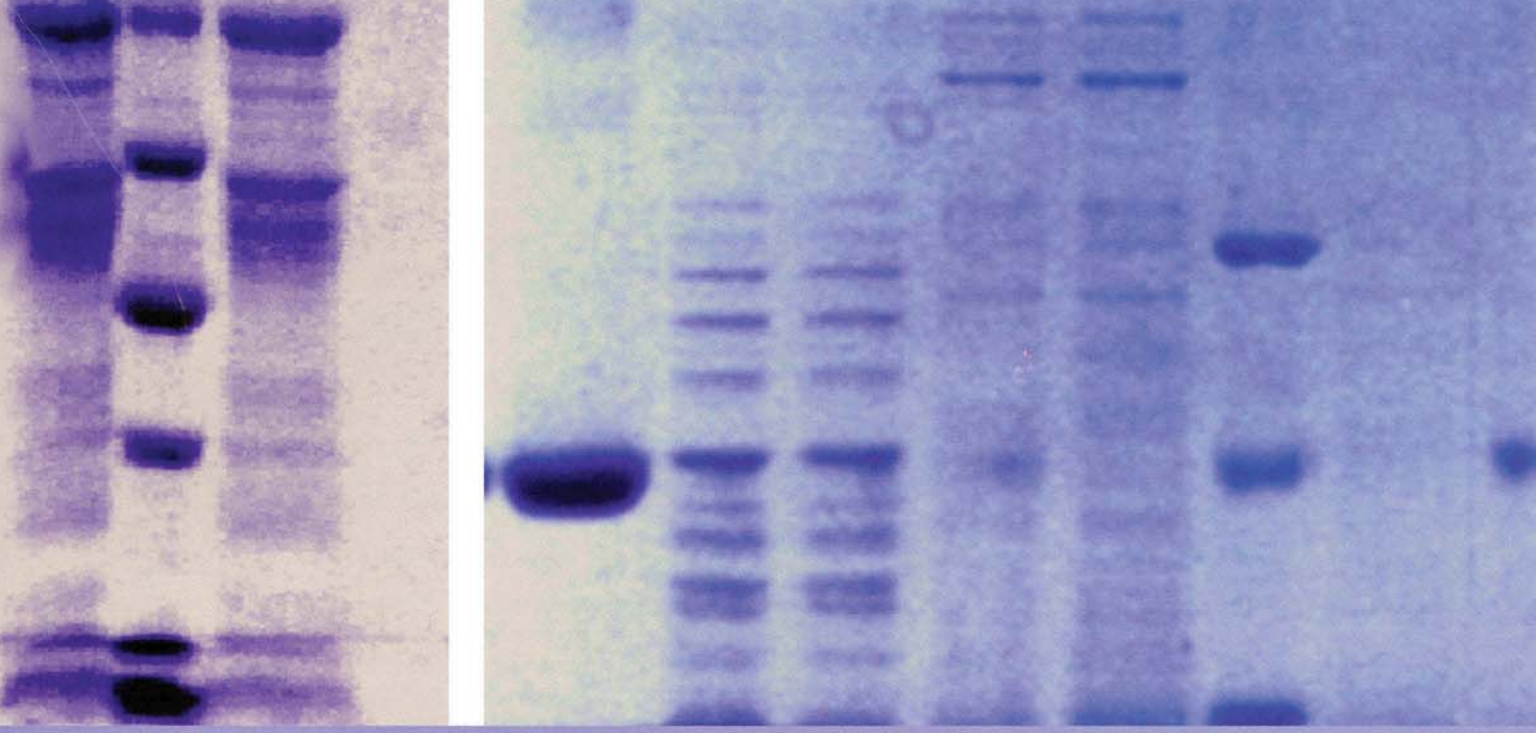

TMC MM I 3.4: Development of farmer friendly diagnostic kits for transgene event seed purity

INTRODUCTION

The CICR has been providing about 12 to 13 thousand ELISA and Bt-Express kits each year to seed testing labs and seed companies. There have been no problems with the quality of the plates or with reference to the performance. Minor operational problems do arise depending on persons using it.

Mostly Cry1 Ac and Cry2Ab detection kits have been used very commonly. Six Bt cotton events have been thus far in India for commercial cultivation. There are four Bt Cotton events expressing Cry1 Ac, one event with Cry1 C, and one event with Cry2Ab2

1. MON531 (Cry1Ac) event of Monsanto
2. JLEvent-1 (Cry1Ac) eventofJK seeds
3. GFM Cry1A (Cry1Ac) event of China, introduced by Nath seeds India
4. BNLA106 (Cry1Ac) event developed by NRCPB and UAS Dharwad
5. Event 9124 (Cry1 C) event developed by Metahelix, India.
6. Mon15985 (Cry2Ab2) event present with Mon531 in Bollgard-II Monsanto.

All the Cry1Ac genes present in the four events released in India are chimeric fusion genes. The Cry1Ac gene in the Bollgard event 531 is a chimeric gene of 3534 bp size, with the first 1398 nucleotides (corresponding to the first 466 amino acids) of *Cry1*Ab gene and rest of the 1399-3534 nucleotides (corresponding to the 467-1178 amino acids) from the Cry1Ac gene. Except for one amino acid at 766 position, the *Cry1Ac* amino acid sequences are identical to that of the wild type Cry1Ac gene. The chimeric gene produces a protein that is 99.4% identical to that of the wild type Cry1Ac. The Cry1Ac genes in JK and BNLA 106 are chimeric fusion genes of 1842 bp with the first 1398 nucleotides (corresponding to the first 466 amino acids) of Cry1Ab gene and rest of the 453 nucleotides (corresponding to 151 amino acids at 467-671 position) from the Cry1Ac gene. The Cry1Ac in Nath seeds is >99% identical to the Cry1Ac used in JK and BNLA 106 events except that the size is smaller at 1824 bp with the first 1377 nucleotides (corresponding to the first 459 amino acids) of *Cry1*Ab gene and rest of the 453 nucleotides (corresponding to 151 amino acids at 460-664 position) from the Cry1Ac gene.

OBJECTIVES

- To develop farmer usable "on-the spot" rapid immunodiagnostic kits (5-min test) to detect specific transgenic products/event released for commercial cultivation in India.
- To develop a ubiquitous test kit for the detection of any GMOs/LMOs at port of entry.
- To develop standard quantifiable parameters for regulatory testing purposes.
- To develop methodology for detecting the presence of transgenes in bulk samples.

SALIENT FINDINGS

Antigen Cry1 F purification

Cry1 F antigen was purified and examined on PAGE gels (Figure 3.4.1). The antigens of Cry1 C and Cry1 B were purified from clones developed specifically for the production ofthe proteins in high expression vectors.

Immunization

Rabbits were immunized and four boosters were administered for Cry1 F, Cry1 C and three for Cry1 B. The Cry1 C antisera was tested and ELISA kits were developed. Cry1 B antisera has now been standardized

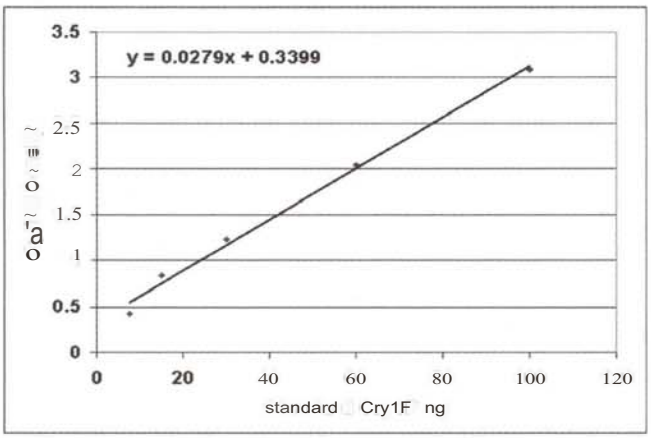

Fig 3.4.1 : Cry1 F standard curve

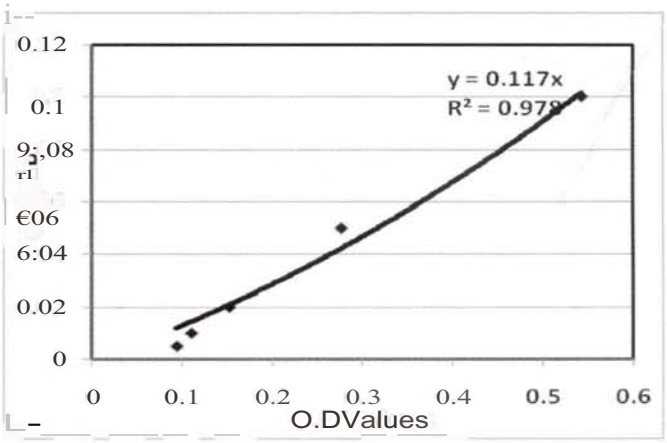

Fig 3.4.2: Standard Curve of Cry1 B

assays were improved. The Cry1 C and Cry1 B Kits developed by CICR accurately detected presence of the insecticidal proteins in putatively transformed plants. The kits were validated, stabilised and produced for use.

Two stock solutions were independently prepared as 10 *mglml* in PBST and diluted further in 1O-fold dilutions. The lyophilized powder containing partially pure protein isolated from the Cry1 F clones, was subjected to Lowry's method. The powder contained 85% protein. 50 IJIof the serially diluted stocks were subjected to ELISA.. The values were interpreted with the regression equation derived from the standard curve of Cry1 F.

The asymptotic and saturated OO values of the lower dilutions, were not considered and the OO values from the linear dilution response were used to calculate the Cry1 F in the sample. Based on the ELISA results the Cry1 F in the sample was estimated as 16.3-19.7%.

Validation

The kits were validated by independent institutions such as NBPGR, New Delhi, Bio-21 institute, Melbourne and Bejosheetal, Aurangabad. Under the TMC-MM-3.4 project ELISA kits have been developed for the four toxins that have been released. ELISA kits that detect Cry1Ac will detect all the four events if the expression levels are more than 10 ppb of fresh wt (leaf tissue). The kits have been tested for stability. A few general problems that are common to ELISA kits were also noticed with the Cry1 C, Cry1 Band Cry1 F kits that were developed last year. These are related to storage conditions of the kits. It was found that the substrate should not be stored at freezing temperatures. After thawing it develops colour. This is construed as positive results with negative samples. The plates should always be stored at 2-6°C. Most of the times, if proper care is not taken by the end user, even a minor contamination can cause positive result with negative samples. This happens most of the times with inexperienced technicians.

GM (Cry1 F) plant samples (14 to 24.4 mg) were crushed in 500 IJIsample buffer. The asymptotic and saturated OO values of the lower dilutions, were not considered and the OO values of 4 times dilution were used to calculate the Cry1 F in the sample from the linear dilution response. Based on the ELISA results the Cry1 F in the sample was estimated as 10.9 to 31.8 ppm (IJg per gm fresh weight).

Table 3.4.1: Quantification of Cry1 F in lypophilised, partially pure protein from clones.

Sample IJg *Iml* buffer	IJgsample loaded per ELISA well	Optical Density	ngCry1F calculated from the std curve	Cry1F *mg/gm*	%Cry1F in the sample
100	5	3.986	131	Saturated OO	
50	2.5	3.695	120	Saturated OO	
25	1.25	3.461	112	Saturated OO	
12.5	0.63	2.729	86	Saturated OO	
6.25	0.31	2.007	60	191.21	19.1
3.13	0.16	1.2	31	197.30	19.7
1.56	0.08	0.696	13	163.37	16.3

Table 3.4.2: ELISA of the putative Cry1 F GM samples

"Sample	ELISA OD 500 IJI (sample from diluted)				Calculated values ng				Sample Wt	Cry1F
Wtmg	2 times	4 times	8 times	10 times	2 times	4 times	8 times	10 times	4 times	ppm
20.2	4.132	3.846	3.24	3.29	13.6	12.6	10.4	10.6	0.051	24.9
21	4.027	3.855	3.24	2.7	13.2	12.6	10.4	8.5	0.053	24
22.5	3.979	3.46	2.7	2.54	13	11.2	8.5	7.9	0.056	19.9
22.9	3.762	3.25	2.3	2.14	12.3	10.4	7	6.5	0.057	18.2
12.5	3.457	2.809	1.4	1.22	11.2	8.8	3.8	3.2	0.031	28.3
21.3	3.648	3.085	1.7	1.73	11.9	9.8	4.9	5	0.053	18.5
21.2	3.928	3.225	2.3	2.75	12.9	10.3	7	8.6	0.053	19.5
14.2	3.273	2.405	1.24	1.98	10.5	7.4	3.2	5.9	0.036	20.9
16.6	3.592	3.069	2.2	2.12	11.7	9.8	6.7	6.4	0.042	23.6
22.5	3.602	3.075	2.16	1.14	11.7	9.8	6.5	2.9	0.056	17.4
14	4.027	3.446	2.44	2.1	13.2	11.1	7.5	6.3	0.035	31.8
14.7	3.745	3.426	2.44	2.06	12.2	11.1	7.5	6.2	0.037	30.1
21.6	3.998	3.422	2.869	1.49	13.1	11	9.1	4.1	0.054	20.5
21.4	3.525	2.973	1.8	31.29	11.4	9.4	5.3	3.4	0.054	17.6
21	3.659	3.021	1.96	1.56	11.9	9.6	5.8	4.4	0.053	18.3
24.4	3.423	2.797	2.13	1.39	11.1	8.8	6.4	3.8	0.061	14.4
19.7	3.662	2.985	2.02	1.35	11.9	9.5	6	3.6	0.049	19.3
22.3	3.536	2.659	1.72	1.04	11.5	8.3	4.9	2.5	0.056	14.9
23.6	3.453	2.134	1.57	1.67	11.2	6.4	4.4	4.8	0.059	10.9
18.8	3.537	3.043	1.9	0.96	11.5	9.7	5.6	2.2	0.047	20.6