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RESEARCH ARTICLE

STRATEGIES FOR IMPROVEMENT FOR CHARACTERIZATION AND QUALITY OF BT COTTON SEEDS

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Abstract

The study highlights diverse methodologies employed for seed quality assessment and characterization, including genetic, biochemical, and physiological analyses. In our present study, PCR and ELISA based techniques was adopted to identify the specific Cry1Ac gene and proteins in MECH 162 and RCH2 transgenic cotton plants. MECH 162 of 21.9ug/g and RCH2 of 17.37ug/g of seed protein was extracted. PCR analysis confirmed that gene specific CRY1Ac F/R primer as amplicon size of 550 bp for Bt gene, 600bp for NptII gene and 200bp for 35s promoter gene. The ELISA method was used for the validation of the developed assay, also ELISA needed simple equipment and took less time. Insights into the integration of these strategies into breeding programs are provided, emphasizing their crucial role in ensuring the production of high-quality Bt cotton seeds with improved agronomic performance and yield potential.

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Introduction:-

The DNA based techniques using PCR, RT-PCR, LAMP, RT-LAMP and Multiplex PCR are currently the major detection methods that are widely used due to their ease and accuracy in detection of GM and Non-GM crops [1]. The area cultivated with Bt-cottons expressing Cry1Ac gene increases year by year globally. The expansion of Bt-cotton cultivation is driven by several factors, including the desire to combat key pests like bollworms effectively, thereby reducing pesticide usage and increasing crop yields. [2]. Advancements in adoption of Bt transgenic cotton hybrids in India have been the most significant event and efficient detection strategies for genetically modified crops need to be in compliance with regulatory frameworks and address consumer concerns need fully [3]. RFLP were the first widely reported means of revealing DNA sequence variations in a diverse range of organisms, including varieties and the potential of RFLP for variety identification purposes has been confirmed [4]. RAPD and AP-PCR have been extensively used to fingerprint different plant species but soon showed limitations in reproducibility across laboratories and a lack of polymorphisms in some important crops such as wheat [5]. Bacillus thuringiensis is a bacterium that naturally produces some proteins that are lethal to insect larvae [6]. By transforming the genes that encode these proteins into cotton balls, scientists have created a type of cotton that produces its own pesticides, making it resistant to insects [7]. Bt cotton plant produces the Cry1Ac insect-control protein and the nptII selectable marker protein [8]. The development of unapproved seeds into the market has been the reason for controversies regarding the acceptance among the farmers made a need for GM detection and testing [9]. The analytic techniques

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are often by the quantitative and qualitative of the target analyte and hence conventional PCR is widely accepted for this purpose because of its specificity, sensitivity and reliability [10]. The findings support the recommendation to improve the existing quality criteria for transgenic cotton variety approval and certification [11].

Materials and Methods:-

Collection of seed material:

For the purpose of characterization and identification of Cry1AC gene in cotton seed samples of MECH–162 and RCH2, two varieties were procured from the retailer shop of Vijayawada, Andhra Pradesh. Cry1AC gene in cotton seed samples of MECH – 162 and RCH 2, two varieties were procured [12].

Preparation of seed smoothie:

Cotton seeds were cleaned thoroughly with distilled water to remove contaminants and dust and were made into a smoothie with the help of a mortar and pestle and collected into a sterile test tube. These tubes were centrifuged at 10,000 rpm for 3-5 minutes. The supernatant was separated and stored for analysis against blood samples [13].

DNA extraction:

Genomic DNA was extracted from cotton seeds incubated with liquid nitrogen by CTAB method using autoclaved mortar and pestle. Extracts were placed in water bath (with gentle shaking) for 60 minutes at 65^0C with periodical shaking at an interval of five minutes. Ten ml of Chloroform: Isoamyl alcohol mixture (24:1) was added to extract and the contents were mixed by shaking then the tubes were centrifuged for 10 minutes at 10,000 rpm at room temperature. Equal quantity of chilled isoproponal was added to each tube and mixed by inverting and incubated at -20^0C for overnight. The content was centrifuged for ten minutes at 10,000 rpm at 40^0C. The supernatant was discarded. The DNA pellet obtained was washed with 70 percent ethanol and the tubes were inverted on blotter paper to dry the pellet. The DNA was dissolved in 100µl TE buffer and stored at -20^0C for further study. The quality and quantity of DNA were estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analyzed by 0.8% agarose gel electrophoresis [14].

PCR Analysis:

PCR analysis of transgenic cotton was carried out by using the primer CRY1AC F/R [15]. The primer sequence and their amplicon size are shown in Table 1 synthesized from Bioserve Biotechnologies, Hyderabad. PCR amplification was carried out using 25 µl reaction mixtures containing Taq DNA polymerase (Genei, Bangalore) 25µM of each forward and reverse primers, 200 mM of each dNTP and 3 µl DNA. The master mix of 25 µl was added to PCR tubes and was given a short spin to mix the contents. The tubes were placed in the thermal cycler for amplification with cycling conditions: Initial denaturation: 94°C for 4 min, Final denaturation: 1cycle of 94°C for 30 s, Annealing: 50°C for 50 s, Primer extension :72°C for 30 sec for 35 cycles. PCR product was subjected to gel electrophoresis containing 2% agarose gel and bands were captured under UV light (254-366 nm) by UV- Gel Documentation System (UVI-Tech, Germany) [16].

Table 1:- Primer used for detection of Cry1AC gene in cotton seed samples.

Bt	Forward Primer	TACTTGGTGGAGAACGCATTGAA
	Reverse Primer	GAGGTCAACTAGTCCGACAACGAA
NptII	Forward Primer	CGCTATGTCCTGATAGCGGTCC
	Reverse Primer	CACAACAGACAATCGGCTGCTC
35S promoter	Forward Primer	GCTCCTACAAATGCCATCA
	Reverse Primer	GATAGTGGGATTGTGCGTCA

Analysis of Cry1AC protein by ELISA

96 wells microtiter plate, precoated with anti-Cry1Ac antibodies (envirologix) was taken. On one side, 50 µl of positive control of Cry1Ac (envirologix) was added to two wells on opposite corners while on the opposite side, 50 µl negative control of Cry1Ac (envirologix) was added to two wells on the opposite corners. Contents were mixed well by gentle shaking and incubated at room temperature for 30 minutes. After washing, to each well 100 µl of substrate solution (TMB) was added and kept for 20 minutes at room temperature for further incubation. On completion of the incubation interval, 100 µl of stop solution (Sulphuric acid) was added to each well immediately. The absorbance of contents from each well was then measured at 450 nm using ELISA reader, (Thermo Multiskan) along with the positive and negative control well [17].

Results and Discussion:-

DNA extraction

The DNA yield was very high ranging from 26 – 50 mg per 100 mg of fresh tissue. The A_{260}/A_{280} was found to be 1.89 ≅2.3, suggesting that the isolated DNA was free of proteins. Comparison of relative DNA yields obtained with the extraction of MECH162 and RCH 2 is shown in figure 1 along with DNA marker (Genei, Bangalore).

Figure 1:- Agarose gel electrophoresis of genomic DNA extracted from MECH162 and RCH2 variants of cotton seeds, M-DNA marker, Lanes 1 & 2 DNA from MECH162 and Lane 3 DNA from RCH2.

PCR Analysis

PCR based approach is carried out for detection, identification and gene stability confirmation of cry1Ac transgene construct in Bt cotton. By using Cry1AC primers for MECH162 as shown in figure 2 and 3 and nptII primers for RCH2 as shown in figure 4 and 5 for the BT gene approximately a 550 bp amplicon was obtained as PCR product.

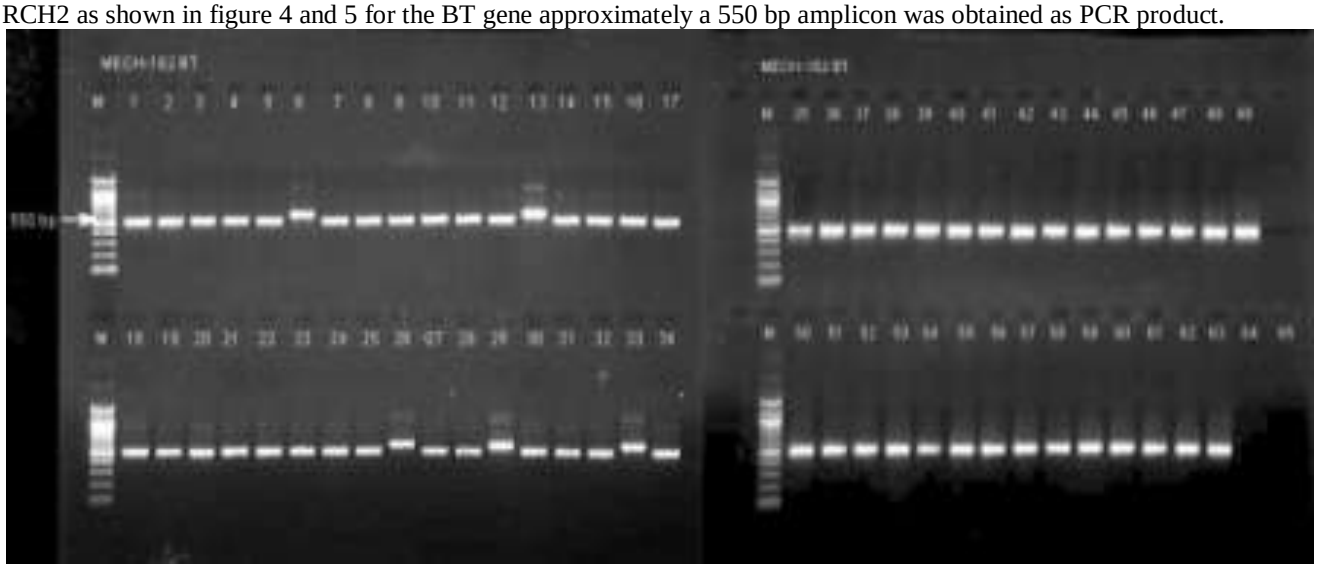

Figure 2:- PCR amplicon patterns for MECH162 generated by Cry1AC primers. M-100 base pair molecular weight marker, Lanes 1 to 65 are BT cotton samples of MECH 162. Lanes 6, 13, 26, 29, 33 were found to be negative. (An amplicon of 550 bp was not generated in these lanes).

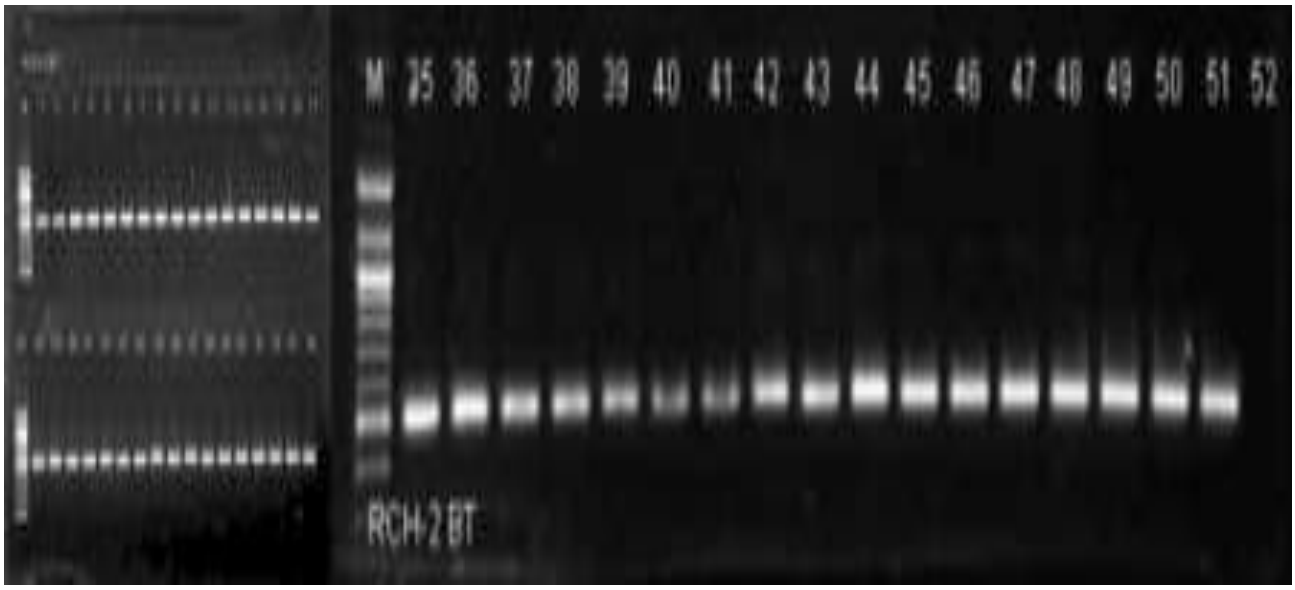

Figure 3:- PCR amplicon patterns for RCH2 generated by Cry1AC primers. M-100 base pair molecular weight marker, Lanes 1 to 65 are BT cotton samples of RCH2. Lane 52 was found to be negative. (An amplicon of 550 bp was not generated in these lanes).

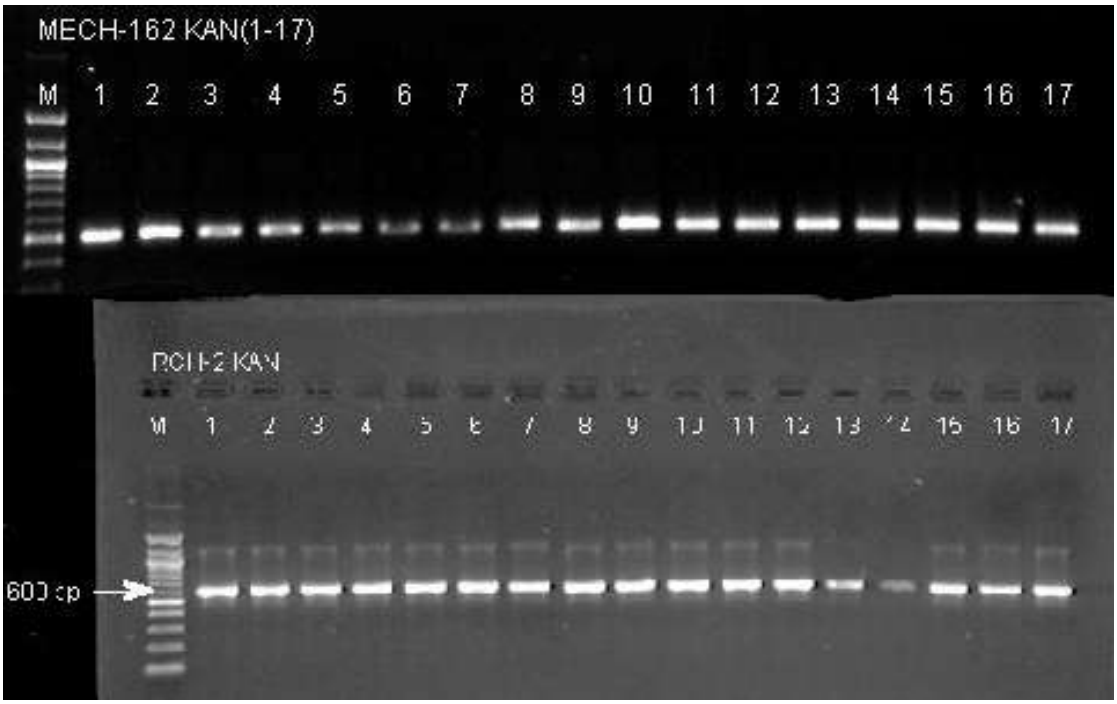

Figure 4:- PCR amplicon patterns for MECH162 and RCH2 generated by nptII primers**.** M-100 base pair molecular weight marker, Lanes 1to 17 are BT cotton samples of MECH162 and RCH 2 respectively. By using primers for nptII promoter approximately a 600 bp amplicon was obtained as PCR product.

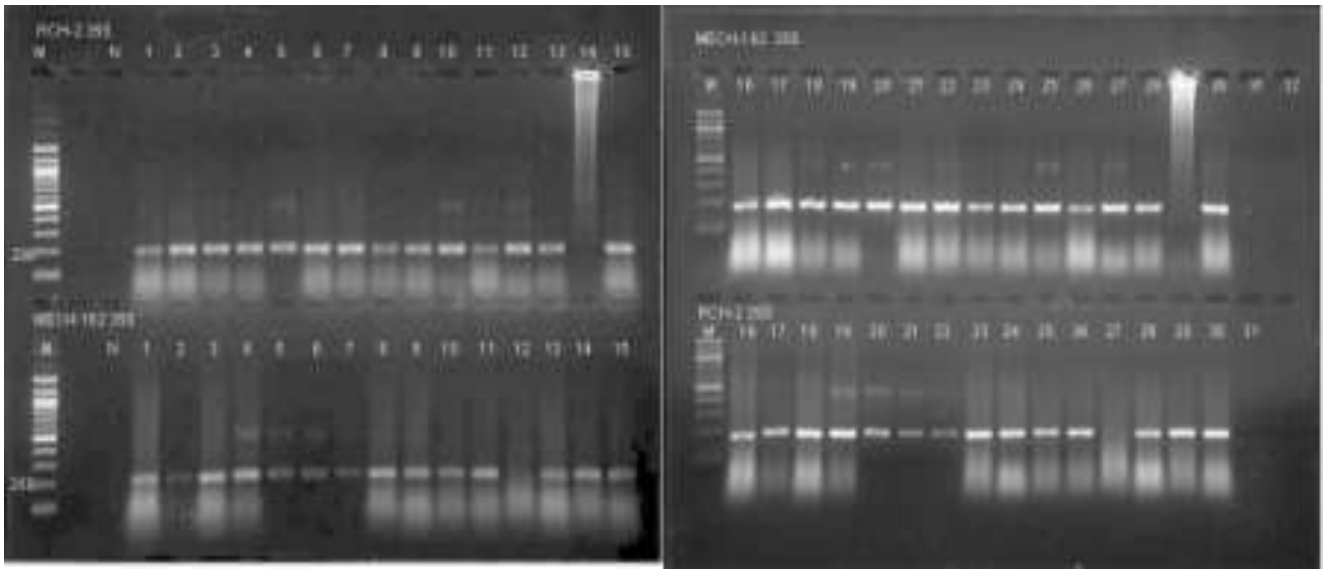

Figure 5:- PCR amplicon patterns for MECH162 and RCH2 generated by 35s promoter primers. M-100 base pair molecular weight marker, Lanes 1to 17 are BT cotton samples of MECH162 and RCH 2 respectively. By using primers for 35S promoter approximately a 200 bp amplicon was obtained as PCR product. Lane 14 of RCH 2 was found to be negative & remaining are positive.

Conclusion:-

It can be concluded that both PCR based and ELISA based screening for the purity of commercial Bt Cotton hybrids was successful. The results indicate that the commercial Bt Cotton seeds sold in the market place are not 100% pure. Enhancing the characterization and quality of Bt cotton seeds is pivotal for sustainable agriculture and the prosperity of farming communities. Through comprehensive strategies, we can achieve significant improvements in this regard. From this study, it can be concluded that RCH 2 samples are pure than MECH 162. It can also be concluded that the technology used in the presented study is perfect and can be used for regular screening of Bt Cotton hybrid seed.

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