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Full Length Article

Spatio-Temporal and Intra-Plant Expression Variability of Insecticidal Gene (*Cry1Ac*) in Upland Cotton

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Abstract

Cotton is cultivated on large area of Punjab and Sindh province of Pakistan. The development of transgenic cotton having *Bt* (*Bacillus thuringiensis* L.) gene producing δ-endotoxin was a success story to get control of bollworm infestation. Dark side of this story was the progressive development of resistant insects against this toxin. The spatio-temporal expression of the transgene is considered as one of the reasons for resistant pests. Though the transgene is under the control of constitutive promoter, but expression is not consistent and stable throughout the growing season. Therefore, the present study was planned to examine the basis of the variable expression of *Bt* gene in the genetic background of local cotton accessions. A set of 10 selected *Bt* genotypes were used to study the season long expression. The plants were sampled for different parts (Leaves and Bolls) at different growth stages (i.e., 30, 60, 90 and 120 days after sowing - DAS). Intra-plant expression variability was also assessed from upper, middle and lower canopy leaves. Enzyme Linked Immuno Sorbent Assay (ELISA) was performed for the quantification of *Cry1Ac* gene in the sampled tissues at protein level. The results revealed that 30DAS leaves had the highest concentration, while 150 DAS had the least expression. A gradual decrease in the expression was observed throughout the growing season among all the genotypes i.e., with the age of the plant and also with active plant growth. Leaves expressed higher expression as compared to bolls and other reproductive parts. Upper canopy leaves had a considerable higher amount of δ-endotoxin protein followed by middle canopy and lower canopy leaves. The study provided guidelines for the development of better performing *Bt* cotton genotypes with standard toxin expression. It is concluded that cultivated genotypes don't have stable expression under local climatic conditions. This situation demands, the development of cotton genotypes expressing Cry1Ac transgene at higher level to maximize the benefit of this technology. © 2018 Friends Science Publishers

Keywords: *Bacillus thuringiensis* L; Bollworms; *Cry1Ac*; Cultivars

Introduction

Cotton is a major fiber and cash crop of Pakistan. It feeds the textile industry of the country and also contributes to domestic oilseed and cattle feed production. It shares, 5.1% value added in agriculture and 1.0% of GDP (Economic Survey of Pakistan, 2015–2016). The area under cotton cultivation is 2.91 million ha with a production of 10.074 million bales and the average yield remained 587 kg/ha during the fiscal year 2015–2016 (Economic Survey of Pakistan, 2015–2016). Pakistan is the 4th largest producer and 3rd largest consumer of cotton in the world. More than 1.5 million farmers (out of 5 million) cultivated cotton over 2.91 million hectares, covering 10–12% of the cultivable area in the country during 2015–2016.

Cotton plant is attacked by almost 15 insect pests. Other than sucking pests, the most devastating pests among them are the American bollworm (*Heliothis armigera*), spotted bollworm (*Earius insulana/vitella*), pink bollworm (*Pectinophora gossypiella*) and army bollworm (*Spodoptera lithura*) (Bakhsh *et al*., 2011). These pests can damage up to 30–70% of crop production. Broad spectrum pesticides have been extensively used for crop protection. These pesticides contain extremely poisonous agro-chemicals that have led to serious environmental as well as health concerns to farmer community.

One of the success stories of genetically engineered crops is transgenic insect resistant cotton, which helped the cotton farmers to combat the challenges of pest infestation to a significant extent. It contains the insecticidal gene, which was taken from *Bacillus thuringiensis* L. producing crystal proteins (Martin and Travers, 1989). Cultivation of transgenic cotton containing genes from *Bacillus thuringiensis* (a soil lodging, gram positive and spore forming bacteria) started in Pakistan during 2010 with the approval of *Bt* cotton varieties harboring single *Cry1Ac* gene and now it is cultivated on 2.9 million acres, which is nearly equal to

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83% of Pakistan's total cotton hectarage and 2% of total global hectarage (James, 2015).

Transgenic cotton with multiple *Cry* genes in a single genotype has been developed for broad spectrum insect resistance (Altman *et al.,* 1996; Sachs *et al.*, 1998). Major Cotton producing countries like USA, Australia, India, and Uzbekistan are using cotton varieties with more than one *Cry* genes. Only two countries (China and Pakistan) are using single gene (*Cry1Ac*) *Bt* cotton varieties. The performance of *Bt* cotton is linked with the expression of tansgene and the amount of toxin protein in the tissues. Single gene *Bt* cotton is performing well in China with successful control of *H. armigera* and *P. gossypiella* (Wan *et al.,* 2012) but multiple reports showed the outbreak of *P. gossypiella* in Pakistan, which can be related to lower level of transgene expression. Another cause might be the variable expression level in different tissues and at various developmental stages.

Variable *Cry1Ac* expression in *Bt* cotton cultivars favors bollworms to develop resistance against this toxin. The expression varies with the age of the plant and within plant parts throughout the cotton growing season (Greenplate *et al.,* 2001; Mahon *et al.,* 2002). The incorporation of *Cry1Ac* gene into high yielding genotypes, with stable transgene expression, is the future target of the breeders. A number of factors are involved in the variable expression of *Cry1Ac* gene among cultivars, but the genetic background of the crop plant in attenuating the expression level has been of great concern (Sachs *et al.,* 1998). This variability in expression between different plant parts and throughout the growing age of the plant is crucial for its effectiveness to control the targeted insect pests. The present study was conducted to evaluate the spatio-temporal performance of single transgene expression in different genetic backgrounds of cotton plant. The expression of *Cry1Ac* gene was analyzed in leaf and bolls of upper, middle and lower canopy of 10 genotypes throughout the growing season.

Materials and Methods

Plant Material and Research Site

Nine selected *Bt* cotton genotypes 01 (IUB-222), 02 (FH-142), 03 (VH-295), 04 (MNH-886), 05 (CRS-456), 06 (IR-4), 07 (FH-114), 08 (FH-182), 09 (SB-149) and one non-*Bt* 10 (MNH-786) control were planted on April 16, 2016, at experimental farms of the Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad.

Field Preparation and Experimental Design

Field was well prepared; all the agronomic and crop management practices were properly carried out for sowing. Environmental conditions like temperature, rainfall, and humidity were suitable for cultivation of cotton crop. Fertilization with DAP (1 bag per acre) was applied at the time of sowing and Urea was applied in three splits i.e., at the time of squaring, once at the time of flowering and last at the time of boll formation. Insecticides/pesticides were timely applied to control whitefly, aphid, jassid and thrips, while bollworms (*lepidopterans*) targeted insecticides were not applied to all plots. Irrigation was carried out at the time of need and with fertilizer application. The experiment was conducted using Randomized Complete Block Design (RCBD) with three replications. Experimental fields were surrounded with five rows of non-*Bt* PB-899 (a locally developed upland cotton line) to serve as refugia to prolong insect resistance. *Sorghum bicolor* was grown around the field to isolate field from surroundings following the recommendations of National Biosafety Committee guidelines (NBC, 1999).

Spatio-temporal Expression of δ-endotoxin Protein (*Cry1Ac*)

The amount of insecticidal protein (C*ry1Ac*) present in nine different transgenic genotypes and one non-transgenic (control) genotype was determined throughout the season. Differential expression of *Cry1Ac* occurs among different plant structures (Greenplate, 1999; Adamczyk *et al.,* 2001), therefore, for temporal expression of *Cry1Ac* a single plant structure was selected for quantification. For each sample date (30, 60, 90, 120 and 150 DAS) and for all transgenic lines, main-stem terminal leaf was collected from three randomly selected plants/transgenic line after every 30 days interval. All the collected samples were assayed on the same day after detachment from plant. For spatial expression two plant parts i.e. leaves and bolls were selected and sampled. Collected samples were transported to the laboratory and were processed the same day.

Intra-plant Expression of δ-endotoxin Protein (*Cry1Ac*)

To determine the intra-plant expression variability of *Cry1Ac* insecticidal protein, the plant leaves were sampled. Leaves were collected from upper, middle and lower canopy of the plant, kept on ice 4°C and transported to the laboratory. Leaf samples were assayed through ELISA on the same day to avoid any change or variability in the protein contents.

Expression of *Cry1Ac* in transgenic lines was quantified by ELISA using Envirologix Kit (Cat # AP051). Negative and positive controls were added to the wells along with test samples. ELISA was performed following the given protocol in the kit (Envirologix, Inc. USA) and quantification of *Cry1Ac* endotoxins was done by plotting absorbance values of *Cry1Ac* test samples on the standard curve generated with purified *Cry1Ac* standards on each of ELISA plates and expressed as microgram of *Cry1Ac* per gram of fresh tissue weight.

Statistical Analysis

Cry1Ac protein expression data were further subjected to Factorial Analysis of Variance (ANOVA) using SPSS software (version 11.0v, SPSS Inc) to evaluate the differences among transgenic lines, sampling dates, interaction of transgenic lines and sampling dates at 5% level of significance.

Results

Temporal Expression of *Cry1Ac* Gene

The toxin concentration was maximum (1.159–3.790 µg/g) in leaf samples taken at 30 DAS (vegetative stage), followed by a continuous decrease in the toxin level. The level of toxin decreased with the age of the plant, as the crop progressed towards the maturity. The genotype 03 remained at top and 04 had the least toxin concentration (Fig. 1). At 60 DAS, the concentrations of *Cry* protein among *Bt* cotton genotypes ranged from 1.030 to 3.285 µg/g on fresh leaf weight basis. Again, the genotype 03 had the highest concentration of 3.285 µg/g and 04 had the lowest concentration of 1.030 µg/g.

Most of the genotypes lost their expression durability after 90 DAS and their expression went below the critical level of 1.9 µg/g (Kranthi *et al.*, 2005). Only two genotypes, 02 (1.897 µg/g) and 03 (2.20 µg/g) had relatively high amount of *Cry1Ac* toxin at 90 DAS. Among the genotypes, 01 was the least expressing genotype at 90 DAS with a toxin concentration of 0.355 µg/g. At 120 DAS, all the genotypes significantly lowered their toxin concentration below lethal level ranging from 0.304–1.702 µg/g. The variety 03 had the highest and 01 had the lowest toxin concentration at this stage (Fig. 1).

Similarly, the concentration of *Cry1Ac* protein at 150 DAS remained between 0.216–0.814 µg/g. The variety 03 was ranked highest with a toxin concentration of 0.814 µg/g and variety 01 was at last position with a concentration of 0.216 µg/g. All the genotypes in this experiment showed significant variations in toxin expression throughout the growing season. The average performance of the varieties during the growing season revealed that 03 was the best performing and 01 was the least performing genotype (Fig. 1). Factorial analysis revealed that the *Cry1Ac* gene expression differences among transgenic lines, sampling dates and interaction of genotypes × sampling dates were all significant at 5% probability (Table 1).

Spatial Expression of *Cry1Ac* Gene

For spatial expression of *Cry1Ac* gene in the entire transgenic genotypes the same quantitative assay (ELISA) was used. For this purpose, two different plant parts (leaves and bolls) were sampled from the field and transported to the laboratory. The collected samples were subjected to ELISA using Envirologix ELISA kit (Cat # AP 003) for the quantification of toxin (Cry1Ac protein) produced in these plant parts. The expression of Cry1Ac protein was variable in leaves and bolls (Fig. 2). Leaves had a higher concentration of Cry1Ac protein as compared to bolls in all the varieties. Two varieties i.e., 03 (2.358 µg/g) and 02 (1.989 µg/g) had significantly higher concentration, whereas, the remaining seven genotypes had a lower concentration of Cry toxin in leaves. In case of bolls, all the genotypes had significantly low level of Cry protein as compared to lethal level (Fig. 2). Factorial analysis of variance for plant parts and genotypes revealed significant differences. Similarly, interaction effects between plant parts and genotypes also revealed significant differences at 5% level of significance (Table 1).

Intra-plant Expression of *Cry1Ac* Gene

Upper canopy leaves: Cry1Ac protein level was adequate (i.e., 1.413–4.621 µg/g at 30 DAS and 1.058–4.014 µg/g at 60 DAS) in the upper canopy leaves early in the season (Fig. 3). After that, a gradual decline was observed in expression of Cry1Ac protein in all the genotypes. This decrease in concentration started relatively early in the season in 01 (1.413 µg/g) and 04 (1.484 µg/g). Both the genotypes had a lower concentration of *Bt* toxin early in the season i.e., at 30 DAS. At 90 DAS, only four genotypes (02, 03, 05 and 09) had a sufficient quantity of this Cry protein. The concentrations of these varieties were 2.747 µg/g, 2.817 µg/g, 1.860 µg/g and 1.890 µg/g, respectively. The rest of the genotypes showed inadequate amount of toxin protein, the genotype 01 expressed minimum toxin concentration i.e., 0.319 µg/g (Fig. 3).

By 120 DAS, Cry1Ac expression decreased to < 1.25 µg/g in almost all the genotypes except 03 (2.324 µg/g) and 02 (1.995 µg/g). The average toxin concentration among genotypes at 120 DAS ranged between 0.418–2.324 µg/g. Interestingly, the Cry1Ac protein expression did not decline completely to an undetectable limit throughout the season in all the genotypes. The genotype 01, 04 and 07 showed the lowest expression (i.e., 0.228, 0.536 and 0.557 µg/g) at 150 DAS (Fig. 3). Factorial analysis of variance for upper canopy leaves expressed that, there were significant differences in toxin expression among genotypes, sampling days and genotype × sampling days interactions (Table 1).

Middle Canopy Leaves

The Cry1Ac protein expression was found variable in middle canopy leaves. The concentration of Cry protein was high at 30 DAS ranging from 1.152 to 3.724 µg/g in all the genotypes, whereas, at 60 DAS sampling the concentration of *Cry1Ac* gene was ranging from 1.062 to 3.228 µg/g. Sampling at 90 DAS revealed that the amount of toxin was in a range of 1.00–1.25 µg/g for most of the genotypes.

Table 1: Means squares of spatio-temporal and intra-plant expression of *Cry1Ac* gene in upland cotton genotypes

SOV	Temporal expression	Spatial expression	Intra-plant expression					
			UCL	MCL	LCL	UCB	MCB	LCB
Genotype	6.860	1.138	11.342	6.443	3.914	0.437	0.288	0.204
DAS	12.800	7.209	13.856	13.379	11.377	0.277	0.384	0.254
Genotype × DAS	0.416	0.379	.461	0.456	0.390	0.007	0.011	0.009
Error	0.015	0.005	.025	0.023	0.016	0.003	0.004	0.005

UCL = Upper canopy leaves **UCB** = Upper canopy bolls
MCL = Middle canopy leaves **MCB** = Middle canopy bolls
LCL = Lower canopy leaves **LCB** = Lower canopy bolls

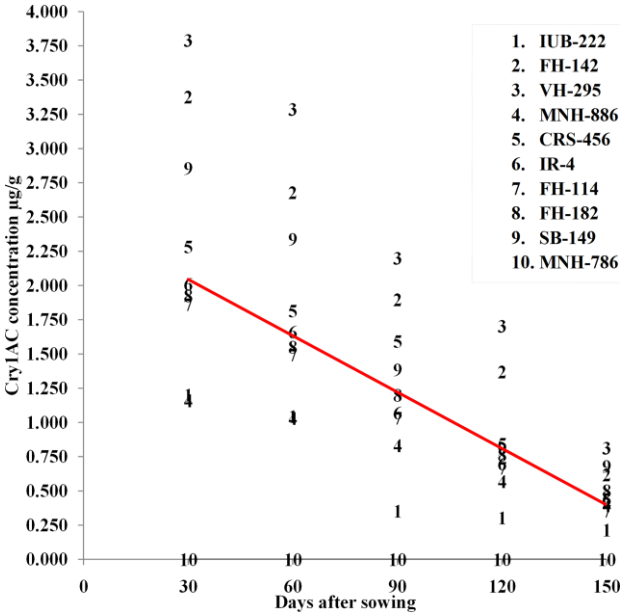

Fig. 1: Temporal expression of C*ry1Ac* gene in *Bt* cotton leaves

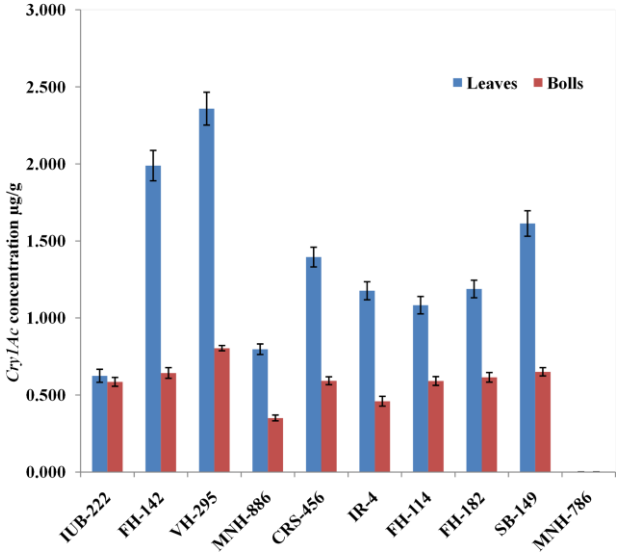

Fig. 2: Spatial expression of *Cry1Ac* gene in *Bt* cotton genotypes

At 120 DAS sampling, the concentration of the endotoxin protein remained below 1 µg/g for all the genotypes except 02 (1.294 µg/g) and 03 (1.611 µg/g), which showed a higher concentration of protein (Fig. 4).

The amount of toxin protein was below 0.5 µg/g at 150 DAS sampling for all the genotypes under study except 03 (0.730 µg/g) and 09 (0.689 µg/g), these genotypes expressed a high concentration of Cry toxin (Fig. 4). The average performance of the genotypes throughout the growing season revealed that 03 was the highest expressing and 01 was the least expressing genotype. Analysis of variance demonstrated that significant differences were present among genotypes and their interaction. Sampling dates also have significant differences at 5% level of significance (Table 1).

Lower Canopy Leaves

The *Cry1Ac* gene expression in the lower canopy leaves ranged between 0.843–3.026 µg/g initially at 30 DAS and 0.721–2.614 µg/gat 60 DAS in all the genotypes. At 60 DAS, the δ-endotoxin protein level declined steadily (i.e., < 1) in 01 (0.923 µg/g) and 04 (0.721 µg/g), while rest of the varieties showed Cry1Ac concentration above 1 µg/g. Similarly, at 90 DAS all the genotypes decreased their expression below 1 µg/g except 03 (1.669 µg/g) and 05 (1.370 µg/g), whereas at 120 DAS and 150 DAS the toxin expression of the genotypes was also less than 1 except 03, which showed a toxin level of 1.171 µg/g at 120 DAS (Fig. 5). Factorial analysis of variance was also performed to determine the significant differences. There were significant differences between the Cry1Ac toxin level, between age intervals and among the genotypes. ANOVA revealed that interaction effects of genotypes and DAS was also significant (Table 1).

Upper Canopy Bolls

The expression of δ-endotoxin protein (Cry1Ac) in bolls was determined at 90 DAS and at 120 DAS. At 90 DAS, the expression of all the varieties ranged between 0.5–1.0 µg/g on fresh weight basis. The genotype 03 (0.974 µg/g) had highest toxin concentration, while the genotype 04 (0.553 µg/g) showed the least Cry toxin (Fig. 6). Similarly, at 120 DAS the concentration of Cry protein ranged between 0.40–0.80 µg/g in most of the entries.

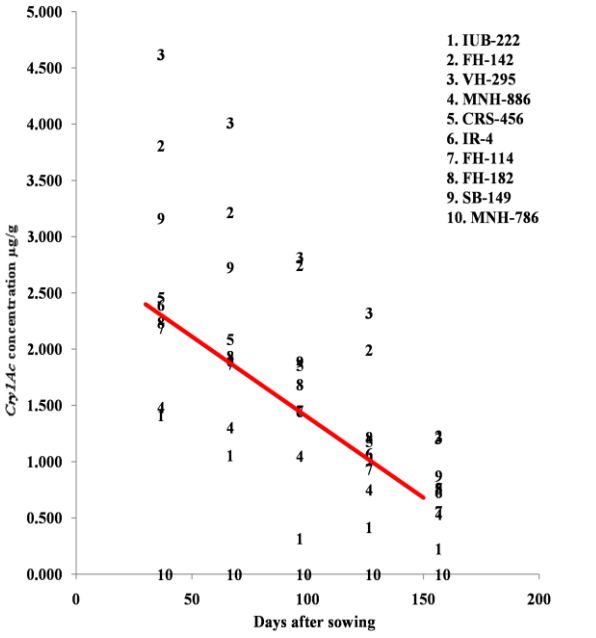

Fig. 3: Temporal expression of *Cry1Ac* gene in upper canopy leaves of *Bt* cotton genotypes

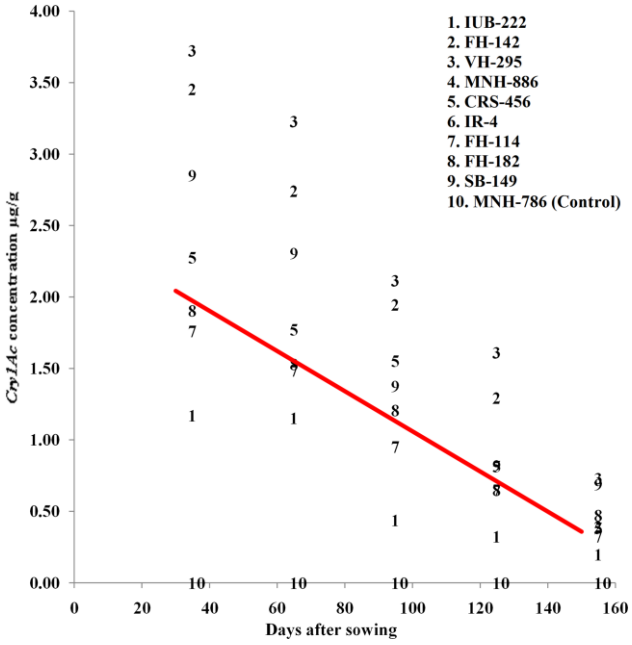

Fig. 4: Temporal expression of *Cry1Ac* gene in middle canopy leaves of Bt cotton genotypes

Mean values with standard deviations indicated that all the genotypes had significant differences. Analysis of variances (ANOVA) among genotypes, days after sowing and their interaction effects indicated significant differences (Table 1).

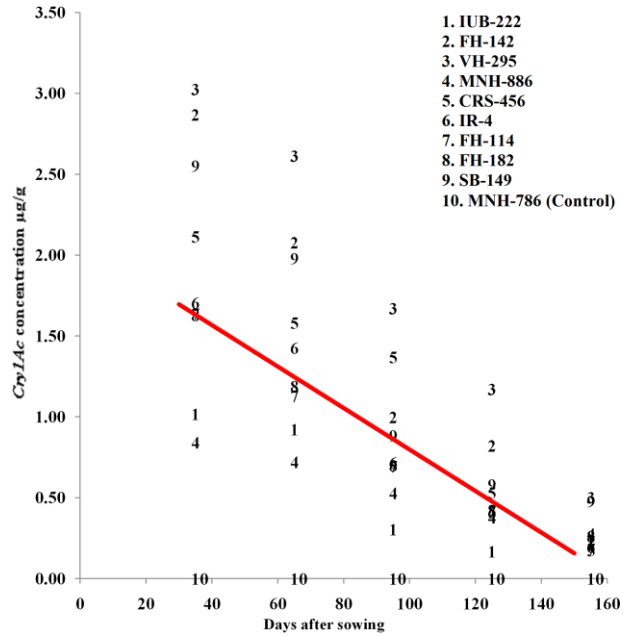

Fig. 5: Temporal expression of *Cry1Ac* gene in lower canopy leaves of Bt cotton genotypes

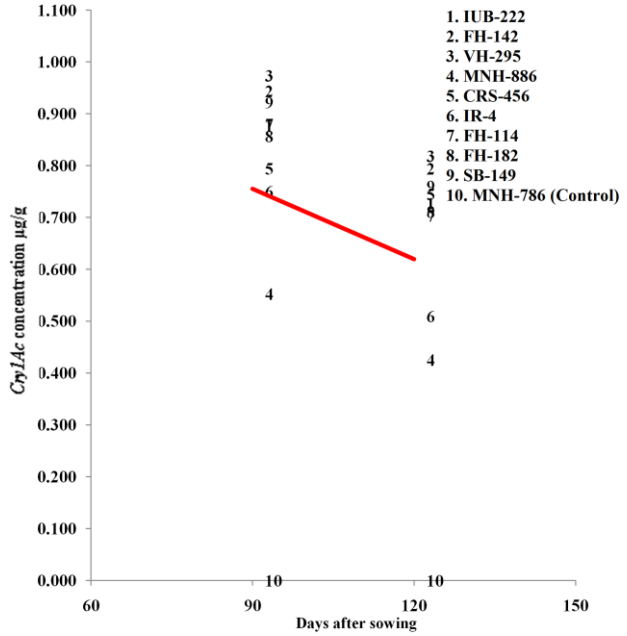

Fig. 6: Temporal expression of *Cry1Ac* gene in upper canopy bolls of *Bt* cotton genotypes

Middle Canopy Bolls

Enzyme Linked Immuno Sorbent Assay (ELISA) was carried out for middle canopy bolls at 90 DAS and 120 DAS. The objective to perform this assay was to quantify the Cry1Ac protein level at different plant growth stages (i.e., at the start of boll formation and at maturity).

It was observed that the concentration of Cry toxin decreased with the age of the plant. The toxin level at 90 DAS ranged between 0.350–0.750 µg/g. Most of the genotypes had toxin concentration above 0.500 µg/g except 04 (0.358 µg/g). Similarly, at 120 DAS sampling concentration ranged between 0.299–0.723 µg/g (Fig. 7). Three genotypes *viz*. 02 (0.553 µg/g), 03 (0.723 µg/g) and 05 (0.529 µg/g) expressed considerably high toxin as compared to other genotypes at 120 DAS. Factorial analysis of variance revealed significant differences among genotypes and DAS. Interaction effects of genotype and days after sowing were also found significant for middle canopy leaves (Table 1).

Lower Canopy Bolls

Concentration of *Cry1Ac* gene decreased significantly in the lower canopy bolls. ELISA demonstrated that at 90 DAS only three genotypes *viz.*03 (0.786 µg/g), 08 (0.555 µg/g) and 09 (0.602 µg/g) had concentration above 0.500 µg/g, while the rest of the genotypes expressed lower concentration. The concentration of the Cry protein at 120 DAS demonstrated that significant variations were present among the genotypes. The amount of Cry1Ac protein at 120 DAS was below 0.500µg/g for all the genotypes under study except 03 (0.638 µg/g) (Fig. 8). Significant differences were observed between genotypes and days of sowing at 5% level of significance (Table 1).

Discussion

Considerable spatio-temporal and intra-plant expression variability of insecticidal gene (*Cry1Ac*) was observed in transgenic cotton genotypes. The expression of *Cry1Ac* gene decreased with the age of the plant (Benedict *et al*., 1996; Chen *et al*., 2000). At vegetative stage (i.e., at 30 DAS) plants had maximum toxin concentration in leaves, which declined during the crop growth and reached at its minimum level (150 DAS) as plant matured (Finnegan *et al*., 1998; Mahon *et al*., 2002; Manjunatha *et al*., 2009; Bakhsh *et al*., 2010; Hussain, 2012).

All the genotypes showed similar decreasing trend of Cry toxin over age. The maximum expression in the nine genotypes was observed at 30 DAS and minimum at 150 DAS (Figs. 1–8). The genotype 03 was the best performing line having maximum Cry1Ac toxin in leaves throughout the growing season. Most of the genotypes lost their efficacy after 90 DAS, which is crucial. The reason behind this decrease in concentration may be due to environmental stresses like temperature (Chen *et al*., 2005) or may be due to promoter methylation (Leeuwen *et al*., 2001; Sunilkumar *et al*., 2002). The type of promoter had been shown to have a significant effect on Cry1Ac concentration (Bakhsh *et al*., 2010). The *Cry1Ac* transgene is controlled by 35S promoter, which is cell-type-specific and developmentally regulated promoter (Nilsson *et al*., 1992; Pauk *et al*., 1995; Yang and Christou, 2005).

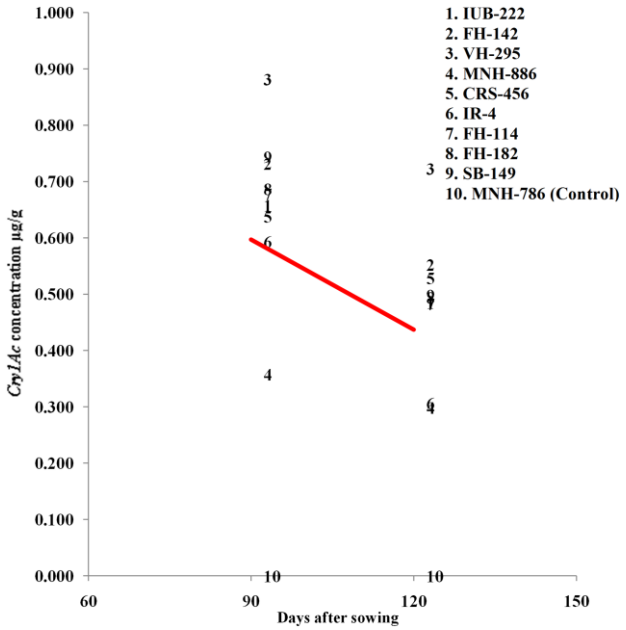

Fig. 7: Temporal expression of *Cry1Ac* gene in middle canopy bolls of *Bt* cotton genotypes

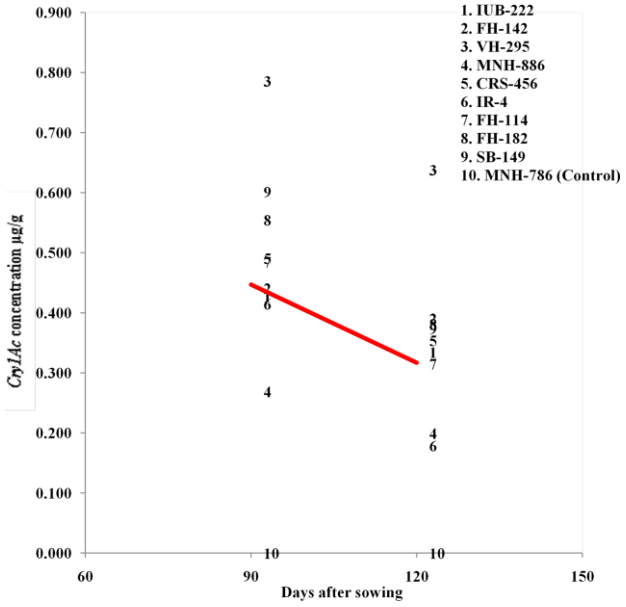

Fig. 8: Temporal expression of *Cry1Ac* gene in lower canopy bolls of *Bt* cotton genotypes

The fluctuations in Cry protein expression throughout the season would help in the development of resistant American bollworms against this toxin.

Similarly, different plant parts (i.e., leaves, bolls, flower etc.) had different toxin level. Significant differences were also observed for Cry1Ac concentration in various plant parts (Chen *et al.*, 2000; Kranthi *et al.*, 2005). Leaves had maximum Cry toxin followed by flowers,

bolls, anthers, squares and ovaries. The δ-endotoxin protein expression was higher in leaves as compared to bolls in all the genotypes. Previously, Greenplate *et al*. (1998) Greenplate (1999) had also reported that cotton plants harboring *Cry1Ac* gene had shown significant declines in efficacy against American bollworm *Helicoverpa armigera* L. Greenplate *et al.* (2000) Adamczyk *et al*. (2001) Gore *et al.* (2001) Olsen *et al*. (2005) Xia *et al.* (2005) Adamczyk *et al*. (2009) have described the spatio temporal expression variation of *Cry1Ac* throughout the crop growth period.

Plant genetic background had also significant effect on Cry1Ac toxin efficacy. Variations in Cry protein expression exists among bollgard genotypes. Genotypes expressing higher amount of Cry1Ac protein provided better and durable resistance against the targeted insect pest (*Helicoverpa armigera* L). The genotypes 03 and 02 were found best in expressing δ-endotoxin protein whereas; 04 and 01 were the least expressing genotypes. Therefore, utilization of high protein expressing genotypes was essential to enhance *Bt* toxin efficiency in new genotypes (Adamczyk and Meredith, 2004). Wu *et al.* (2003) noticed that a commercial Chinese cotton genotype GK-12 carrying *Cry1Ac* gene had significantly reduced toxin concentration at maturity, while in another experiment Wu *et al.* (2005) studied a genotype named GK-19, a transgenic *Bt* cotton cultivar carrying a *Cry1Ac*/*Cry1Ab* fused gene. The expression of the insecticidal proteins was higher during the early growth stages and significantly declined thereafter, behaving more variably than in GK-12.

Sustainable expression of δ-endotoxin protein in *Bt* cotton genotypes is essential for its efficacy in controlling the American bollworms (*Helicoverpa armigera* L.). Intra-plant expression variability of Cry toxin also expressed significant differences in the *Bt* cotton genotypes. Upper canopy leaves had a higher toxin concentration as compared to middle and lower canopy leaves. In case of upper canopy leaves the genotype 03 remained the best performing line with highest toxin expression and genotype 01 remained the poor performing line with least toxin expression. Whereas, in case of middle and lower canopy, the genotype 03 was the best performing and the genotype 04performed the least (Figs. 3–5). Kranthi *et al.* (2005) also studied intra-plant and in-season variability for *Cry1Ac* gene in cotton hybrids under Nagpur, India climatic conditions. The finding of his studies revealed that significant variations exist within plant canopies and between different plant parts. Upper plant canopy and among parts "leaves" had the highest *Cry1Ac* gene expression among *Bt* cotton hybrids, whereas lower plant canopy and ovary of the flower had the least Cry protein concentration. Upper, middle and lower canopy bolls had also variable *Cry1Ac* gene expression. The current experiment revealed that upper canopy bolls had a higher concentration as compared to middle and lower canopy bolls. The genotype 03 and 04 were the best and poor performing genotypes respectively (Figs. 6–8).

Expression of *Cry1Ac* gene varies with two types of factors; one is external and the second is internal. External factors include temperature, humidity, rainfall, nitrogen availability, plant density, plant spacing, drought and water lodging conditions, while internal factors are plant genetic background, transgene copy number, internal cell environment, point of insertion of transgene, promoter and nucleotide sequence of the gene (Hobbs *et al.,* 1993; Guo *et al.,* 2001; Mahon *et al.,* 2002; Rao *et al*., 2009). Several factors affect the *Cry* toxin concentration in transgenic cotton genotypes. Therefore, the need of the day is to identify those transgenic cotton genotypes having high and stable *Cry1Ac* gene expression during the whole growing season. The genotype 03 and 02 expressed significant toxin concentration and can be used in further varietal development programs.

Conclusion

Principally on the selection of *Bt* varieties for approval of general cultivation on the basis of quantitative level of Cry toxin in leaves and bolls at three defined growth stages and also at the canopy levels. In other words, the minimum levels of Cry protein at the 3 stages of development and the three parts of canopy need to be set for varietal approval to avoid insect resistance to Bt toxin.

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