Confirmation of the absence of 'terminator gene' i.e. a patented embryogenesis deactivation system in Mahyco Bt cotton hybrids

Chapter III

Annexure 3

Report on the study titled : "Confirmation of the absence of Terminator gene i.e., a patented embryogenesis deactivation system in MAHYCO Bt cotton hybrids"

Objective :

The purpose of the present study was to ascertain the presence/absence of *cre* recombinase gene (using molecular approaches) in Bt cotton hybrids provided by the Maharashtra Hybrid Seed Company Limited (MAHYCO). Absence of the above gene, which is an integral component of the "terminator technology", would in turn, indicate the absence of the "terminator genes" in the supplied hybrids. The plants would also be tested for the presence of *cry1Ac* gene.

Methodology :

(1) Growth and Maintenance of cotton plants:

Seed samples of three Bt cotton hybrid lines namely, MECH 12, MECH 162 and MECH 184 were provided to us by MAHYCO. These three Bt cotton hybrids along with the non-transformed cotton line were germinated in an autoclaved mixture of soil and soil rite in a 1:1 ratio and grown under containment in growth chambers (Conviron) under the following conditions: 16 hours Day, 8 hours Night; Day temperature : 30°C; Night temperature : 24°C; Humidity : 70%.

(2) Isolation of DNA and PCR conditions:

DNA samples from individual seedlings of each transgenic line and from the non-transformed line were isolated from young expanded leaves using the DNeasy Plant Mini Kit (Qiagen) according to manufacturer's instructions. The presence/absence of the *cry1Ac* and *cre* gene(s) was determined by PCR using appropriate primers and amplification conditions as outlined below.

1. **Amplification of *cry1Ac* gene:**

   Forward primer: 5' - GGT CTC CAC CAG TGA ATC CTG G - 3'

Reverse primer: 5' - CTG CTC AGC GAG TTC GTG CC - 3'

Expected product size: 1356 bp

A plasmid containing the *cry1Ac* gene (available in our laboratory) was used as a positive control in all amplification reactions. Optimized reaction conditions for each amplification included ~500ng of template genomic DNA, 50pmol of each primer, 200µM of each dNTP, 2U of Taq DNA polymerase (Finnzyme) and the supplied reaction buffer at 1X concentration in a final reaction volume of 100µl. Cycling parameters were as follows: initial denaturation at 94°C, 5min followed by 40 cycles each of 94°C, 30sec; 52.5°C, 30sec; 72°C, 1min 40seconds. A final extension at 72°C for 5min was given.

2. Amplification of *cre* gene

Forward primer: 5' – ATG TCC AAT TTA CTG ACC GTA - 3'

Reverse primer: 5' – CTA ATC GCC ATC TTC CAG CAG - 3'

Expected product size: 1031 bp

Amplifications using *cre* primers were restricted to lines that showed an amplified *cry1Ac* product. DNA derived from the bacteriophage P1 (the natural source of *cre* gene) as well as a plasmid containing the *cre* gene (available in our laboratory) were used as positive controls in all amplification reactions. Optimized reaction conditions for each amplification included ~500ng of template genomic DNA, 50pmol of each primer, 200µM of each dNTP, 2U of Taq DNA polymerase (Finnzyme) and the supplied reaction buffer at 1X concentration in a final reaction volume of 100µl. Cycling parameters were as follows: initial denaturation at 94°C, 5min followed by 40 cycles each of 94°C, 30sec; 60°C, 30sec; 72°C, 1min. A final extension at 72°C for 2min was given.

PCR products were electrophoresed on a 1% agarose gel with appropriate markers to determine size(s) of the amplified products.

Results :

Results obtained from PCR analysis of various transgenic lines and the non-transformed line are summarized in Table 1 below.

Table 1

S.No.	Transgenic line	Plant No.	Amplification using *cry1Ac* primers	Amplification using *cre* primers
1.	MECH 12	1	+	-
		2	+	-
		3	+	-
2.	MECH 162	1	+	-
		2	+	-
		3	-	-
		4	+	-
3.	MECH 184	1	-	NT
		2	+	-
		3	-	NT
		4	+	-
4.	Non-transformed cotton line		-	-

'+' : positive; '-' : negative; NT : not tested.

t was observed from PCR analyses that seedlings from the Bt cotton hybrid lines which tested ›ositive for *cry1Ac* amplification did not show any amplification product using the *cre* primers. he non-transformed cotton line tested negative for *cry1Ac* and *cre* amplification. Plasmids ontaining the *cry1Ac* and the *cre* genes, which were used as positive controls, showed mplification products for *cry1Ac* and *cre*, respectively. Amplification profiles of various lines :sted using *cry1Ac* and *cre* primers are shown below in Figure 1 and Figure 2 respectively.

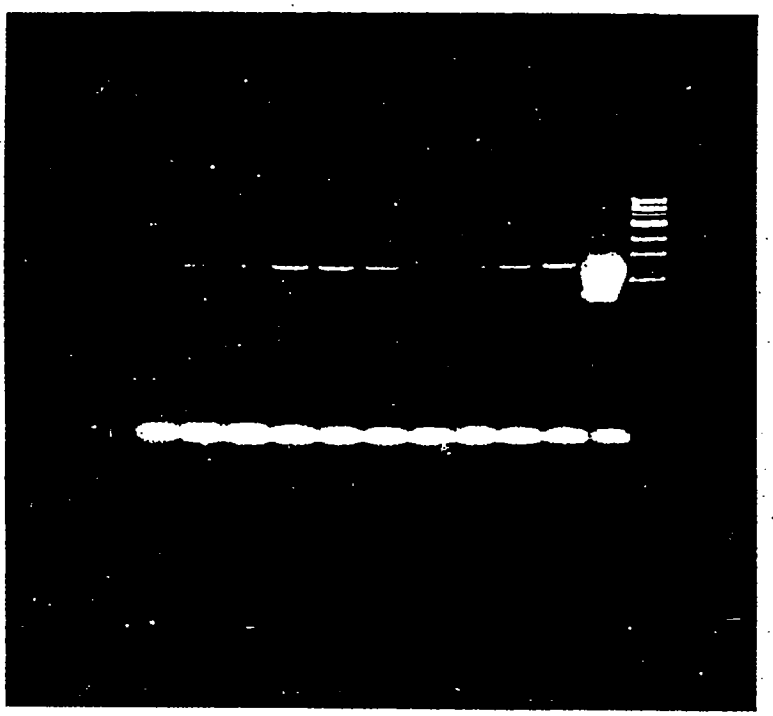

Figure 1: amplification profiles using *Cry1Ac* primers: lane 1: non-transformed cotton plant, lane 2: MECH 12 plant no. 1, lane 3: MECH 12 plant no. 2, lane 4: MECH 12 plant no. 3, lane 5: MECH 162 plant no. 1, lane 6: MECH 162 plant no. 2, lane 7: MECH 162 plant no. 3, lane 8: MECH 162 plant no. 4, lane 9: MECH 184 plant no. 2, lane 10: MECH 184 plant no. 4, lane 11: positive control, lane 12: 1kb ladder marker (NEB).

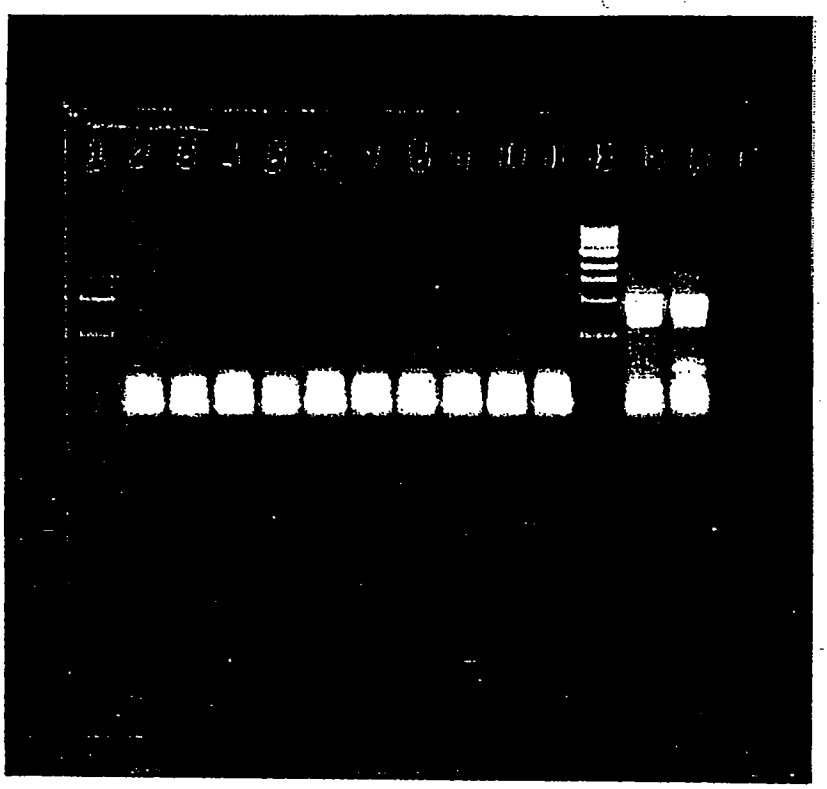

Figure 2: amplification profiles using *cre* primers: lane 1: 100bp ladder marker (NEB), lane 2: non-transformed cotton plant, lane 3: MECH 12 plant no. 1, lane 4: MECH 12 plant no. 2, lane 5: MECH 12 plant no. 3, lane 6: MECH 162 plant no. 1, lane 7: MECH 162 plant no. 2, lane 8: MECH 162 plant no. 3, lane 9: MECH 162 plant no. 4, lane 10: MECH 184 plant no. 2, lane 11: MECH 184 plant no. 4, lane 12: 1 kb ladder marker (NEB), lane 13: positive control (plasmid containing *cre* gene), lane 14: positive control (eluate of *cre* gene)

To confirm our observations in plants which did not show any amplification with the cry1Ac primers, genomic DNA was re-isolated from the above plants [MECH 162, plant no. 3; MECH 184, plant nos. 1 and 3] along with plants MECH 162, plant no.2 and MECH 184, plant no. 2, which had earlier shown amplification with the *cry1Ac* primers (used as positive controls). We found our earlier observations to be reproducible in all the above events and the amplification profile using *cry1Ac* primers is shown in Figure 3 below.

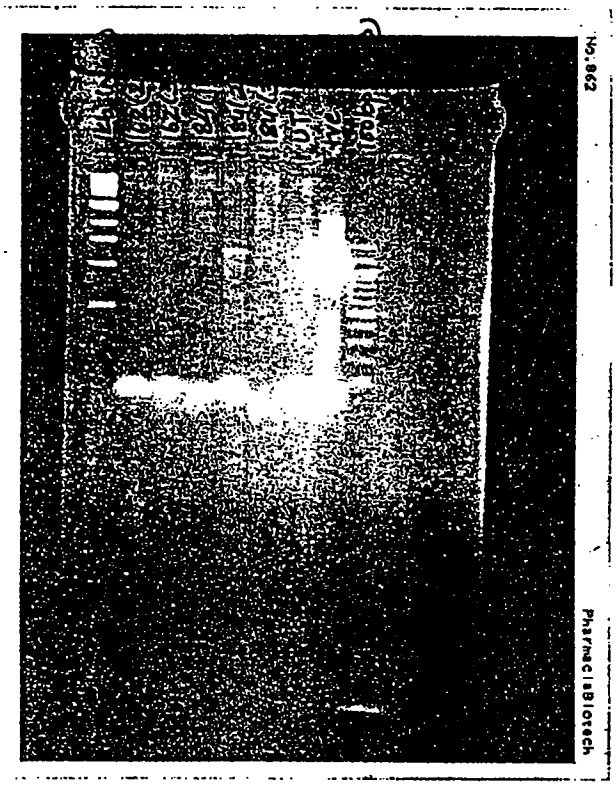

Figure 3: amplification profiles using *cry1Ac* primers: lane 1: 1kb ladder marker (NEB), lane 2: MECH 162 plant no. 2, lane 3: MECH 162 plant no. 3, lane 4: MECH 184 plant no. 1, lane 5: MECH 184 plant no. 2, lane 6: MECH 184 plant no. 3, lane 7: non-transformed cotton plant, lane 8: positive control, lane 9: 100 bp ladder marker (NEB).

Conclusion :

Based on absence of *cre* gene in the plants derived from Bt cotton hybrid lines MECH 12, MECH 162 and MECH 184, it can be concluded that the lines containing the Bt *cry1Ac* gene are devoid of the "terminator technology". Since these Bt cotton hybrid seeds were produced by traditional plant breeding methods from the original Bt cotton transformant, it is expected that all Bt cotton hybrids are devoid of the "terminator technology".

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