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DETECTION OF *CRY*1AC AND *CRY*2AB PROTEINS WITH ELISA AND PCR TECHNIQUES IN TRANSGENIC COTTON (*GOSSYPIUM HIRSUTUM* L.)

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ABSTRACT

To develop BGII hybrids/varieties, the elite lines which were proven for high yield with jassid resistance were selected and converted under BGII background for further usage in BGII hybrid development programme. Hence, the study was aimed to detect the presence of *Cry*1Ac and *Cry*2Ab proteins/genes through qualitative ELISA, PCR techniques and to select the homozygous population from the segregating generations consisting of 1663 samples from different segregating generations as well as from stabilized populations from OYT/PYT/AYT yield trials. The study revealed that out of 9125 samples, 8760 (96%) of samples showed the presence of *cry* 1Ac and *cry* 2Ab proteins in the transgenic leaf samples and also demonstrated that the technique of ELISA for identification of c*ry* 1Ac and c*ry* 2Ab proteins is quite handy and easily adoptable. Using PCR technique, the zygosity status of the samples were known for c*ry*1Ac and c*ry*2Ab genes; used for selection and advancement to next generations in the breeding programme.

Keywords: Cotton (*G.hirsutum* L.), c*ry*1Ac, c*ry*2Ab, ELISA, PCR and Primer

INTRODUCTION

Till date no GM crop/products are allowed in India to be marketed. However, Bt-cotton is the only transgenic crop, approved for commercial cultivation in India. In India, since 2002 the cultivation of Bt cotton hybrids consisting of the *Cry*1Ac gene (BG I with the event MON 531 developed by Monsanto company) played a vital role in the effective control of boll worms like *Helicoverpa armigera* (Hubner), *Erias vitella* (Hubner) and *Pectinophora gossypiella* Saunders) (Naik *et al*., 2018, Arain *et al*., 2021 Likhitha *et al*., 2023). To maintain delay bollworm resistance to BGI, second-generation Bt cotton, BGII with the event MON 15985 was developed by Monsanto company that expressed two *Cry* toxins (*Cry*1 Ac and *Cry*2Ab) and was commercialized in India (2006), occupied > 95 per cent of traditional non-Bt cotton cultivation in India (Kumar *et al*., 2015, Venugopalan and Reddy, 2017). This invention protected the cotton crop from bollworms and lead to reduced pesticide usage up to 20 to 40% whereas in past five to six years pink bollworm became a menace (Kranthi, 2016 and Naik *et al*., 2018). Hence, plant breeders focussed more on developing BGII cotton varieties as it is

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providing resistance to the pests *Helicoverpa armigera* (Hubner) and *Erias vitella* (Hubner). To identify the trait positive plants in segregating generations and to select the homozygous samples ELISA followed by PCR techniques are very much effective while breeding for BGII cotton elite lines with high yield and resistance biotic stresses especially boll worms. The ELISA technique includes the identification of proteins produced by the introduced trait gene through the detection of its specific antibody, such as by Enzyme Linked Immunosorbent Assay (ELISA) (Dohare and Tank, 2014; Khetarpal and Kumar, 2017), while second technique employs the identification of specific DNA sequence used for gene modification by Polymerase Chain Reaction (PCR) (Hussain *et al*., 2014; Cheema *et al*., 2016). Based on the above advantages the research work was proposed to test the BC_3F_4 generation lines developed through back cross breeding as well as homozygous populations from different yield trials'

MATERIAL AND METHODS

Sample preparation for Qualitative assay:

Leaf sample preparation: Taken 3 leaf disc in 2 mL centrifuge tube and then added 1 autoclaved stainless-steel bead to the tube. After that dipped all the sample tubes in liquid nitrogen for 10 min. Added 500 µL of 1X Extraction Buffer to the tube. Proceed all the tubes for crushing on tissue homogenizer for 30 sec. Transferred all the content from 2 mL tube to 1.5 mL tube followed by centrifugation of all the tubes at 10000 RPM for 10 min. at 4 ºC. Kept all the tubes to 4 ºC until use.

Protocol for Qualitative Assay:

Added 50 µL leaf sample extract to the precoated plate followed by 50 µL Conjugate was added. Later 50 µL of Blank, Controls, to respective wells were added. Incubated the plate at room temperature for 45 minutes. Decantated the content from the plate after incubation. Washed the plate 4 times and pat dry the plate after last wash . Added 100 µL of Substrate to the plate. Incubate the plate at room temperature for 15 min. strictly in dark. Added 100 µL of Stop solution to the plate . Measured the absorbance at 450 nm using an ELISA plate reader (Multiskan FC ESW version 1.01.16, make Thermofisher). The absorbance of a blank well must be subtracted from absorbance values of samples and controls. Optical density (OD) values were used to determine the presence or absence of a specific antigen or antibody in a sample. The OD of the wells is then measured using a microplate reader at a specific wavelength, typically 450 nm for most ELISA. The OD value is a measure of the intensity of the color developed in each well.

Assay Acceptance Criteria and Interpretation:

The OD value of blank well should be below 0.200 for cry1Ac and 0.250 for cry2Ab. Subtracted the OD value of blank from all samples including positive and negative control. The mean OD value of negative control(s) should be below 0.150. If negative control showed negative (minus) readings then considered that reading as zero. The OD value of 2 positive controls should be within 15% of each other. Mean OD value of positive control should be at least 0.50. The cutoff value was calculated by adding the mean O.D. of negative control to the numerical value 0.2 for cry1Ac detection and 0.1 for cry2Ab detection. The positive sample are the one the samples showing absorbance above cutoff value while the samples showing absorbance below cutoff value ate negative samples.

DNA Extraction Procedure:

DNA extraction was performed using CTAB method as suggested by (Doyle & Doyle 1990). Two to three punches of leaf sample in

PCR Method to detect *cry*1Ac and *cry*2Ab zygosity status:

The PCR analysis carried out in a proflex thermal cycler, Thermofisher, U.S.A. A total reaction volume of 20 µl was prepared using the following protocol

Event/ Trade Name	Genes	PCR Primers	PCR Reaction Conditions		Source
MON531	*cry*1Ac	SQ95 (1075 bp) SQ525 SQ524 (810bp)	Each primer concentration (1µM)	0.4 µl of 0.2 µM concentration	GMO METHODS
			dNTP mix (10mM) 10x Reaction Buffer (with MgCl2) Taq	0.4 µl 2µl 1µl	
			Template DNA	50 ng	
			DD water	10.4 µl	
MON15985	*cry*2Ab	ESQ119 (1056bp) SQ121 SSQ120 (1760bp)	Each primer concentration (1µM)	0.4 µl of 0.2 µM concentration	GMO METHODS
			dNTP mix (10mM) 10x Reaction Buffer (with MgCl2) Taq	0.4 µl 2µl 1µl	
			Template DNA	50 ng	
			DD water	10.4 µl	

2 mL centrifuge tubes were collected and kept all sample tubes in ice. Added small and big sized steel beads to each tube and then placed all the sample tubes in tray and dumped them in container containing Liquid Nitrogen and left it in container for 10 minutes. After 10 minutes, took out the tray and proceeded to crushing for 1 minute on machine crusher. Added 850 µL of prewarmed CTAB Buffer (having 300 µL of beta mercaptoethanol to 10 mL of CTAB Buffer) to all the tubes as soon as possible (before sample thawing). Shook the tubes well so as to mix the content. After that all the contents were transferred to new 2 mL centrifuge tubes and incubated all the tubes to 65°C for 1 hour (Inverted the tubes occasionally). Later added 850 µL of Chloroform: Isoamyl Alcohol (24: 1) to the tube sand inverted properly for 10-15 times and centrifuge all the tubes at 9000 RPM for 20 min. at room temperature. In a new 1.5 mL centrifuge tube supernatant was taken and added 10 µL of RNAse enzyme (10mg/mL stock) to all the tubes. Incubated the tubes at 37°C for 20 minutes. Later added Chloroform: Isoamyl Alcohol (24: 1) in ratio equivalent to volume of supernatant. Invert the tubes properly for 10 to 15 times and centrifuged all the tubes at 9000 RPM for 20 minutes at room temperature. Took the supernatant

Number of Cycles	Settings (°c) of Proflex PCR	Time
1	94	3 minutes
38	94	15 seconds
	60	30 seconds
	72	1 minute
1	72	5 minutes

The expected band size of the amplicons was as follows:

Amplicon name	Expected band size (bp)
Non-transgenic cotton	1075
531 homozygous cotton	810
531 heterozygous cotton	1075 and 810

The expected band size of the amplicons was as follows:

Amplicon name	**Expected band size (bp)**
Non-transgenic cotton	1050
15985 homozygous cotton	1760
15985 heterozygous cotton	1050 and 1760

(approximately 410 µL) in new 1.5 mL centrifuge tubes and the added 2.5 volumes of prechilled absolute ethanol. Kept all the tubes in -20°C for 10 min (or overnight). The next day centrifuged all the tubes at 9000 RPM for 20 minutes at room temperature. After that discarded the absolute ethanol and added 500 µL of 70% Ethanol. Tapped the pellet of DNA in each tube well. Centrifuged all the tubes at 10000 RPM for 20 min. at room temperature and discarded the 70% Ethanol without disturbing the pellet followed by added 500 µL of 70% Ethanol. Tapped the pellet of DNA in each tube well and centrifuged all the tubes at 10000 RPM for 15 min. at room temperature and discarded the 70% Ethanol without disturbing the pellet. After that air dried the DNA pellet in DNA Concentrator (Eppendorf make) set at 37°C for 15 minutes. Added approximately 100 µL of TE buffer and dissolved the DNA well in all the tubes and kept all the tubes at -20°C for long term storage.

The sequences of primers for, MON531 for cry1Ac and MON15985 for cry2Ab were recovered from the GMO Detection Method Database (GMDD; www.gmdd.shgmo.org).

The Proflex PCR (make: Thermofisher) was used to perform the PCR and the following were the PCR conditions https://patents.google.com/patent/WO2002016588A3/en :

Agarose gel electrophoresis

Eight ìl of PCR products (including PCR amplification products, positive and negative controls) were electrophoresed on 3% agarose gel containing 2 ìl DNA safe stain at 80 Voltage in 1× TBE (Himedia make) running buffer (containing 600 ml dH_2O, 48.4 g Tris base, 11.42 ml glacial acetic acid and 40 ml EDTA

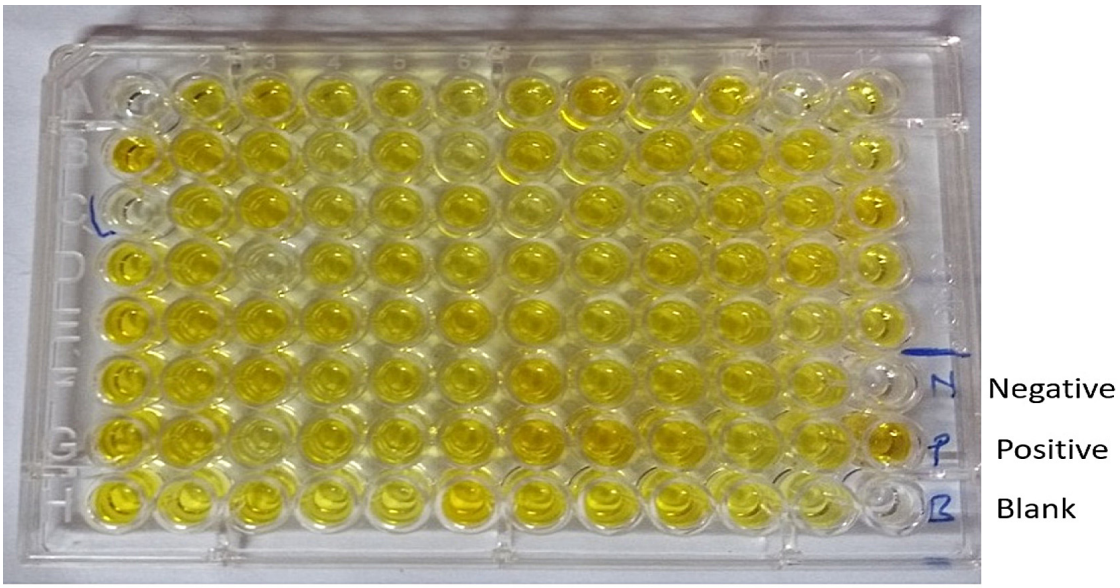


Figure 1. Detection of *Cry*1Ac proteins in cotton leaf samples through qualitative ELISA

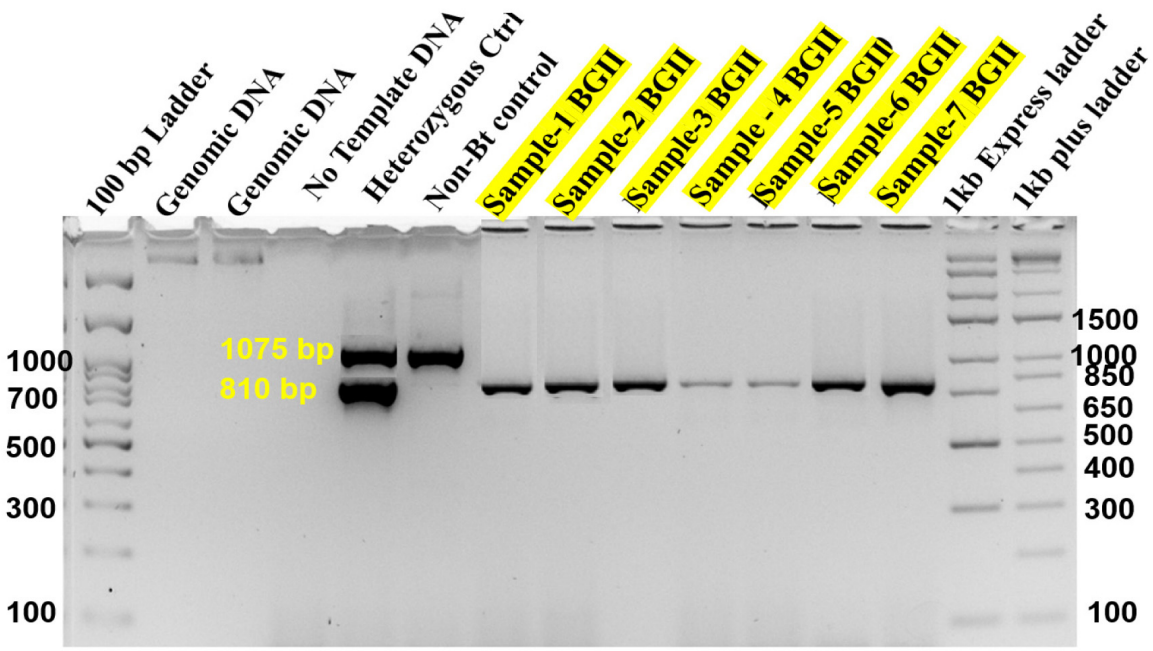


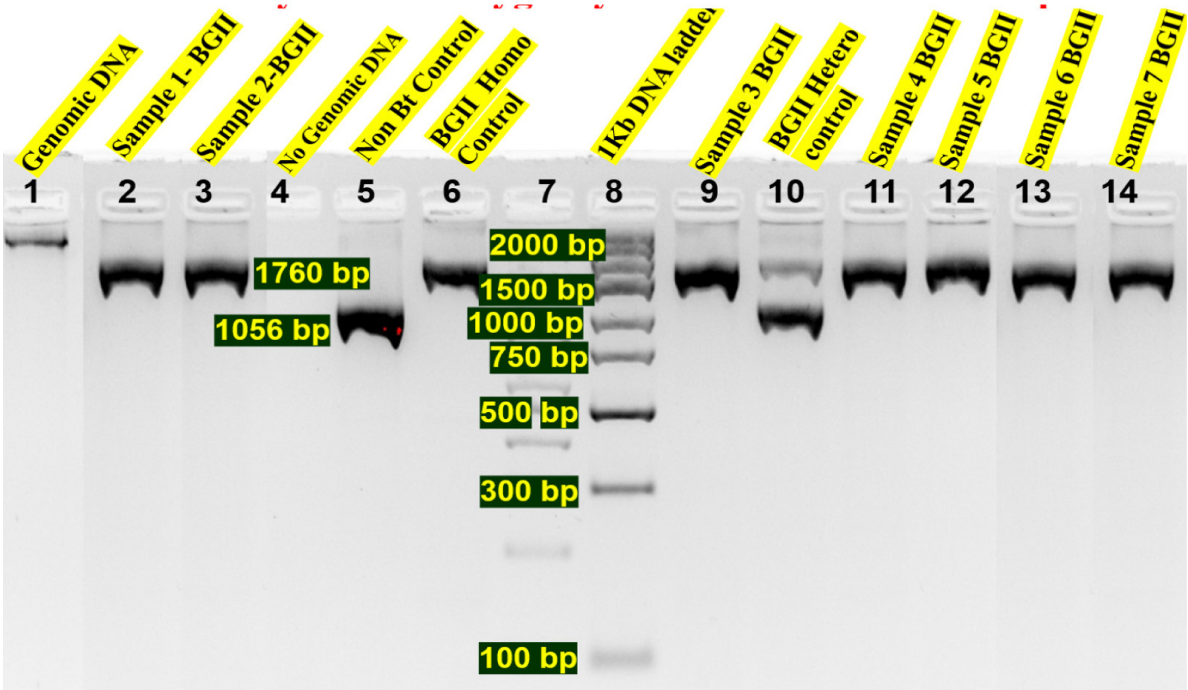
Figure 2. Detection of *cry* genes homozygosity status in cotton leaf samples. a) *cry* 1Ac

(0.5 M), PH 8.0, then dilute with dH2O to obtain a final volume of 1 L) for 60 min. 4 ìl of 100 bp DNA ladder was used as a reference marker. DNA fragments were separated through a gel based on size and then visualized using chemidoc (Thermofisher make).

RESULTS AND DISCUSSION

From different yield trials like OYT (2208 samples), PYT (2004 samples), AYT (1465 samples), uniform bulks (3448 samples) and segregating generation of BC_3F_4 (957 samples) were tested for confirmatory study of *cry*1Ac

b) c*ry* 2Ab



and *cry*2Ab proteins in cotton leaf samples. The ELISA plates were prepared with blank, positive and negative controls as shown in Figure 1. The mean absorbance values were detected using ELISA reader, Multiskan FC ESW version 1.01.16 (make Thermofisher) at 450 nm and presented in the Table 6. SkanIt Software 4.1 for Microplate Readers RE, ver. 4.1.0.43 was used to calculate cut off value for the presence of c*ry*1Ac and c*ry*2Ab. The cutoff value was calculated by adding the negative control value with 0.2. Always the negative control values would be less than 0.1 and blank values were to be 0.05. The samples exhibiting the absorbance values of less than 0.3 were considered as negative for the presence of both c*ry*1Ac and c*ry*2Ab proteins, whereas the samples exhibited absorbance vales of e" '+0.3 were considered to be as positive for the traits. The mean absorbance values of the BGII samples evidently displayed the occurrence of proteins in the samples while some samples showed negative mean absorbence values as they did not have the genes (non bt cotton lines). The samples which exhibited both trait positive was subjected to one more time confirmation and those samples were tested with PCR for homozygosity using zygosity primers for stabilization of the lines under c*ry*1Ac and c*ry*2Ab background as shown in Figure 2. The samples which possessed homozygous for both the traits were selfed and advanced to BC_3F_4 /F_3 to F_4 generation/maintaince of bulks. Similar reports were reported by Alka and Tank, 2014, Ramanjali *et al*., 2015, Shahid *et al*., 2015, Cheema *et al*., 2016, Likhitha *et al*., 2023. Out of 9125 samples from yield trials, 8760 (96%) samples exhibited positiveness for both the traits while from the segregating generations out of 1663 test samples 1297 (78%) only showed positiveness for *cry*1Ac and *cry*2Ab proteins.

CONCLUSIONS

From the above study, it was evident that ELISA technique was a handy technique for identification of c*ry*1Ac and c*ry*2Ab proteins in the cotton leaf samples for the advancement of trait specific positive selections in cotton crop improvement programme and easily adoptable. Few limitations observed from the assay were high light sensitivity, required more time for assay coupled with high costs for purchase of precoated plates. In cotton aged plants (more than 120 days) showed less protein expression for c*ry*1Ac and c*ry*2Ab hence using ELISA technique at this stage did not provide accurate results. The early detection of positive samples for c*ry*1Ac and c*ry*2Ab in the segregating generations with ELISA technique saved many resources in the breeding programme. The ELISA technique followed by detection of events with PCR for homozygosity practiced for efficient conversion of cotton elite lines under c*ry*1Ac and c*ry*2Ab background. This procedure may also be applied in identification of the purity issues for varietal samples.

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