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Mutational Analysis of Cadherin and v-ATPase Genes in Pink Bollworm and Resistance Towards Bt Cotton Through *In-Vitro* Insect Feeding Assay

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ABSTRACT

Insect pest attack is one of the major problems in the agriculture sector. Bt crops were found effective in providing resistance against pink bollworm in cotton but recent reports on insect-evolved resistance have highlighted mutations in pink bollworm (PBW) receptor genes. We investigated cadherin and v-ATPase genes from local isolates of PBW for possible mutations. We amplified ~1224bp and ~1149bp cadherin and v-ATPase genes respectively and cloned them in the pJET2.1 vector. The deduced sequence of cadherin didn't exhibit any nucleotide change while v-ATPase gene showed mutations and was submitted in GenBank under accession No. OR231877. The CDS of the v-ATPase showed four mutations; three silent and one missense mutation. At position 593, thymine was replaced with cytosine due to which valine was replaced by alanine at position 198 (593 T>C, p. V198A). Similarly, at position 648, the thymine was replaced by cytosine (648 T>C. Y216Y), at position 663, cytosine was replaced by thymine (663 C>T, Y221Y), and at position 666 the guanine was replaced by adenine (666 G>A, G222G). These changes have no effects on amino acid sequences and protein structures and were confirmed as silent mutations. In-vitro plant feeding assay revealed a significant difference in PBW mortality fed on Bt transgenic and non-transgenic cotton plants. In conclusion, our findings suggested good efficiency of Bt cotton while lacking any alarming mutation in cadherin and v-ATPase genes of local PBW isolate.

INTRODUCTION

Insect pest is one of the biggest threat to agriculture, and it causes a loss of almost 15-22% to economically important cash crops (Singh *et al*., 2021). Biological, chemical, physical, and genetic methods are used to protect crops from these pests. The biological and genetic methods are more effective than other methods. In the biological control method, a living organism is used to combat the pests. These organisms protect the host by producing compounds that have toxic effects on the other organisms (Anderson *et al*., 2019). *Gossypium hirsutum*, cotton, known as the king of fiber, is one of the major cash crops in Pakistan. The country ranks 4th in cotton production and 5th in consumption. It contributes 10% to the GDP and 55% to the foreign exchange earnings of the country. The annual yield is decreasing due to different factors, including insect pest attacks. More than 1326 insects attack cotton, causing a 15% loss of the total cotton production in the country. The pink bollworm contributes 40% of these losses (Bhute *et al*., 2023; Karar *et al*., 2020).

Bacillus thuringiensis (Bt), a Gram-positive bacterium, has been used as a microbial insecticide due to the production of Cry and Cyt proteins, also known as Bt toxins. These proteins are toxic to many insects, especially lepidopterans, hymenopterans, dipterans, and coleopterans. Bt is used worldwide due to its higher specificity to target insects and non-toxic to beneficial insects (Kumar *et al*., 2021). The Cry proteins are activated in the insect's midgut due to an alkaline environment and proteases. The activated Bt toxins bind with the receptors on the epithelial cells of the midgut of the target insects, resulting in pore formation in the midgut. The cell lyses, and the contents of the cells leak out, leading to nutrient absorption and digestion malfunction. These proteins move to the body cavity and damage other tissues and

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organs. These damages and nutrient deprivation caused the death of the target insects (Paul and Das, 2021).

Pectinophora gossypiella (pink bollworm) is an insect belonging to the order Lepidoptera and a major pest of cotton plants (Dou *et al*., 2021). It is thought to be originated from the sub-continent, India and Pakistan. The larva feeds on the seed of the cotton. Controlling through spray is difficult as they reside inside the boll. This invasive organism is a global threat to crops. Different control mechanisms have been introduced. The Bt can produce Cry protein which controls these pests (Bhatti *et al*., 2023). It is more threatening to regions like India and China, where they developed resistance against Bt toxins (Fabrick *et al*., 2023).

The insecticidal proteins are receptor-specific and target-specific sites. The mutations in the receptor sites change the receptor's shape, which causes resistance development against the insecticides (Boaventura *et al*., 2020). The receptor site mutation in different species of Lepidoptera caused resistance development against the insecticides (Huang *et al*., 2020; Shabbir *et al*., 2021; Sun *et al*., 2023). It has been reported that the pink bollworm got resistance against Cry proteins (Fabrick *et al*., 2023). Cadherin receptor mutations are the main cause of resistance development in the pink bollworm (Fabrick *et al*., 2023; Hussain *et al*., 2020; Wang *et al*., 2019, 2020, 2022). The v-ATPase subunit A in the midgut plays an important role in mediating the Cry proteins (Qiu *et al*., 2019). The alteration in v-ATPase subunits A and C in the midgut through RNAi techniques, and gene silencing to control the pest shows the importance of the target sites in insect pest management (Mohammed, 2016).

This study aims to find out the mutation in the coding sequence of the cadherin and v-ATPase gene in local PBW isolates and analysis of the protein structure to find out the possible structural changes of the protein that may trigger resistance development in PBW. It also aims to find out the mortality rate in non-Bt cotton and Bt cotton through in-vitro insect feeding assay.

MATERIALS AND METHODS

Gene amplification

The coding sequence of both cadherin and v-ATPase genes was retrieved from NCBI with accession numbers AY198374.1 and KJ854407.1. Primers were designed through Primer 3 and presented in Table I. The The samples of pink bollworms were collected from the fields of Centre of Excellence in Molecular Biology, CEMB, University of Punjab, Lahore. The insect samples (~100 mg) were ground in TRIzol reagent (Thermo Fisher) for RNA isolation. To remove any potential DNA impurities, the isolated RNA samples were treated with DNaseI (Thermo Scientific) to digest single- and double-stranded DNA and hydrolyze phosphodiester bonds releasing mono- and oligo-deoxyribonucleotides. The cDNA was prepared from 1µg RNA by using the Revertaid cDNA synthesis kit (Thermo Fisher). The reaction mixture contained PCR master mix, forward and reverse primers, cDNA, Taq polymerase, and RNase-free water. For amplification, the denaturation step was set at 95°C for 5 min and 95°C for 1 min. The annealing step was set between 54-58°C for 30 sec; the extension step was set at 72°C for 45 sec, and the post-extension step at 72°C for 10 min. The last step was set at 4 C for infinity time. The PCR amplification was done in a Thermal Cycler (Veriti, Applied Biosystem). The PCR products were resolved on 1% agarose gel and the amplified gene fragments were eluted from the gel and used for cloning in pJET2.1 vector. The ligated products were moved in *E. coli* Top10 strain through transformation. The bacterial colonies obtained after transformation were selected and cultured with LB broth for 24 h. The plasmid was extracted through a Miniprep kit (Thermo Fisher) and sent for sequencing.

Table I. Primer list used for amplification of cadherin and v-ATPase genes from local pink bollworm isolate.

Gene name	Primer sequence (5′ - 3′)	Product size
Cadherin-CADH	F GACCGGGACATAGGAGACAG R GTTCTCCACGTACACCTGGC	~1224 bp
V-ATPase-VATP	F ACTGGTTGATTAGCGCCCC R CTATGTATGCGTGTCGTGGC	~1149 bp

Sequencing and mutational analysis

Sanger sequencing of the amplified coding sequences of both (Cadherin and v-ATPase) genes from local PBW isolate was performed. The sequencing results were received in ABI format, and the peaks were analyzed through Chromas software. The sequences were extracted in FASTA format for mutation analysis and blast through NCBI blastn. Chromas software was used to analyze the peaks. The software mutation surveyor (https://softgenetics.com/products/mutation-surveyor/) was used to detect and analyze the mutations. The mutations were confirmed by NCBI blast while query and source were aligned through NCBI blastn to confirm the mutations.

In-silico *predicted protein structure*

The deduced sequences of both v-ATPase and cadherin genes amplified from local PBW isolate were translated into amino acids through an online translation tool, sequence manipulation suite. Both amino acid sequences of query and source were aligned through "blastp" to confirm amino acid change due to mutation.

The mutation and amino acid changes were noted. The structure of the proteins was predicted by the online tool Phyre2. The protein structures of both Query and Source were analyzed through the software PyMOL. Both structures were compared, and changes were highlighted.

In-vitro *insect feeding assay*

The Bt cotton variety (double gene) was used for *in-vitro* feeding assay along with non-transgenic cotton plants to evaluate the efficiency of Bt toxins towards PBW. Young, upper leaves were taken from transgenic and non-transgenic cotton plants and thoroughly washed with sterilized distilled water. Later, the leaves were excised in ~3-4mm long section while immature cotton bolls were also included in the feeding assay as short pieces as shown in Figure 4. Later, the leaves were dried on blot paper and the assay was setup with 03 biological replicates separately for each of the transgenic and non-transgneic samples. Moreover, for each of the biological replicate, 11 age-synchronised pink bollworm 2nd instar larvae were fed for a period of 5 days while the leaf and immature cotton boll diet was changed after every 36 h. The assay dishes were covered with muslin cloth and placed at 30°C with 60-70% relative humidity. The insect mortality was recorded in both control, non-transgenic and Bt transgenic cotton plants after 5 davs. The mortality rate was calculated by the fo

$$\%\ age\ Mortality = \frac{Number\ of\ dead\ insects}{Total\ insects} \times 100$$

The data obtained post feeding assay was subjected to one-way ANOVA (Analysis of Variance) and Tukey's multiple comparison test aspost test in GraphPad Prism software to reveal the significance ($p \leq 0.05$).

RESULTS

The main aim of the research was to reveal any potential mutation in the Cadherin and v-ATPase gene of pink bollworm. Moreover, to investigate any significant difference in PBW mortality among Bt and non-transgenic, an in-vitro insect feeding assay was executed.

Mutational analysis of cadherin and v-ATPase genes

The PBW isolates collected from local cotton fields were used to amplify the v-ATPase and cadherin gene. The v-ATPase gene fragment of ~1149bp was successfully amplified as shown in Figure 1. Moreover, a ~1224bp gene corresponding to the cadherin gene from the PBW isolate was amplified as depicted in Figure 1. Both of the amplified genes were cloned in the pJET2.1 vector and used to sequence.

The amplified ~1224bp sequence of the cadherin gene derived from the local PBW isolate was analyzed through the Mutation Surveyor showed no mutation in the coding sequence (CDS) of the cadherin gene depicting no change in the amino acid sequence was found when compared with cadherin sequence available at NCBI GenBank (Fig. 2A, B). The in-silico predicted protein structure of the amplified cadherin gene depicted a non-significant difference (Fig. 2C, D).

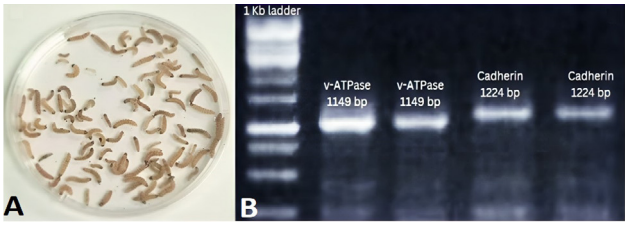

Fig. 1. Amplification of Cadherin and v-ATPase gene fragments from PBW local isolate. (A) The selected pink bollworms for screening (B) The amplified fragments of v-ATPase (~1149 bp) and Cadherin ~1224 bp.

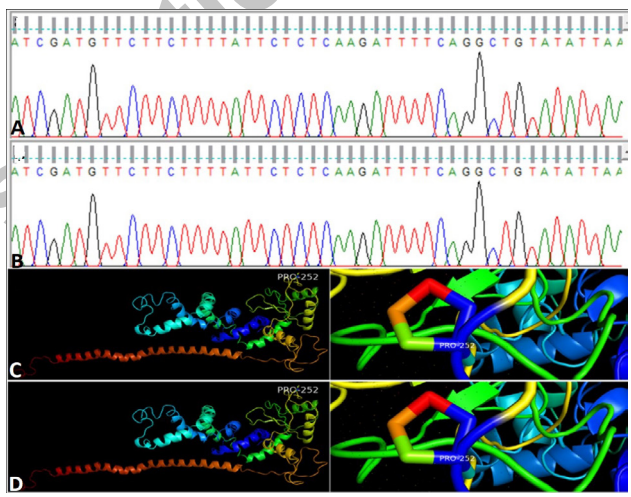

Fig. 2. Mutational analysis of the cadherin gene sequence deduced from PBW and in-silico structure predictions. (A, B) deduced nucleotide sequence of reference cadherin sequence available in GenBank and query sequence depicting no change (C, D) protein structure of reference sequence and deduced sequence of cadherin gene from local PBW isolate showing no change in protein structure.

Moreover, the sequencing result of the CDS of the v-ATPase gene amplified from local PBW isolate showed four mutations, three silent and one missense. At position 593, the cytosine replaced thymine (Fig. 3A) resulting in the substitution of the amino acid valine with alanine at position 198 (Fig. 3B, C). Similarly, at position 648, the cytosine replaced thymine (Fig. 3D), at position 663, position 198 (Fig. 3B, C). Similarly, at position 648, the cytosine replaced thymine (Fig. 3D), at position 663, thymine replaced cytosine, and at position 666 adenine replaced guanine (Fig. 3F). These changes don't affect the

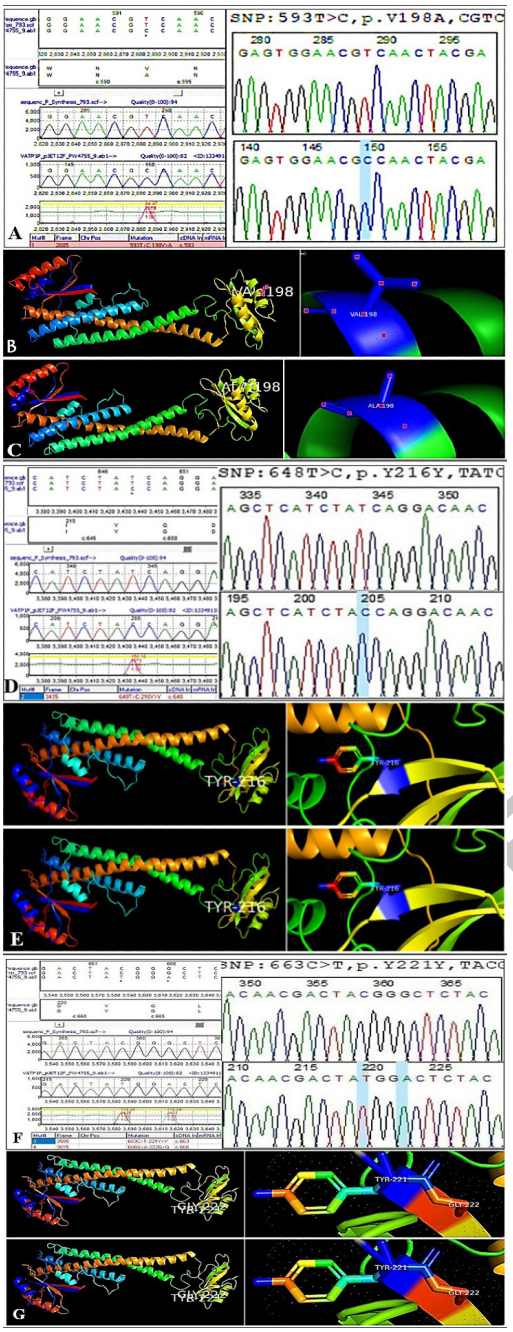

Fig. 3. Mutational analysis of the v-ATPase gene sequence deduced from PBW and in-silico structure predictions. (A) shows SNP 593 T>C, p.V198A (B) the protein structure of control with amino acid valine at 198 (C) shows the protein structure of a sample with amino acid alanine at 198. (D) shows SNP 648 T>C, p.Y216Y. (E) The protein structure of the control and sample with no changes in the amino acid at 216. (F) shows SNP 663 C>T, p.Y221Y, and SNP 666 G>A, p. G222G. (G) shows the protein structures of control and samples with no amino acid changes at positions 221 and 222.

amino acid sequence and protein structure, confirming them as silent mutations (Fig. 3E, G). The sequence with these multiple mutations at different positions was submitted to NCBI and accession # OR231877 was granted.

In-vitro *insect feeding assay reveals a significant difference in PBW mortality*

To investigate any insect-evolved resistance in local PBW isolate, the *in-vitro* feeding assay was executed by feeding on Bt and non-Bt cotton. The percentage mortality was calculated 5 days post-feeding on leaf and immature boll fiber. It was observed that the PBW larvae feeding on non-transgenic cotton variety were active and their morphological growth was found normal while the PBW larvae feeding on Bt cotton were less active and consumed relatively small amounts of leaves and immature fiber (Fig. 4A). Moreover, significant mortality of PBW larvae was observed in the Bt cotton in comparison to non-Bt plants 5 days post-feeding assay (Fig. 4C). The average mortality found in non-Bt cotton (control) was 11.11%, while in Bt cotton (transgenic), the mortality of PBW was found to be 76% (Fig. 4B). The significant difference in mortality among non-Bt and Bt cotton indicates that Bt cotton provides sufficient resistance against local PBW isolates.

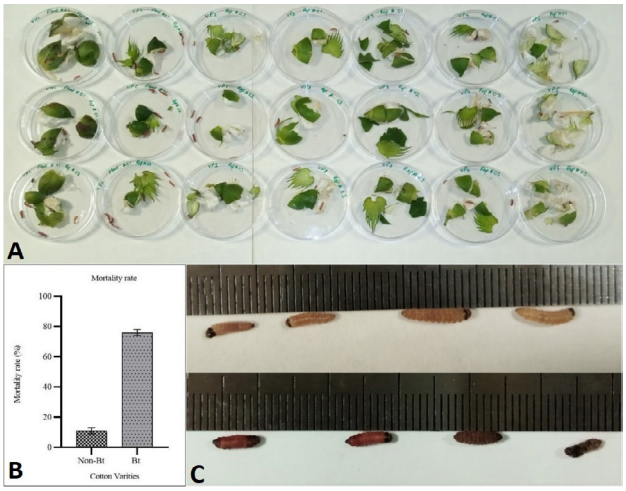

Fig. 4. *In-vitro* feeding assay of pink bollworm on non-Bt-cotton and Bt-cotton. (A) Second instar larvae of PBW fed on leaf and immature cotton boll samples collected from Bt and non-Bt cotton plants, (B) Percentage mortality of pink bollworm while fed on non-Bt-and Bt-cotton, (C) Morphology of PBW fed on Bt cotton before and after 5-days feeding trial. The alive larvae and dead larvae are clearly visible.

DISCUSSION

Insect resistance against the BT toxin was reported

many years ago. The resistance is developed more in Asian countries (Naik *et al*., 2020). Practical resistance in the pink bollworm against BT toxins was reported in India in 2002 and 2006, and in Pakistan in 2010 (Fabrick *et al*., 2023). The mutation in the cadherin gene has been reported to be involved in resistance development (Tabashnik and Carrière, 2019). The factor related to the resistance development was different; one of the major reported factors was the mutations in the receptor genes of the midgut. Mutations in the cadherin and v-ATPase genes were reported that cause resistance development in pink bollworms (Wang *et al*., 2020). In China, the resistance was increased initially but decreased when the non-Bt cotton refugee was introduced (Fabrick *et al*., 2023).

In Pakistan, moderate field-evolved resistance against Bt toxin was reported (Akhtar *et al*., 2016). We found no mutation in the cadherin coding sequence that depict no change in the protein structure as revealed through in-silico prediction tools. cadherin gene has multiple alleles responsible for the resistance development (Wang *et al*., 2022) and we have amplified one of such portions so there might be the possibility of potential mutations in the remaining coding sequences of the cadherin gene. Similarly, we found one missense mutation and three silent mutations in the v-ATPase gene. The v-ATPase gene has 14 subunits and two domains (Mohammed, 2016). Mutational screening of other alleles and coding sequences of both cadherin and v-ATPase genes in pink bollworms from different regions of Pakistan is required to confirm the resistance development due to mutations. In this study, the in-vitro feeding assay showed significant mortality in the Bt cotton compared to non-Bt.

The possible solutions to overcome the resistance are the refugee strategy, crop rotation, farmer's training, CRISPR Cas technique, and RNA interference (Adeyinka *et al*., 2023; Akhtar *et al*., 2016). This study would help to determine the cause of resistance development. In Pakistan, the mutational cause is not well studied yet. Only a few genes were studied and no significant mutations were reported before in Pakistan. The mutations in the present study are novel in the pink bollworm population of Pakistan. The resistance development against Bt due to mutations in cadherin and v-ATPase genes needs to be studied more with multiple gene screening in both laboratory-reared and field insects. The laboratory and field-evolved resistance can be determined after the comparison of resistance development in both groups.

CONCLUSION

Conclusively we found no mutations in coding regions of cadherin while three silent and one missense mutation were found in v-ATPase genes amplified from local PBW isolates. Significant mortality of the PBW in Bt-cotton suggests resistance to Cry toxins. A significant difference in mortality rates between the control and experimental groups was observed suggesting no significant resistance development in the Pakistani PBW population against Bt toxin.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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