ase precursor and the expression of this gene is found to be associated with disease resistance and suppression of autonecrosis[16], and in leaf senescence in tomato[17]. The suggested role of cysteine protease in fruit senescence is a novel hypothesis, which needs to be investigated further. Apart from these, integral membrane protein genes, vacuolar ATPase gene and other putative expressed protein genes have been identified, whose function remains enigmatic. A list of differentially expressed cDNA clones is given in Table 3.

In order to ascertain the validity of EST array-based results, RT–PCR strategy was used to analyse the differential expression of six selected genes, three that showed up-regulation (CD002149, CD002721, CD002690) and three that were down-regulated (CD002885, CD002453, CD002549) in the two cultivars (Table 2). The transcript abundance of the three up-regulated genes, viz. unknown protein (CD002149), integral membrane protein (CD002721), and low-molecular weight HSP (CD002690), was more in the cultivar Pusa Ruby as against Pusa Uphar (Figure 2). Similarly, the expression of the three down-regulated genes, viz. a putative monooxygenase cDNA (CD002885), Ids-4-like protein (CD002453), and another unknown protein (CD002549) was lower in Pusa Ruby compared to Pusa Uphar (Figure 3). The above results confirmed that the respective transcript abundance of the selected genes is in agreement with the results obtained using EST arrays in the two cultivars.

Further experiments are needed to elucidate the biological role of the isolated cDNA clones and to know how exactly they fit into the overall scheme of ripening. The nature of regulation of these cDNA clones will provide further insights into the complex molecular events associated with ripening regulation.

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ACKNOWLEDGEMENTS. K.N.A. acknowledges financial assistance received in the form of Senior Research Fellowship from the Council of Scientific and Industrial Research, New Delhi. We thank Dr Bal Raj, IARI, New Delhi, for help in raising tomato plants in the greenhouse and Dr D. C. Pachauri, IARI, for valuable suggestions.

Received 26 December 2003; revised accepted 17 November 2004

Bt-cotton seed as a source of *Bacillus thuringiensis* insecticidal Cry1Ac toxin for bioassays to detect and monitor bollworm resistance to *Bt*-cotton

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A simple bioassay on *Helicoverpa armigera*, utilizing *Bt*-cotton seed as a source of Cry1Ac toxin is described. The Cry1Ac content in seeds was found to be 1.77 ± 0.23 µg/g and the variability between individual seeds and seed lots was minimal. Bioassays on *H. armigera* using *Bt*-seeds stored at room temperature for 2 years showed that there was no significant reduction in bioactivity of the toxin present in the seeds. A discrimina-

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tion dose assay utilizing 160 g *Bt* seeds in 1.3 l diet is proposed for detection and monitoring of *H. armigera* resistance to *Bt*-cotton in India.

TRANSGENIC *Bt*-cotton as currently commercialized in India incorporates a *cry1Ac* gene derived from the soil bacterium *Bacillus thuringiensis*. The gene expresses a crystal protein delta-endotoxin called Cry1Ac, in all parts of the transgenic plants. The protein is toxic to many lepidopteran caterpillars that feed on the transgenic plants. Three *Bt*-cotton hybrid varieties, Bollgard-MECH-12, Bollgard-MECH-162 and Bollgard-MECH 184 were released in India during 2002 by Mahyco, India Ltd, Aurangabad for commercial cultivation. The hybrids are descents of a transgenic *Bt*-cotton variety developed by Monsanto, and express Cry1Ac under the influence of a constitutive promoter. Thus the target insect pests, which are affected by the toxic crop, are subjected to selection pressure. This is likely to result in the selection, propagation and increase in the frequency of resistant genotypes in field populations. The cotton bollworm *Helicoverpa armigera* (Hübner) has a history of demonstrated potential in developing resistance to virtually all the insecticide molecules used against it[1]. It is important to detect resistance in its early developmental phase, so that proper management measures can be initiated in time.

The methods of resistance detection and monitoring, developed thus far have been based on the use of purified Cry1Ac toxin or MVP-II (Cry1Ac formulation from Dow Agrosciences San Diego, CA, USA) as lyophilized powders incorporated in semi-synthetic diet. The Cry1Ac toxin and MVP lyophilized powders require to be stored frozen for sustained bioactivity. However, frequent freezing and thawing and long-term storage affect the bioactivity of both the toxin sources. The production of Cry1Ac toxin from recombinant clones is a specialized task and not many laboratories have access to the clones or have the facilities required for toxin production. The *Pseudomonas* encapsulated Cry1Ac is a proprietary product of Dow Agrosciences, USA and is no longer available in the market for use by researchers in India and elsewhere. Moreover, though the Bt protoxin expressed in bollgard is generically called Cry1Ac, it is actually encoded by a chimeric gene comprising 1–1398 nucleotides of *cry1Ab* gene and 1399–3534 nucleotides of *cry1Ac* gene[2]. Hence the use of Cry1Ac either purified from over-expressing clones or from MVP, is unlikely to be as authentic as the toxin present in the transgenic seed itself, for resistance monitoring purposes. We report the development of a semi-synthetic diet based bioassay in which *Bt*-cotton seed flour has been used as the toxin source. The bioassay being described herein is simple and robust, and can be easily used in laboratories with moderate facilities. Since the toxin source is from the *Bt*-cotton seed itself and not from closely related toxins such as the purified Cry1Ac or MVP-II, it is arguably more authentic for resistance detection and monitoring purposes.

Monsanto–Mahyco Biotech, Mumbai kindly provided seeds of Bollgard *Bt*-cotton hybrids, MECH-12 and MECH-162. ELISA (Enzyme linked immunosorbent assay) was carried out to determine the Cry1Ac content in Bt-cotton seeds using the commercially available 'Bt-Quant' ELISA kit (Innovative Biosciences, Nagpur). Cry1Ac was estimated in the bulk seed flour and also in 91 seeds each from MECH-12 and MECH-162 separately to examine the variability of Cry1Ac in individual seeds. ELISA and bioassays were carried out for two separate seed lots to examine the effect of seed storage on the Cry1Ac contained in the seed. One of the seed lots was obtained in 2003 and the other was in storage from 2001. Seeds were de-corticated and ground to flour in an electric grinder. Three aliquots of 50 mg samples were drawn randomly from the seed flour and used to estimate the Cry1Ac content by ELISA. Individual seeds were crushed to discard the seed coat and homogenized in 0.5 ml 0.05 M sodium carbonate buffer pH 9.0, using a teflon pestle in a 1.5 ml micro centrifuge vial. Cry1Ac toxin protein standards were prepared according to Albert and co-workers[3] from *E. coli* strains containing hyper expressing recombinant plasmid vector pKK 223-3, kindly provided by Zeigler, Ohio State University, USA. The toxin was purified from over-expressing cells by sonication and extensive washing with 10 % sodium bromide. Proteins were quantified according to Lowry's method[4] and the toxin was quantified on SDS–PAGE densitometry as described by Kranthi *et al.*[5]. Cry1Ac standards were solubilized in alkaline sodium carbonate buffer, 0.05 M, pH 9.0 and diluted in 6–7 concentrations before being used for ELISA. The samples were centrifuged at 10,000 *g* at 4°C for 5 min. The supernatant was used either directly or diluted to 1 : 10 with homogenization buffer and 0.1 ml was dispensed into each of the wells of a 96-well Nunc-maxisorp assay plate pre-coated with anti-Cry1Ac polyclonal IgG. The plates were incubated for 1 h at RT, washed twice with PBST (phosphate buffer saline with 0.1% Tween 20) and 0.1 ml of anti-Cry1Ac IgG-HRP (horse radish peroxidase) conjugate was added to each well. The plates were washed twice with PBST after 1 h incubation and 0.1 ml of the substrate solution, TMB (3′,3′,5′,5′-tetramethylbenzidine) was added to each well. The assay was terminated after 30 min with the addition of 0.050 ml 7% H_2SO_4 to each well. Absorbance was recorded at 450 nm on an ELISA reader POWER WAVE Select$_x$ (Labtech, USA). Quantification of Cry1Ac was done by plotting the absorbance values of the test samples on the standard curve generated with Cry1Ac standards on each of the ELISA plates.

Bioassays were carried out on a susceptible strain of *H. armigera*, which was derived from cotton fields and isolated from F_2 progeny of single-pair mated isofemale moths from *Bt*-susceptible populations using methods[6] described by Andow and Alstad[6]. The strain was maintained in the laboratory on a wheatgerm-based semi-synthetic diet[7]. The following diet recipe was used for the bioassays: 160 g

cotton seed-flour, 60 g wheatgerm, 3.3 g methyl parabenzoate, 1.7 g sorbic acid, 5.3 g ascorbic acid, 2.5 g Aureomycin, 16 g agar, 53 g dried active yeast and 1000 ml water. Diet prepared with non-*Bt*-cotton seed flour was found suitable to rear *H. armigera* for at least eight generations under laboratory conditions. For diet preparation, measured quantities of cotton seed-flour, wheatgerm, methyl parabenzoate, sorbic acid, ascorbic acid and Aureomycin (Cynamid India, Mumbai) were added to 500 ml water in a large bowl and mixed thoroughly. Active dried yeast (53 g) was added to 500 ml water and heated to dissolve completely, after which 16 g agar was added slowly and heated until complete uniform melting without the formation of clods. The yeast–agar mixture was boiled for 5 min, allowed to cool for 3–4 min at 27°C, and added to the bowl containing the rest of the diet ingredients. Next 13.5 ml 10% formaldehyde solution was added to the diet contents and mixed thoroughly using a blender. The hot diet was poured into soft plastic squeeze-dispenser bottles with lids having spouts trimmed to 1 cm and the diet was dispensed into wells of the multi-cell insect rearing trays. The trays were allowed to cool in laminar airflow under UV lamp for 2–3 h to sterilize the diet surface. The diet thus prepared could be stored at 4–8°C for 7–8 days.

In a separate experiment, the yeast–agar mixture was divided into four parts immediately after boiling and cooled at 27°C for variable time intervals of 1, 4, 7 and 10 min before adding it to a proportionate amount of rest of the diet ingredients containing *Bt*-cotton seed flour, in order to determine the effect of temperature on the bioactivity of Cry1Ac present in the seed flour. The immediate initial temperature of the diet was 64, 53, 47 and 42°C when the yeast–agar solution was added after variable incubation time intervals of 1, 4, 7 and 10 min respectively. When the yeast–agar solution was cooled for 7 and 10 min before adding to rest of the diet ingredients, the diet solidified almost immediately within 2–3 min after addition. Hence, instead of using squeeze bottles for dispensing, the diet was poured in trays, cooled and 1 cm^3 cubes were cut using a clean knife. The cubes were placed in the wells of the multi-cell trays before releasing larvae for bioassays. The temperature effect on Cry1Ac bioactivity was examined using discriminating dose diets (160 g *Bt* seed flour in 1.3 l diet) prepared with yeast–agar mixture pre-incubated at four different time intervals of 1, 4, 7 and 10 min. Control diets were prepared under identical conditions as those of the experimental diet, with the exception of using non-*Bt*-cotton seed flour instead of *Bt*-cotton seed flour. Bioassays were conducted with each of the diets using 100 first instar larvae per diet. The larvae were transferred onto freshly prepared experimental diet after 3–4 days and mortality observations and weight of surviving larvae were recorded on the 7th day.

Differential dose–response bioassays were conducted to determine median lethal concentration (LC_{50}) and median growth inhibition concentration (IC_{50}), and their 95% fiducial limits (FL) through probit analysis[8]. Cotton seed-flour was substituted with variable quantities of *Bt*-cotton seed flour for the differential dose bioassay to prepare diets containing Cry1Ac in a range 0.003 to 0.20 µg/ml diet. The mixture was vortexed thoroughly and poured into 25-well insect-rearing trays (Innovative Biosciences) approximately at 2 ml per well. The diet was cooled and first instar larvae (2-day-old) were released @ one per well. The plates were incubated at 25°C at 70% RH. The assays were done with at least five concentrations of the toxin and with 2–3 replicates. A total of 25 larvae were used per concentration. The larvae were transferred onto fresh toxin-incorporated diet after 3–4 days and incubated until they were seven days old. Mortality observations and weight of surviving larvae were recorded on the 7th day. The growth inhibition concentrations IC_{50}, IC_{90} and IC_{99} were derived based on the concentration of Cry1Ac in the diet that inhibited 50, 90 and 99% of the test insects respectively, from reaching third-instar stage. The major advantage with the IC method is that it is based on the observation of number of larvae not molting to third instar in each of the concentrations and not on weights of individual larvae as is done for the EC (effective growth inhibition concentration) calculations.

Results from ELISA of individual seeds indicated that there was little variability in the expression of Cry1Ac in seeds ($F = 1.89$; df = 3, 348; $P = 0.130$). The seeds of MECH-12 and MECH-162 contained an average ± SD (standard deviation) of 1.823 ± 0.366 and 1.743 ± 0.118 µg/g Cry1Ac respectively, from the seed lots obtained in 2003. The seed lots of MECH-12 and MECH-162 that were obtained in 2001 contained Cry1Ac at an average of 1.769 ± 0.238 and 1.761 ± 113 µg/g respectively. The content of Cry1Ac in the bulked seed flour of MECH-162 was 1.770 and 1.750 µg/g in the lots of 2001 and 2003 respectively, thereby indicating that the seed lots that were obtained in 2001 and were stored for two years had levels of Cry1Ac similar to those of the relatively fresh lot. Thus both the ELISA results on individual seeds and the measurement of bulked seed showed that there was no significant difference in the Cry1Ac content between the samples tested.

The results (Tables 1 and 2) indicated a consistent regression response of *H. armigera* to Cry1Ac present in *Bt*-cotton seeds. The IC_{50} values ranged from 0.012 to 0.013 µg/ml and LC_{50} from 0.115 to 0.125 µg/ml diet. The fiducial limits of LC_{50} values overlapped extensively for both the bioassays using *Bt*-seed sources from 2001 and 2003, thereby indicating that there were no significant differences in the bioassay results. Similarly, FL of the IC_{50} values of all the bioassays overlapped, and there were therefore no significant differences between the IC_{50} values (Table 2) of the bioassays. The similarity in toxic effect of the two-year-old seed lot in comparison to the fresh lot indicated that the bioactivity of the Cry1Ac toxin did not decline despite the seeds having been stored at room temperature for two years. The results clearly showed that *Bt*-

Table 1. Toxicity of Cry1Ac present in MECH-162 seed meal-based semi-synthetic diet to *Helicoverpa armigera*

Toxin source	LC_{50}	95% FL	LC_{90}	Slope	χ^2
Bt-seed 2001	0.115	0.085–0.209	0.365	2.6 ± 0.6	1.98
Bt-seed 2003	0.125	0.083–0.314	0.505	2.1 ± 0.5	0.55

LC_{50} and LC_{90} represent lethal concentrations in µg/ml diet that kill 50 and 90% of the larvae respectively. FL are the fiducial limits at 95% confidence intervals.

Table 2. Effective growth-inhibiting concentrations of Cry1Ac present in the MECH-162 seed meal-based semi-synthetic diet on *H. armigera*

Toxin source	IC_{50}	95% FL	IC_{90}	IC_{99}	Slope	χ^2
Bt-seed 2001	0.012	0.009–0.016	0.037	0.091	2.7 ± 0.5	2.16
Bt-seed 2003	0.013	0.009–0.017	0.034	0.074	3.1 ± 0.6	0.65

IC_{50}, IC_{90} and IC_{99} represent effective concentrations in µg/ml diet that prevent 50, 90 and 99% of the larvae respectively, from reaching the third-instar stage. FL are the fiducial limits at 95% confidence intervals.

cotton seeds were a good source of Cry1Ac for bioassays on *H. armigera*.

We examined the effect of maximum initial temperature during the process of diet preparation on the toxicity of Cry1Ac present in *Bt*-cotton seed flour, on *H. armigera*. The results presented in Table 3, clearly show that the influence of maximum initial heat of 64°C during diet preparation had minimal detrimental effect on the bioactivity of Cry1Ac on *H. armigera*. There was no significant difference in the growth inhibition response and weights of surviving larvae ($F = 1.24$; df = 3, 155; $P = 0.29$) between the treatments. However, it must be pointed out here that the average weight of the larvae surviving the diet prepared at initial maximum temperature of 64°C was higher compared to that from rest of the treatments. It is thus possible that the initial heat of 64°C may have affected the bioactivity of Cry1Ac, albeit to a minor extent. Hence, we suggest that it is advisable to allow the yeast–agar mixture to cool for 3–4 min before adding it to rest of the diet ingredients containing *Bt*-cotton seed flour.

The LC_{50} and IC_{50} values of Cry1Ac from *Bt*-cotton seed source reported here are similar to those reported previously for *H. armigera* strains from India[9–12], Australia[13,14] and China[15,16]. However, the baseline LC_{50} susceptibility values[15] of *H. armigera* to Cry1Ac in China, were found to be variable with a range from 0.091 to 9.073 µg/ml diet. The baseline LC_{50} values of 0.01 to 0.67 µg/ml reported by us[11] previously, and 0.11 to 0.71 µg/ml reported recently by Jalali *et al.*[12] for Indian strains indicate that the Chinese *H. armigera* strains are inherently more tolerant to Cry1Ac than the Indian strains. The baseline range of EC_{50} values at 0.003 to 0.008 µg/ml and EC_{90} at 0.009 to 0.076 µg/ml diet, published by Jalali *et al.*[12]; our[10] previous EC_{50} data of 0.014, and the current values of the *Bt* seed-based bioassays at IC_{50}, 0.012 to 0.013 µg/ml diet, showed that the results of the *Bt*-seed bioassay were similar to the published data using Cry1Ac and MVP-II based assays on the Indian *H. armigera* population.

Baseline studies provide a benchmark for the susceptible response of insect species. However, detection of resistant genotypes and monitoring the increase in their frequencies is crucial for resistance management. Dose–mortality regression assays indicate shifts in the baseline, but may not assist in detecting resistant genotypes or even indicate the onset of resistance. Discriminating dose bioassays clearly define the toxic level of the insecticide that can detect resistant genotypes. Thus a single dose would then enable monitoring of an increase in the frequencies of resistant alleles in field populations, as influenced by selection pressure. However, discriminating dose assays using mortality as the bioassay response were found to be unreliable in clearly differentiating resistant and susceptible populations of *Heliothis virescens* and *Helicoverpa zea*[17]. Therefore, the correct discriminating assay was designated to assess growth-regulating effects based on the capability of a single dose (IC_{99}) to prevent at least 99% larvae from reaching the third instar stage in susceptible populations[15]. Thus the discriminating dose would be capable of detecting heterozygous individuals having single resistant allele with partial dominance. Wu *et al.*[16], monitored resistance using the discriminating dose method and showed that IC_{99} was a preferred indicator of resistance development.

Based on the data that have been published thus far from India[9–12], the results presented here, and our recent resistance monitoring data (unpublished), we found that a Cry1Ac concentration of 0.2 µg/ml diet would prevent at least 99% *H. armigera* larvae from reaching third instar stage (30–40 mg) in the 7-day bioassay. In the control diet, *H. armigera* molted to third instar by the fifth day of the bioassay and the larvae weighed 32 ± 6 mg. Therefore, ideally, any larva molting to the third instar on fifth day of the bioassay containing 0.2 µg/ml diet can be considered as

Table 3. Influence of initial temperature during diet preparation on the bioactivity of Cry1Ac on *H. armigera*

Maximum initial temperature (°C) of diet during preparation	*Bt*-cotton* seed based diet		Non-*Bt* cotton seed based diet	
	Percentage growth inhibition response	Weight (mg) of surviving larvae (mean ± SD)	Percentage growth inhibition response	Weight (mg) of surviving larvae (mean ± SD)
64	100	8.48 ± 2.85	0	81.16 ± 10.32
53	100	7.53 ± 1.89	1	82.52 ± 6.06
47	100	7.97 ± 2.42	1	78.98 ± 7.18
42	100	7.69 ± 2.29	0	80.46 ± 8.84

*Seeds of Bollgard MECH-162 were used as the source of Cry1Ac. Growth inhibition response was recorded as number of test larvae unable to reach the third-instar stage.

truly resistant. We found that none of the larvae in the treatments of >0.2 µg Cry1Ac/ml diet, molted to third instar even by the seventh day in any of our laboratory-susceptible populations.

We propose a *Bt*-seed-based assay incorporating 0.2 µg Cry1Ac per ml diet as a reliable resistance diagnostic assay using 160 g of *Bt* seed-flour in the diet recipe (described earlier in this communication) for a resistance diagnostic diet. The *Bt*-seeds contained Cry1Ac at an overall average ± SD of 1.774 ± 0.234 µg/g. Hence, the average Cry1Ac content in 160 g *Bt* seed would be 284 ± 0.37 µg, which when added to the other ingredients to make 1.3 l diet would result in a minimum assured quantity of 0.20 ± 0.01 µg Cry1Ac/ml diet. Thus, the *Bt*-seed-based diet reported here can be used in discriminating dose assays to detect and monitor changes in the frequencies of resistant *H. armigera* genotypes. The *Bt* seeds can also be used as a source of Cry1Ac for log dose probit assays that can be used to determine changes in the baseline susceptibility of *H. armigera* to Cry1Ac.

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ACKNOWLEDGEMENTS. The work was supported by grants from the Department of Biotechnology, New Delhi. We thank Dr C. D. Mayee, Agricultural Commissioner, GOI and ex-Director, CICR, Nagpur for having provided facilities and encouragement. We thank Dr M. K. Sharma, Mahyco–Monsanto Biotech, Mumbai for kindly providing us Bollgard hybrid seeds. We also thank Prof. Derek Russell, NRI, UK (currently at the CESAR, LaTrobe University, Melbourne, Australia) for critical review of the manuscript.

Received 31 March 2004; revised accepted 25 October 2004