.,* 1982; Bai *et al.,* 2019; Heu *et al.,* 2020; Liu *et al.,* 2021; Han *et al.,* 2024), though knockouts can be lethal in particular species (Khan *et al.,* 2017; Reding and Pick, 2020). Laccase genes, particularly *LAC1* and *LAC2*, are also important targets. While *LAC1* is associated with metal ion metabolism and detoxification of plant secondary compounds (Dittmer *et al.,* 2004; Yang *et al*., 2018), *LAC2* is critical for cuticle sclerotization, pigmentation, andeggshell formation (Elias-Neto *et al.,* 2010; Yang *et al.,* 2022). RNAi studies further confirm the essential role of *LAC2* in structural development (Arakane *et al.,* 2005; Niu *et al.,* 2008; Futahashi *et al.,* 2011), with its dysfunction leading to pale, fragile eggs and embryonic lethality in mosquitoes and aphids (Wu *et al.,* 2013).

CRISPR-Cas system is a powerful, precise, and versatile genome-editing tool for understanding gene functions in insect species. It holds great potential for generating insect progeny with morphological, reproductive or behavioural deficiencies, which can be strategically utilized for the suppression of pest populations. CRISPR-Cas9 technology utilizes the Cas9 nuclease and a guide RNA (gRNA), which is typically composed of crRNA and tracrRNA (Deltcheva *et al.,* 2011); however, a synthetic single-guide RNA (sgRNA) is often used for enhanced targeting (Jinek *et al.,* 2012). The Cas9-sgRNA ribonucleoprotein (RNP) complex enables efficient gene editing in embryos (Zhang *et al.,* 2023), with direct delivery reducing off-target effects by ensuring rapid DNA cleavage and degradation (Ramakrishna *et al.,* 2014). Cas9 induces double-strand breaks (DSBs) at target sites adjacent to a PAM sequence via its HNH and RuvC-like nuclease domains, which cut complementary and non-complementary strands, respectively (Gasiunas *et al.,* 2012). DNA repair follows through non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Lin *et al.,* 2014). By guiding Cas9 to specific loci, sgRNAs allow precise gene knockouts (Liu *et al.,* 2017). Evaluating RNP activity *in-vivo* remains a significant challenge, primarily due to the technical complexities associated with delivering the CRISPR-Cas9 system to target cells, the risk of unintended off-target effects, and the necessity for precise DNA repair mechanisms. These factors make in-vivo validation both time-consuming and resource-intensive. Moreover, in many non-model organisms, including agricultural pests such as *B. tabaci*, the lack of well-established genetic tools and optimized delivery methods further complicates functional genomic studies. There is a need for efficient, scalable prescreening methods to evaluate RNP efficacy prior to in-vivo application.

In this context, in -vitro cleavage assays provide a practical and efficient alternative. These assays enable the rapid screening of RNP activity across various organisms, eliminating the need for cell line development or complex embryo microinjection protocols at the initial stage. In-vitro assays are instrumental for optimizing and validating the CRISPR-Cas9 system before its application in living cells or whole organisms. They enable researchers to prescreen gRNAs for on-target cleavage efficiency, assess potential off-target interactions, and verify the functional integrity of the Cas9 protein. This pre-validation step not only conserves resources but also minimizes the risk of deleterious or non-specific genomic alterations *in-vivo.* In view of the above, this study demonstrates the feasibility of genome engineering in *B. tabaci* and highlights the potential of CRISPR-based tools in advancing pest management strategies through targeted genetic interventions.

Materials and Methods

Maintenance of *Bemisia tabaci* Asia II 1 population: An isofemale population of *B. tabaci*, maintained since 2018, was used throughout the study. The population was maintained under controlled environmental conditions at 28 ± 2°C, 60 ± 5% relative humidity, and a 16:8 hrlight: dark photoperiod. For the confirmation of genetic identity, total DNA was extracted from the adult specimens following the method of Rehman *et al.* (2021). The mitochondrial cytochrome oxidase I *(mtCOI)* gene was PCR-amplified and sequenced, confirming the colony as *B. tabaci* Asia II 1.

Amplification, sequencing and phylogenetic analysis of the *white* and *laccase* 2 genes: Based on the previous studies (Arakane *et al.,* 2005; Niu *et al.,* 2008; Futahashi *et al.,* 2011; Grubbs *et al.,* 2015; Figon and Casas, 2019), the *B. tabaci LAC2* and *w* genes were selected for knock out using RNP complexes. To amplify the target genes, total RNA was extracted from 50 adult *B. tabaci* using TRIzol™ reagent (Invitrogen, USA).

Complementary DNA (cDNA) was synthesized using a cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. The *LAC2* and *w* genes of *B. tabaci* were amplified using the primer pairs AG855F-AG856R and AG896-AG897R, respectively (Table 1). The PCR product was analyzed on a 1% agarose gel and visualized using a gel-doc system (Bio-Rad, USA). The purified amplicons were cloned into the pGEM®-T Easy vector (Promega, USA). The recombinant plasmids were purified and subjected to bidirectional sequencing for verification.

For the phylogenetic analysis of *LAC2* and *w* genes, orthologous sequences from various insect orders were retrieved from the NCBI database. *LAC2* sequences included *B. tabaci* (XM_0190576345, XM_019057635) and species from Hemiptera (XM_022318196, XM_027989094, XM_001950753, XM_026953764), Hymenoptera (XM_006562254, F470292), Lepidoptera (XM_022963884, MZ998903, KF479388), Diptera (XM_001970801, XM_033295842, XM_016167502), and Thysanoptera (XM_034375390, XM_026423410). The *lac2* sequence of Crustacean *Daphnia pulicaria* (XM_046776564) (Phylum: Arthropoda, Class: Branchiopoda) was used as the outgroup. For w gene, a putative assembled *B. tabaci* sequence was used to identify orthologs from Hemiptera (XM_014429735), Blattodea (XM_023858539, XM_069823334), Coleoptera (XM_044405709, XM_044405717, XM_023454486, XM_050448796), Diptera (XM_036371618, XM_040106531), Hymenoptera (XM_031916231, XM_035089061, XM_062081104), and Lepidoptera (XM_034984274, XM_069509419, XM_022964124). The *Penaeus chinensis* Crustacean sequence (XM_047636688) (Phylum: Arthropoda, Class: Malacostraca) served as the out group. Multiple sequence alignment was performed using Clustal W, and phylogenetic trees were constructed with MEGA X and visualized usingi TOL. The Neighbor-Joining method was applied with 1,000 bootstrap replications and pairwise deletion to ensure accuracy and robustness (Thompson *et al.,* 1994; Kumar *et al.,* 2018).

Designing, synthesizing, and preparing sgRNA for RNP assembly: The design of sgRNAs begins with identifying PAM sites (5'-NGG-3' for SpCas9) that are adjacent to 20-nt crRNA sequences. In this study, the sgRNAs targeting the *LAC2* and *w* genes were deisgned using the CRISPOR online tool. Factors such as GC content, MIT scores (≥ 50 on a 0–100 scale), and predicted efficacy were prioritized to minimize off-target effects and reduce mismatches. The Doench and Moreno-Mateos scores were also considered for T7 promoter-based *in-vitro* sgRNA synthesis, targeting a GC content of 20-80% for optimal cleavage. Additionally, PAM region mismatches were taken into account to further lower the potential for off-target activity.

A free online sgRNA design tool from NEB (https://sgrna.neb. com/#!/sgrna) was used to design target-specific DNA oligos containing a T7 promoter for sgRNA synthesis. These target-specific DNA oligos consisted of a 5' T7 promoter, followed by an additional G nucleotide, the 20 nt of crRNA sequence (without the PAM), followed by a 14-nucleotide overlap complementary to the *Streptococcus pyogenes* Cas9 scaffold oligo at 3' end. The oligos (Table 3) were obtained from GCC Biotech Pvt. Ltd. sgRNA synthesis was performed using the EnGen sgRNA Synthesis Kit (New England Biolabs, USA) according to the manufacturer's protocol. The reaction mixture contained NTPs, dNTPs, the Cas9 scaffold oligo, DTT, and DNA/RNA polymerases. The reaction was assembled at room temperature in a PCR tube and then transferred to a PCR machine (Bio-Rad) for incubation at 37°C for 30 min to synthesize sgRNA. At 37°C, the DNA polymerase present in the mixture extended the oligos into double-stranded DNA (dsDNA). Subsequently, RNA polymerase transcribed this template into sgRNA, which comprised the target-specific crRNA and tracrRNA. The synthesized sgRNA was purified using the Monarch RNA Cleanup Kit (New England Biolabs) according to the manufacturer's protocol, and its concentration was determined using a Nano-300 Micro Spectrophotometer (Genetix Biotech, India). Integrity of the purified *in-vitro* transcribed sgRNA was visualized on a 2% agarose gel. The sgRNA sequences targeting the *LAC2* and *w* genes, listed in Table 4, were

Table 1: List of primer pairs used in the study

Name of the primer	Primer sequence (5'→3')	Annealing temperature in PCR (°C)	Amplicon size (bp)	Target gene	Purpose	Reference
C1-J-2195	TTGATTTTTTG GTCATCCAGAAGT	53	860	*mtCOI*	Biotype characterization	Simon *et al.,* (1994)
L2-N-3014	TCCAATGCACTAAT CTGCCATATTA					
AG855F	CTCTACACGTCTCT GGACTTTGACACTGT	55	1879	*laccase2*	Gene amplification	This study
AG856R	GGGGTAAGAAGTCT CCACATCTAAGAT					
AG896F	AAGAAGAACAGAA ACCGCTCC	60	2085	*white*	Gene amplification	This study
AG897R	ATTTTCGCAACGT CCGGTA					

Table 2: CrRNA sequence targeting *Bemisia tabaci LAC2* and wgene

crRNA sequence (5'→3') PAM
CGTTCGCGTACCATTGATAC**CGG**
TGTAGACGTCAGGAACCAGA**TGG**
CGTGCTCCCACCTACGATAT**TGG**
CGTCCGCGCCAAATTCCCGG**AGG**
GGATTCCATTGACTGCTCGC**TGG**
TATAGGAACCTTAACCGTTA**GGG**

subsequently used for RNP complex assembly, as outlined in the following section.

Preparation of RNP complex and *in-vitro* DNA cleavage assay: To evaluate RNP-mediated cleavage activity, the *in-vitro* assays were performed using purified PCR amplicons of the *LAC2* and *w* genes as target templates. Each amplicon was diluted to a final concentration of 0.5 µg µl^{-1}.Three separate RNP complexes were prepared by assembling the target gene-specific sgRNAs with Cas9 nuclease (*Streptococcus pyogenes*; New England Biolabs, USA). To reduce potential secondary structure heterogeneity and aggregation, sgRNAs were pre-heated at 70 °C for 10 min and allowed to cool to room temperature. Each RNP complex was assembled in a 27 µl reaction volume containing 1.5 µg of sgRNA, 0.5 µg of Cas9, and 3 µl of NE Buffer r3.1. The mixture was thoroughly mixed, pulse-spun, and then incubated at room temperature for 10 min. Subsequently, 1.5 µg of purified PCR-amplified double-stranded DNA (dsDNA) was added to the reaction mixture. The mixture was again pulse-spun, thoroughly mixed, and incubated at 37 °C for 2 hr to facilitate DNA cleavage. The reaction was terminated by incubating the mixture at 80 °C for 10 min, followed by immediate cooling on ice. Cleaved DNA fragments were resolved on a 2% agarose gel and visualized using a Gel Doc imaging system (Bio-Rad).

Results and Discussion

PCR amplification of the *mtCOI* gene of *B. tabaci* using primers C1-J-2195 and L2-N-3014 produced an ~860 bp amplicon. The resulting 596 bp sequence exhibited 99.6% identity with known *B. tabaci* Asia II 1 sequences in NCBI (LN897440; MN329162). These assembled sequences were submitted to NCBI and retrieved with accession number PP907972. The LAC2 and w genes of *B. tabaci* were successfully amplified and characterized. PCR amplification of the LAC2 gene, using primers AG855F and AG856R, resulted in a 1875 bp

Table 3: List of target-specific DNA oligos used in the study

Name of the crRNA	Target-specific DNA oligo template (5'→3')
AG898-Bt laccase2-cr RNA	**TTCTAATACGACTCACTATA**G**CGTTCGCGTACCATTGATAC***GTTTTAGAGCTAGA*
AG899-Bt laccase2-cr RNA	**TTCTAATACGACTCACTATA**G**TGTAGACGTCAGGAACCAGA***GTTTTAGAGCTAGA*
AG900-Bt laccase2-cr RNA	**TTCTAATACGACTCACTATA**G**CGTGCTCCCACCTACGATAT***GTTTTAGAGCTAGA*
AG908-BtWhite-cr RNA	**TTCTAATACGACTCACTATA**G**CGTCCGCGCCAAATTCCCGG***GTTTTAGAGCTAGA*
AG909-BtWhite -cr RNA	**TTCTAATACGACTCACTATA**G***GATTCCATTGACTGCTCGCG****TTTTAGAGCTAGA*
AG910-BtWhite -cr RNA	**TTCTAATACGACTCACTATA**G**TATAGGAACCTTAACCGTTAG***TTTTAGAGCTAGA*

T7 promoter sequence is marked in bold letters. An additional G was added at the 5' of the crRNA sequence. The crRNA sequence is marked by an underline. 14-nt overlap complementary to the *S. pyogenes* Cas9 Scaffold Oligo is marked in italic letters

Table 4: List of *in-vitro* transcribed sgRNAs used in the study

Name of the sgRNA	Transcribed sgRNA sequence (5'→3′)
AG898-Bt laccase2-sgRNA	**GCGUUCGCGUACCAUUGAUAC***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*
AG899-Bt laccase2-sgRNA	**GUGUAGACGUCAGGAACCAGA***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*
AG900-Bt laccase2-sgRNA	**GCGUGCUCCCACCUACGAUAU***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*
AG908-BtWhite -sgRNA	**GCGUCCGCGCCAAAUUCCCGG***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*
AG909-BtWhite -sgRNA	**GGAUUCCAUUGACUGCUCGC***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*
AG910-BtWhite -sgRNA	**GUAUAGGAACCUUAACCGUUA***GUUUUAGAGCUAGAAAUAGCAAGUU* *AAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU*

In the transcribed sgRNA, crRNA sequence is marked by underlined letters. TracrRNA sequence is marked by italic letters

amplicon. Subsequent bidirectional sequencing of the cloned product allowed for assembly and BLASTn analysis revealed 98.67% similarity to known *B. tabaci LAC2* sequences (XM_019057634.1; XM_019057635.1).

Similarly, the amplification of the *w* gene using primersAG896 and AG897R successfully yielded a 2085 bp fragment. The assembled bidirectional sequence showed 98.61% identity to the known *B. tabaci w* gene sequence (XM_019053144.1) in the BLASTn analysis. These assembled sequences have been submitted to NCBI and are readily accessible under accession numbers PV012220 (*LAC2*) and PV012221 (*w*). Phylogenetic analysis of *B. tabaci LAC2* and w gene sequences revealed high similarity to characterized sequences from other *B. tabaci* cryptic species, as well as diverse insect orders **(Fig. 1, 2)**. The *LAC2* phylogeny revealed five distinct clusters, with the *B. tabaci* sequences grouping tightly within the Hemipteran clade, while forming separate clusters with sequences from other insect orders. Similarly, the *w* gene phylogenetic analysis identified six clusters corresponding to distinct insect orders, with *B. tabaci* sequences clustering closely within the Hemipteran group. These patterns highlight the high conservation of both *LAC2* and w genes across insect taxa.

Three crRNAs targeting the *LAC2* gene of *B. tabaci* (PV012220) were identified using CRISPOR with NGG as the PAM (Table 2). AG898 and AG899 target exon 5, while AG900 targets exon 8. AG898 was on the antisense strand with a CGG PAM at position 876, while AG899 and AG900 were on the sense strand with TGG PAMs at positions 764 and 1521, respectively. AG898 and AG899 had a GC content of 50%, while AG900 had a GC content of 55%. MIT specificity scores were 100 for AG898 and AG900, and 99 for AG899, indicating high on-target specificity. Three crRNAs targeting the white gene of *B. tabaci* (PV012221) were designed using CRISPOR. AG908 targets exon 2, while AG909 and AG910 target exon 3. AG908 and Ag910 were on the sense strand with AGG and GGG PAMs at positions 515 and 884, respectively; AG909 was on the antisense strand with a TGG PAM at position 783. Their GC content was 70%, 55% and 35%, respectively and all had MIT specificity scores of 99. Doench and Moreno-Mateos efficiency scores indicated strong performance, especially for AG910 (57%). Predicted efficiency scores for AG908 and AG909 were 45 and 11%, respectively.

Off-target analysis (GCF_001854935.1) showed no potential off-targets for any crRNA. Target-specific DNA oligonucleotides (55 nucleotides in length) were designed and synthesized, comprising a 20-nt T7 promoter sequence at the 5' end, a 21-nt crRNA (including an additional G nucleotide at the 5' end), and a 14-nt overlap sequence complementary to the 3' end of the Cas9 scaffold oligo. Using these 55-nt target-specific oligos and an 80-nt *Streptococcus pyogenes* Cas9 scaffold oligo in the reaction mix, a 121-bp double-stranded DNA (dsDNA) template was generated via DNA polymerase. Due to the presence of the T7 promoter, this dsDNA template was immediately transcribed *in-vitro* by RNA polymerase to produce a 101-nt single-guide RNA (sgRNA), which included both crRNA and tracrRNA sequences (Table 4). Following purification, the *in-vitro* transcribed sgRNAs yielded an average of 20 µg of each sgRNA for *LAC2* and 22 µg for the wgene, respectively. In the present study, three distinct sgRNAs were synthesized through *in-vitro* transcription to target the *B. tabaci* laccase and white genes. The use of two or more sgRNAs for a single gene target has been shown to significantly enhance CRISPR-Cas9-mediated editing in both mammals and insects, resulting in higher editing efficiency (Zhou *et al.,* 2014; Zhu *et al.,* 2017; Hunter *et al.,* 2018).

In-vitro Cas9 assays with RNP complexes targeting *LAC2* efficiently cleaved the 1879 bp dsDNA amplicon. Cas9-AG898 produced 1433 bp and 446 bp fragments; Cas9-AG899 yielded 1553 bp and 326 bp fragments; Cas9-AG900 resulted in 1083 bp and 796 bp fragments. For the wgene (2085 bp amplicon), Cas9-AG908 generated 1887 bp and 198 bp fragments, Cas9-AG909 produced 1598 bp and 487 bp fragments, and Cas9-AG910 yielded 1518 bp and 567 bp fragments. No digestion occurred in the negative controls, which lacked either sgRNA or Cas9. These results indicate that the selected RNP complexes successfully digested the targeted *LAC2* and w gene amplicon at the expected positions, as evidenced by the band patterns observed in the following gel electrophoresis. These findings validate that the RNP complexes specifically and efficiently recognize and cleave the wand *LAC2* genes under *in-vitro* conditions. These results are consistent with the previous reports (Jinek *et al.,* 2012; Sternberg *et al.,* 2014; Wang *et al.,* 2016; Bhargava *et al.,* 2024; Ye *et al.,* 2025), who demonstrated that Cas9 mediates DNA cleavage at specific genomic loci through precise base pairing between the sgRNA and target DNA adjacent to a protospacer-adjacent motif (PAM), resulting in the cleavage of the targeted DNA.

In the gel electrophoresis, distinct banding patterns were observed following genomic DNA cleavage when different sgRNAs targeting each gene were tested individually, providing specific insights into their efficiency in directing Cas9 to the corresponding DNA sequences. Notably, the cleavage efficiency of the Cas9 ribonucleo protein complex often varied depending on the sgRNA employed. These findings were consistent with the reults of Javaid and Choi (2021), who reported that Cas9 cleavage efficiency was influenced by various factors such as buffer composition, reaction temperature, sgRNA and the enzymeintrinsic properties. The cleavage activity depends Cas9 Intrinsic nuclease activity, and the affinity of the sgRNA to the DNA template. Similar findings were reported in earlier studies (Javaid and Choi, 2021; Konstantakos *et al.,* 2022). The sgRNA utilized in this study contained both crRNA and tracrRNA within a single frame, offering considerable potential for the development of a versatile and straight forward RNA-directed genome editing system compared to tracrRNA and crRNA separately (Hsu *et al.,* 2013; Bassett *et al.,* 2013; Hu *et al.,* 2019; Di Cristina *et al.,* 2025). sgRNA simplifies expression and transcription while maintaining comparable editing efficiency (Jinek *et al.,* 2012; Dang *et al.,* 2015). RNP complexes were synthesized by incubating the sgRNAs with Cas9 at room temperature. Similar results were reported (Nyberg *et al.,* 2020; De Rouck *et al.,* 2024).

Fig. 2: A phylogenetic tree of the *w* gene for different insect orders was constructed using Neighbor-Joining methods. The *w* gene sequences of varying insect orders were retrieved from NCBI. The figure has been presented using iTOL. The phylogenetic analysis of the *w* gene revealed high conservation across taxa, with *B. tabaci* clustering closely within the Hemipteran clade and exhibiting distinct evolutionary divergence from other insect orders.

Fig. 1: A phylogenetic tree of the *LAC2* gene for different insect orders was constructed using Neighbor-Joining methods. The *LAC2* sequences of varying insect orders were retrieved from NCBI. The figure has been presented using iTOL. The phylogenetic analysis of the *LAC2* gene revealed high conservation across taxa, with *B. tabaci* clustering closely within the Hemipteran clade and exhibiting distinct evolutionary divergence from other insect orders.

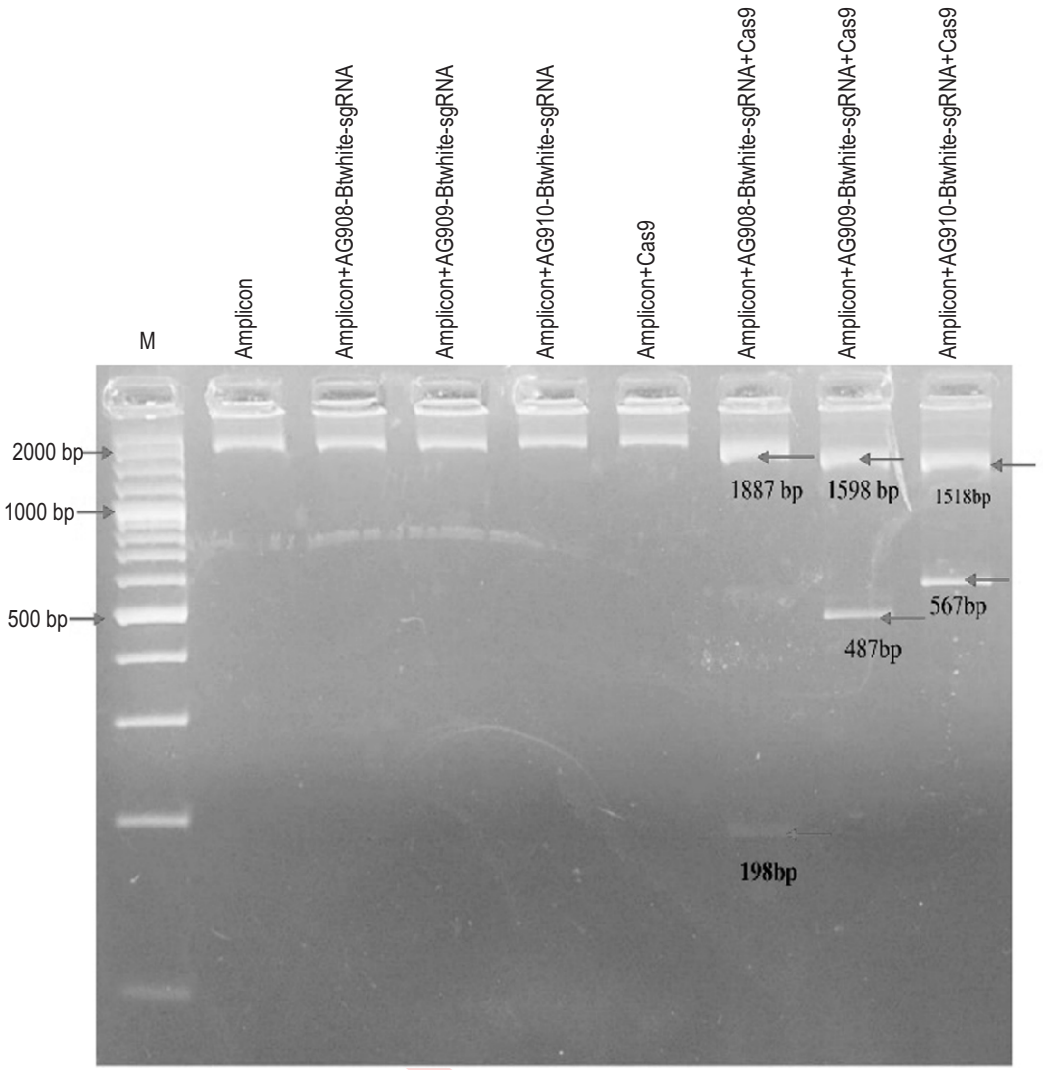

Fig. 3: *In-vitro* Cas9 cleavage assay. In the *in-vitro* cleavage assay, the RNP complexes containing Cas9 and sgRNAs cleaved a 2085 bp amplicon of *w* gene into 1887 and 198 bp, 1598 and 487, and 1518 bp and 567bp fragments, respectively. Red arrows on the agarose gel mark the digested products.

In this study, readily available ribonucleo protein (RNP) complexes were synthesized and prescreened for in-vivo gene editing. These pre-assembled sgRNA-Cas9 complexes enable direct application, thereby minimizing the risk of foreign genetic integration by avoiding plasmid vector-based *in-vivo* transcription and translation of sgRNA and Cas9 (Bassett *et al.,* 2013; Farboud *et al.,* 2018). This strategy provides a safer and more precise approach to genome editing. On the other hand, using the negative control lacking either sgRNA or Cas9, no digestion was noticed in the treatments, which helped in confirming that the presence of the RNP Complex is the cause, not any other factors such as degradation or contaminants. However, these results are similar to *in-vitro* RNP assays conducted for optimizing genome editing in various insect species of the orders Diptera, Hemiptera, and Hymenoptera (Martin-Martin *et al.,* 2018; Le Trionnaire *et al.*, 2018; Lester *et al.,* 2020). In-vitro screening of RNP complexes remains a critical step for optimizing subsequent *in-vivo* genome editing in target organisms. However, by using prescreened optimal sgRNA-Cas9 Ribonucleoprotein complexes,future efforts should focus on developing the mutant population and performing a comprehensive phenotypic assessment to establish the efficacy of this technology in mitigating the threats posed by *B. tabaci.* The research highlights the potential use of CRISPR-Cas as a pioneering genetic tool for studying essential gene functions, aiming to manage pest populations of *B.tabaci*.

In conclusion, *in-vitro* cleavage of target DNA using the CRISPR-Cas9 ribonucleoprotein (RNP) complex provides an efficient method for prescreening the functionality and relative efficiency of the CRISPR system in targeting the *LAC2* and *w* in *B.*

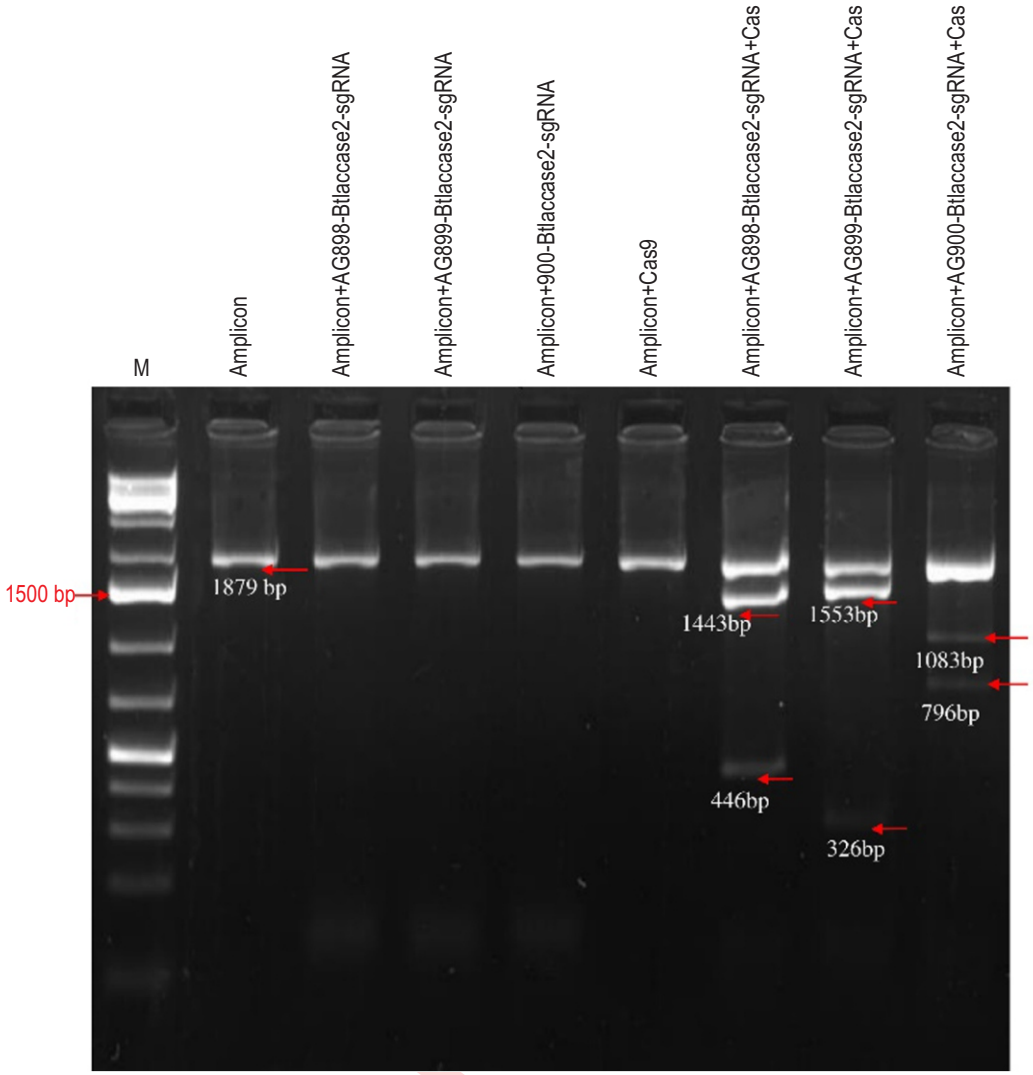

Fig. 4: *In-vitro* Cas9 cleavage assay. In the *in-vitro* cleavage assay, the RNP complexes containing Cas9 and sgRNAs cleaved a 1878 bp amplicon of *LAC2* into 1443 and 446 bp, 1553 and 446, and 1083bp and 796bp fragments, respectively. Red arrows on the agarose gel mark the digested products.

tabaci. This rapid screening approach eliminates the need for initial cell line testing, positioning Cas9 RNP complexes as the most efficient, transient, and convenient format for CRISPR-Cas9 gene editing. It reduces time, costs and labour, making it an ideal method for evaluating editing efficiency under *in-vitro* conditions. The approach also offers a practical foundation for subsequent *in-vivo* applications, facilitating successful genome editing in targeted organisms.

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