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GENOTYPE-DEPENDENT REGULATION OF δ-ENDOTOXIN REVEALS THAT INSECT-RESISTANT COTTON CONTAINS TOXIN PREDOMINANTLY IN SEEDS

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Engineered insecticidal δ-toxins protect cotton genotypes against devastating insects but the accumulated amount of toxin varies during the growth period, generating conflicting results. Here we report varied effects of *cry1Ac* gene, driven by CaMV 35S promoter, in aerial parts of cotton plant including seed under different growth and environmental regimes. Data is recorded after every 20 days, starting from 60 to 120 DAS at temperatures 25°C, 35°C, 40°C and 50°C. An increasing trend in the concentration of δ-toxin in leaves is observed from 60 to 100 DAS and at temperature from 25°C to 40°C, and is decreased at 120 DAS and 50°C. The levels of accumulated toxin are significantly higher in MNH-886 compared to FH-142, irrespective to temperature and age of the plants, demonstrating that MNH-886 is more stable genotype for transgene expression and for effective insect control. Interestingly, transprotein is accumulated 28% and 130% higher in seeds (3.6 µg g^{-1}) than in the leaves (2.8 µg g^{-1}) and 15 DAP locules (1.59 µg g^{-1}); respectively, suggesting that varied expression is due to interaction of 35S promoter with environmental factors, plant growth and development. Insects *Pectinophora gossypiella*, *Helicoverpa armigera* and *Spodoptera litura* are serious threats to cotton crop. First two damage bolls whereas later attacks on leaves thereby results in loss of photosynthetic area on leaves consequently lower the yield. This calls for tissue-specific or inducible engineering of toxins in cotton for effective control of insects and biosafety of non-target animals and human being since they feed on seedcake and cotton seed oil respectively.

Keywords: *Bacillus thuringiensis*, cry1Ac endotoxin, cotton, seeds, ELISA

INTRODUCTION

Engineering plant genomes for useful traits leads toward sustainable agriculture. Amongst useful traits, resistance against insects is developed by using cry proteins from *Bacillus thuringiensis* (*Bt*). The *Bacillus thuringiensis* is an endospore forming, aerobic and gram-positive bacterium found in soil having crystal (cry) proteins in the cytoplasm of its sporulating cells. The cry proteins are extremely toxic to some chewing and sucking insects belonging to orders Lepidoptera and dipteral (Soberon *et al*., 2016).

Crossbred cotton varieties expressing BT toxins are being grown on more than 80% of the agricultural land in Pakistan, irrespective to formal approval by the statutory bodies. Cotton was engineered using *cry1Ac* gene to produce its own insecticide, limiting the insecticidal sprays to reduce burden on farmers' budget. Unfortunately, farmers have to spray their fields to protect crop from insect attack, though number is almost half of the number of sprays on non-Bt-cotton varieties, because of variable accumulation of toxin in different genotypes throughout the growing season (Kranthi *et al.,* 2005b; Olsen *et al.,* 2005; Adamczyk *et al.,* 2009; Bakhsh *et al.,* 2010). As the crop growth and yield performance was much influenced by environmental factors, the abiotic factors could play important role in gene expression and thus performance of Bt cotton. The experimental evidences for the influence of abiotic factors on cry gene expression are meager compared to influence of biotic factors. Among these, effect of soil, nutrition, temperature, CO_2 and various stresses are found to be important (Chen *et al.,* 2005; Adamczyk and Sumerford, 2001). In addition to temperature stress, the age of the plant also plays important role in the expression of cry genes. Toxic accumulation levels vary with the age of plant as well (Kranthi *et al.,* 2005a). Hence, it is assumed that the changed efficiency might be linked with high temperature. To make sure that resistance management designed for the use of Bt cotton successful, the estimation of the insecticidal toxic proteins expression by high temperature is required (Chen *et al.,* 2005). Since cake is developed for animals from seeds therefore, it is imperative to quantify the toxin levels in seeds. Though CaMV 35S promoter directs constitutive expression of transgenes but varied expression levels may result from its interaction with environmental factors and physiological state

of plant development. Here we report varied effects of *cry1Ac* gene, driven by CaMV 35S promoter, in aerial parts of the cotton plant including seed under different growth and environmental regimes. The objective of the experiment was to investigate the accumulation of cry1Ac toxic protein in various parts of Bt cotton at variable temperatures and different growth stages.

MATERIALS AND METHODS

***Plant material and growth conditions*:** Seedlings from uniformly germinated seeds were transferred into pots. Single plant per pot was maintained for further studies. Well established 40 days old plants were exposed to four different temperatures under controlled conditions. One set of plants was allowed to grow in the field where maximum day temperature was recorded as 35°C±3 and second set was grown at 25°C±2 in incubation room. The third and fourth sets of plants were placed at 40°C±2 and 50°C±2 respectively in the glasshouse. Under controlled conditions, temperatures were maintained using cooling and heating facilities.

***Genomic DNA isolation and PCR analysis*:** Total genomic DNA was isolated from plants using CTAB method with modifications (Sambrook and Russel, 2001). This isolated DNA was used in PCR for the confirmation of transformation event using event-specific primer for Mon-531. PCR reactions were set up using 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 30 ng/µl each forward and reverse primers, 0.2 µl Taq DNA polymerase, 15 ng/µl template DNA and autoclaved double distilled water. PCR profile used to amplify the desired fragment as follows. 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and initial extension at 72°C for 45 sec with a final extension step at 72ºC for 7 min. Amplified PCR product was separated on 1.5 % agarose gel and visualized under UV equipped gel documentation system (Photonyx Ultra, NYXTecnhnik).

***Total RNA isolation and cDNA synthesis*:** Freeze-dried 1.0 gram leaf tissue of cotton was used to isolate total RNA using GeneJET Plant RNA Purification kit (Thermo Scientific, Lithuania). The isolated RNA was treated with DNase-I enzyme (Thermo Scientific, Lithuania) for the removal of genomic DNA contamination. The integrity of purified RNA was analyzed by electrophoresis on a 1.0% agarose gel stained with ethidium bromide. Purified RNA was quantified using Nanodrop Spectrophotometer (NanoDrop Technologies, USA) and 1.0 µg RNA was used to generate cDNA using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania). The cDNA synthesis was carried out by reverse transcription using 1.0 µg of total RNA from each sample in a reaction volume of 20 µl using $Oligo(dT)_{18}$ primers provided with cDNA synthesis kit following manufacturer's instructions. Reaction was incubated for 60 min at 42°C and reverse transcriptase enzyme was inactivated by heating at 70°C for 5 min and the cDNA was stored at -20°C until it was used in qPCR reactions.

***Semi-quantitative PCR*:** Semi-quantitative PCR expression analysis was carried out using ABI 7500 (Applied Biosystems, Inc.) using SYBER-Green Super Mix (Thermo Scientific, Lithuania). Each 25 µl of reaction having 12.5 µl of 2x master mix (provided in kit), 2 µl of cDNA, 1.25 µl of forward and reverse primers and 9.25 µl of d_3H_2O each concentration in duplicate fashion. The PCR conditions were used as initial denaturation at 95°C for 5 min followed by 40 cycles denaturation at 95°C for 30 sec, annealing at 55°C for 1.0 min and extension at 72°C for 1:30 min with final extension at 72ºC for 7 min. Semi-quantitative analysis of results was carried out with the help of 7500 software version 2.0.1 on basis of Ct values of genes in different samples converted to their linear form normalized with *GAPDH* gene used as an internal control.

***Qualitative and quantitative analyses of toxins in samples*:** The presence of toxin was detected using Cry1Ac protein specific Immuno-strips (Envirologix Inc. USA). Reaction results were recorded as positive (+) and negative (-) on the basis of test line appeared on the strips. Expression level of Cry1Ac endotoxin was quantitatively verified for each temperature treatment at 60, 80, 100 and 120 DAS through quantitative ELISA. Samples, 20 mg each was used in powder form in protein extraction buffer according to manufacturer's protocol (Envirologix Inc. USA). In addition, at 80 DAS, accumulation of Cry1Ac toxin was also quantified in different plant parts of field-grown Bt cotton by using ELISA plate reader (uQuantBioTek, USA) attached with computer and Gen-5 software was used to calculate the expression level of Cry1Ac protein in the targeted tissues.

***Statistical analysis*:** Completely Randomized Design (CRD) was used to conduct experiments and samples from three replications were used to collect data. The experimental data was analyzed using analysis of variance (ANOVA) and Statistix v. 8.1.

RESULTS

***Genomic analysis of transgenic plants to verify the transformation event*:** Two sets of primers were used to investigate whether undertaken insect-resistant crossbred cotton varieties carry *cry1Ac* gene. Total cellular DNA was extracted from both transgenic as well as non-transgenic cotton plants using CTAB method (Rogers and Bendich, 1998) and was used in PCR reactions. PCR reactions were performed using event-specific primers 5'-AAGAGAAACCCCAATCATAAAA-3' and 5'-GAGAATGCGGTAAAGATACGTC-3' (Yang *et al.,* 2005). Further, an indigenous control fragment was amplified using primers 5'-CCAGATTAGAACCTATGAAAC-3' and 5'-TAGCCCATTTCTTACCAC-3'. Since, size of the amplified fragments was different hence multiplex PCR was performed

to analyze the transgenic cotton plants along with non-transgenic as control. Transformation event was verified by using genomic DNA from 4 representative plants from each Bt cotton genotype. Amplification of a fragment of 346 bp size from transgenic plants (Fig. 1, lanes 1-8 and +ve) whereas absence of the same fragment from non-transgenic control plant (Fig. 1, lane C) confirmed the presence of the *cry1Ac* gene in the crossbred transgenic plants. Nevertheless, a fragment of 183 bp size that was amplified from genomic DNA of transgenic and non-transgenic plant (as an internal control). These results confirmed that both varieties MNH-886 and FH-142 contain stably integrated *cry1Ac* gene in their genomes.

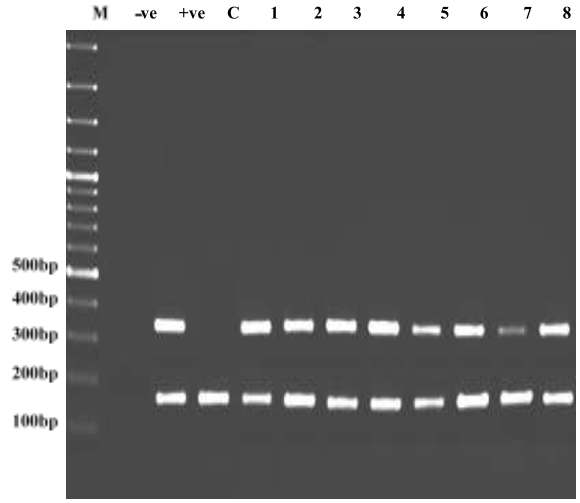


Figure 1. Multiplex PCR using event-specific and internal control primer for cotton to confirm MON-531 transformation event. **Lane M** 100bp DNA marker, **Lane –ve** is PCR control without any template DNA, **Lane +ve** is DNA from known Bt cotton, **Lane C** reflects DNA from Non-Bt cotton plant, **Lanes 1-4** having template DNA from MNH-886, **Lanes 5-8** from FH-142 plants

***Quantitative analysis for transcripts of cry1Ac gene in the transgenic plants*:** Quantitative transcript analysis of *cry1Ac* gene was carried out in FH-142 and MNH-886 varieties under variable temperature and plant developmental stages. The cDNA of *cry1Ac* and *GAPDH* was exponentially amplified using gene-specific primers. The concentration of amplified product in reaction was monitored using Syber green dye. A significant difference in the expression level of *cry1Ac* gene was observed at different growth levels of the plant at variable temperature. An increasing trend in the accumulation of transcripts of *cry1Ac* gene in leaves was recorded at temperatures from 30°C to 40°C, which was decreased from 40ºC to 45ºC temperatures (Fig. 2). As far as number of days of growth is concerned, maximum transcripts accumulated in leaves of 100 DAS plants that significantly reduced in leaves of 120 DAS plants, indicating that transcripts accumulate variably at different growth stages (Fig. 2).

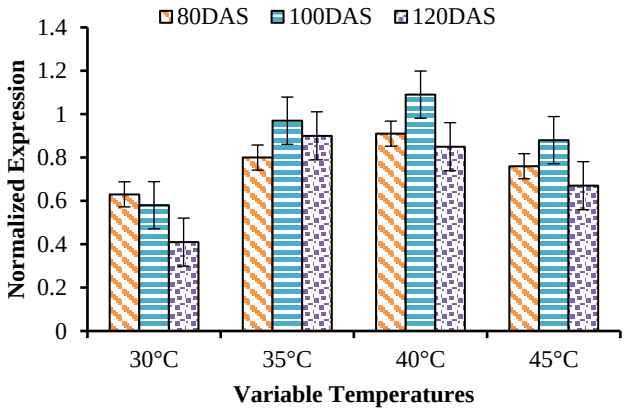


Figure 2. Real Time PCR expression analysis of *cry1Ac* gene in *Bt* cotton genotype under variable temperatures and at different growth stags. Results were normalized using *GAPDH* as an internal control.

***Qualitative and quantitative analyses of accumulating δ-toxin in the transgenic plants*:** PCR positive *Bt* cotton plants were used to detect Cry1Ac protein qualitatively and quantitatively. Cry1Ac endotoxin-specific Immuno-Strips were used and strong signals were detected in representative samples of crossbred varieties, FH-142 and MNH-886 (Fig. 3) whereas no signal was detected in the control non-transgenic cotton sample, confirming the expression of the transgene in the genome of crossbred cotton varieties.

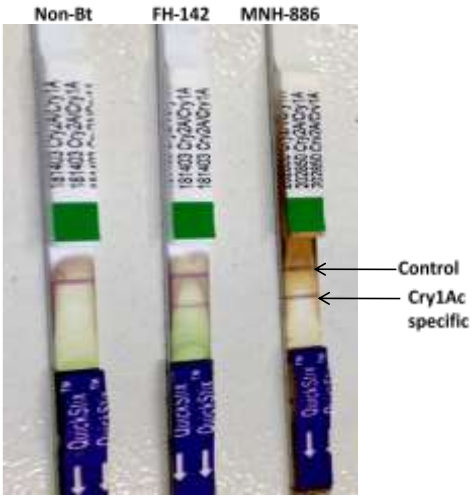


Figure 3. Qualitative Immuno-Strip test for the detection of Cry1Ac protein in leaf tissues of Bt cotton genotypes.

After qualitative analysis, plants were subjected to quantitative analysis of the *Bt* δ-endotoxin when plants reached to the level of 80 DAS (Days After Sowing). Highest levels of transprotein were observed in leaves (2.13 µg g^{-1} and 2.85 µg g^{-1} of total soluble proteins) compared to the lowest levels in fibrous roots (0.05 µg g^{-1} and 0.03 µg g^{-1}) of the representative plants of both genotypes, FH-142 and MNH-886. Further, levels of transprotein accumulation were significantly higher in different parts starting from anther, stigma, ovary, petals, brackets, locules, leaves, pedicel, stem and main to fibrous roots of the representative plants of MNH-886 compared to FH-142 (Table 1).

Table 1. Quantification of Cry1Ac endotoxin (µg g^{-1} fresh weight basis) in different tissues from field-grown Bt cotton plants at 80DAS.

Plant Tissues	Toxic Protein (µg g^{-1} FW)	
	FH-142	MNH-886
Leaves		
Top Third	2.13±0.080	2.85±0.085
Middle	1.49±0.061	1.61±0.056
Bottom	0.29±0.025	0.94±0.028
Anthers	0.29±0.011	0.45±0.012
Stigma	0.62±0.012	0.85±0.012
Ovary	1.02±0.057	1.41±0.057
Pedicel	1.11±0.051	1.29±0.050
Petals	0.76±0.013	0.81±0.013
Brackets	1.33±0.071	1.53±0.073
Stem	0.48±0.061	0.59±0.060
Locule		
15DAP	1.46±0.058	1.59±0.060
30DAP	1.14±0.048	1.33±0.052
Seed	3.36±0.091	3.68±0.091
Roots		
Main Root	0.16±0.021	0.12±0.017
Secondary Root	0.05±0.010	0.03±0.010

***Accumulation of δ-endotoxin is regulated by genetic makeup and age of the plant*:** Since the levels of accumulated toxin were different in different parts of the plant then it was of interest to know whether these levels vary with position or age of the leaves of the plant. To address the question, plants were divided into three canopies i.e. upper, middle and lower where each canopy was comprised of six (06) leaves (Fig. 4), starting from top to bottom of the representative plant. Leaves from each canopy were analyzed and δ-endotoxin levels were recorded using DAS-ELISA, following the manufacturer's instructions (uQuantBioTek, USA). Transprotein was accumulated to the levels between 3.67 µg g^{-1} to 0.11 µg g^{-1} total soluble proteins in leaves from top to bottom. The highest level of transprotein were observed in top first leaf which gradually reduced to the lowest level in the last leaf (Fig. 5), suggesting that accumulation of toxin was reduced with the age of the leaves. These results also indicate that the expression and accumulation of Cry1Ac transprotein is inversely proportional to the age of the leaves, as the maximum toxin was found in young leaves which decreased with the maturity.

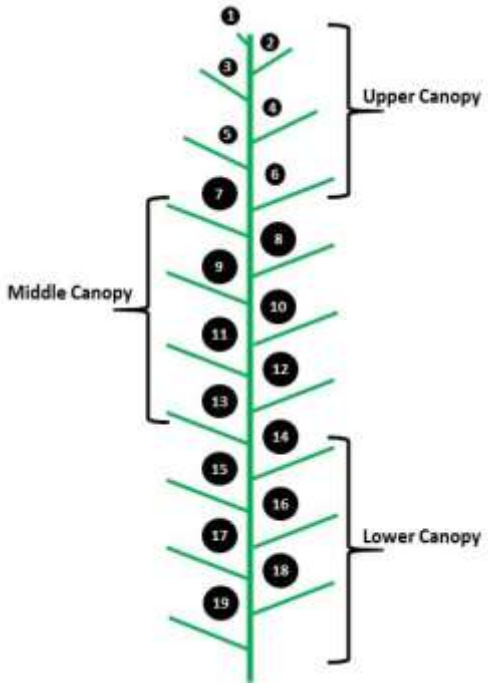


Figure 4. Schematic diagram of cotton plant divided into three canopies; top, middle and bottom, each canopy was comprised on six leaves starting from top towards downward position.

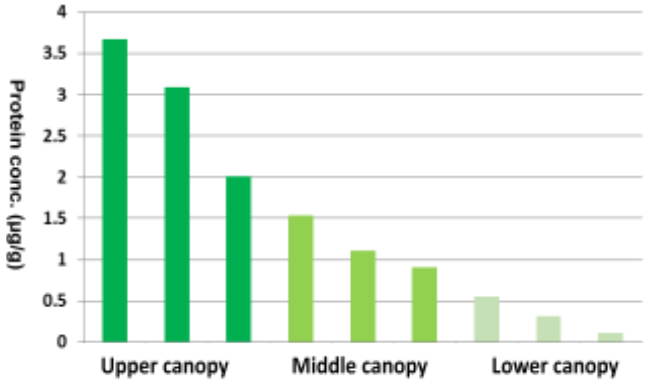


Figure 5. Accumulation of Cry1Ac endotoxin in Bt cotton leaves from top to downward position at 80DAS.

To know whether cell development has any role in transprotein accumulation, top third leaf on the plant was divided into three parts and transprotein levels were recorded. No significant change in the accumulation of transprotein

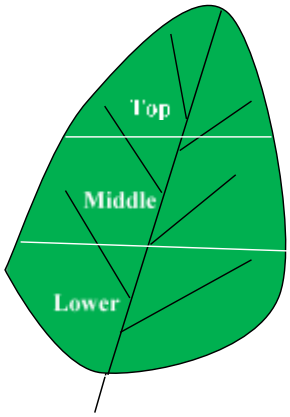
between three different parts of the leaf was observed (Table 2 (a,b), confirming that cell development has no role in the accumulation of the protein.

Table 2. (a) Quantification of Bt endotoxin (Cry1Ac protein µg/g fresh weight basis) in three parts of a third leaf on Bt cotton genotypes at 95DAS; (b) is the representative leaf divided into three parts.

(a)

Third Leaf	Toxic Protein (µg g^{-1} FW)	
	FH-142	MNH-886
Three Parts		
Top	3.09±0.072	3.33±0.043
Middle	3.14±0.044	3.26±0.072
Bottom	3.11±0.079	3.30±0.057

(b)



***Accumulation of δ-toxin in leaves is regulated by temperature and growth of the plant*:** Since the amount of the transprotein in leaves was reduced with the age of the leaves. It was imperative to know whether accumulated amount of toxin vary in leaves with the growth of the plant. Data were recorded for 60 to 120 DAS for the third leaf of the plant. An increasing trend in the accumulation of toxin in leaves from 2.01 µg g^{-1} to 3.4 µg g^{-1} for 60 to 100 DAS was observed, which was declined to 2.33 µg g^{-1} at 120 days of sowing (Fig. 6).

As significant variations in the accumulation of transprotein in leaves of the *Bt* cotton from 60 to 120 DAS were observed, it was decided to know the effects of temperature on transprotein accumulation in leaves. Toxin levels appeared to be increased from 2.01 µg g^{-1} to 3.6 µg g^{-1} at 25°C to 40°C, respectively at 60 DAS. An increasing trend in the accumulation of toxin was observed with increasing temperatures from 25°C to 40°C at 80, 100 and 120 DAS. Nevertheless, the levels were reduced at 50°C for all age plants (60 to 120 DAS), indicating that temperature affects the accumulation of toxin in leaves of the transgenic cotton. Interestingly, accumulation levels were less affected in MNH-886 compared to FH-142, confirming that expression of *cry1Ac* gene under 35S promoter allows genotype-dependent accumulation of toxin in plants.

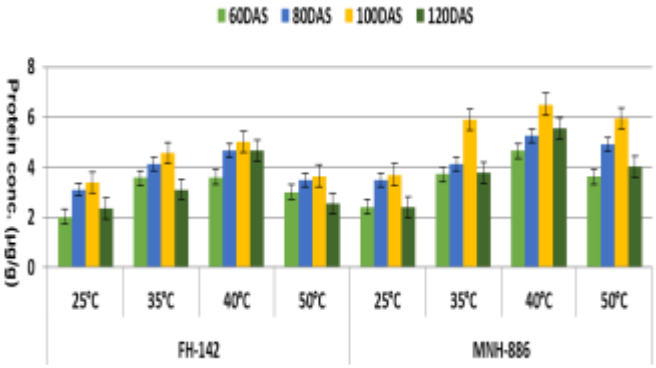


Figure 6. Expression level of Cry1Ac endotoxin in leaves of two Bt cotton genotypes at different growth stages and under variable temperatures.

Transgenic *Bt* cotton plants after successful screening were subjected to quantitative analysis of Cry1Ac endotoxin. Protein was isolated from various parts of field grown cotton at 80 DAS and quantified using DAS-ELISA following the manufacturer's instructions. It was observed that the highest level of cry protein was accumulated in seeds followed by leaves, locules, bracts, pedicel and ovary (Table 1). Low level of transprotein was also found in anthers, stem, stigma and roots.

***Accumulation of δ-endotoxin in seeds*:** Since the accumulated amount of transprotein decreased with the age of the plant in both genotypes, it was of interest to investigate that how much δ-endotoxin accumulates in the seeds while considering the commercial feasibility of transgenic *Bt* δ-endotoxin-containing seedcake and oil. Surprisingly, there are no reports in the literature regarding *Bt* δ-endotoxin levels in seeds for *Bt* cotton plants expressing either *cry1Ac* or *cry1Ab* genes from *Bacillus thuringiensis*. To address this highly important question, 15 and 30 DAP (Days After Pollination) locules and post-harvested delinted seeds from both genotypes were analyzed. Transprotein accumulation levels were 130 (1.46 µg g^{-1}) to 131% (1.59 µg g^{-1}) and 152 (1.14 µg g^{-1}) to 194% (1.33 µg g^{-1}) low in 15- and 30 DAP locules than leaves (2.13 µg g^{-1} and 2.85 µg g^{-1}) of MNH-886 and FH-142 genotypes, respectively (Table 1). Interestingly, transprotein was accumulated 28% and 130% higher in seeds (3.68 µg g^{-1}) than in the leaves (2.85 µg g^{-1}) and 15 DAP locules (1.59 µg g^{-1}), respectively, suggesting that varied expression is due to interaction of 35S promoter with environmental factors and plant development. Toxin level was also tested in field grown plants using 5 consecutive leaf from top towards bottom and seeds from different bolls of same plant at 95 DAS (Table 3).

Table 3. Quantification of Bt endotoxin (Cry1Ac protein µg g^{-1} fresh weight basis) in leaf and seeds of Bt cotton genotypes at 95DAS.

Plant Tissues	Toxic Protein (µg g^{-1} FW)	
	FH-142	MNH-886
Leaves		
Leaf-1	3.36±0.075	3.49±0.055
Leaf-2	3.21±0.073	3.38±0.044
Leaf-3	3.13±0.058	3.25±0.079
Leaf-4	2.88±0.072	3.03±0.107
Leaf-5	2.24±0.058	2.73±0.050
Seeds		
Seed-1	3.95±0.076	3.87±0.089
Seed-2	3.88±0.064	4.06±0.055
Seed-3	3.90±0.067	4.00±0.072

DISCUSSION

Cotton crop that carries *cry1Ac* gene offers season-long, complete plant protection against insects during its growth, allowing plants to stay healthy to withstand environmental pressures. Nevertheless, several reports have been published claiming differences in expression levels during the growth cycle of the plant, leading towards inadequate control of invading insects. The difference in the efficiency might be due to the inactivation of transgene leading to decrease in quantity of the toxin developed. This effect has been described in other crops as a response to environmental stresses, like high light concentration or elevated temperatures and also growth stages (Mahon *et al.,* 2002). Since toxin level in cotton plant is a crucial factor for effective control of bollworms hence, quantity of toxin should not be less than 1.9 µg g^{-1} (on fresh weight basis) to control insects efficiently (Kranthi *et al.,* 2005b). Prior to the quantification of endotoxin expression, confirmation of the type of *Bt* gene is another essential factor. All existing *Bt* cotton hybrids are descended from a widespread parent with a single transformation event Monsanto-531 i.e. Bollgard cotton (Iqbal *et al.,* 2013). Initially every genetic transformation experiment is exposed to genomic analysis, and the PCR is the primary technique which is being successfully used for verification of the transgene integration and confirmation. The amplification of 346 bp fragment indicated that both genotypes MNH-886 and FH-142 have Mon-531 transformation event. Amount of toxin varied in different parts of the plant at different growth stages. Since a number of internal and external factors affect the crop growth and yield and hence could play an important role in gene expression (Chen *et al.,* 2005; Bakhsh *et al.,* 2011).

We have examined the accumulation of Cry1Ac toxin in various parts of cotton plants during growth season in the field as well as under controlled conditions in the greenhouses. Our experiments show that depending on the stage of the plant, detectable endotoxins levels vary from top to bottom. The highest levels of transprotein were observed in top first leaf that gradually reduced to the lowest level in the last leaf on the plant, suggesting that accumulation of toxin was reduced with the age of the leaves. Further, an increasing trend in the accumulation of toxin from 60 to 100 DAS was observed, which was declined at 120 days of sowing, indicating that the accumulation of Cry1Ac transprotein is inversely proportional with the age of the cotton leaves, as the maximum toxin was found in young leaves and it was decreased with the maturity.

Earlier studies indicated that Bt toxin levels in cotton genotypes are highly variable and it is due to the poor performance of transgenic technology in Pakistan, and this could foster resistance in target pests (Cheema *et al.,* 2015). The technology is performing well and the GM crop is adopted overwhelmingly by the farmers not only in Pakistan but in the entire region however; generated conflicting results are due to the poor planning of the lead investigator due to many known reasons. Position and age of the leaf on a plant and environmental cues greatly affect accumulation levels of the transcripts a well as of the transprotein. The role of the regulatory elements, changing climate, age, growth of the plant and position of leaves on the plant were totally ignored while collecting the samples. Further, *Bt* gene was transferred from 'Coker 312' to elite cotton lines through backcrossing. Crossbred lines were released in the environment without purification and stability of the trait, consequently segregation and spread of heterogeneous population in the field was observed. Seed mixing of Bt and Non-Bt cotton varieties is another explanation of heterogeneous population in the field. Insects feeding on heterogeneous population may manage to survive, and that may lead to resistance development against endotoxin.

As significant variations in the accumulation of transprotein in leaves of the *Bt* cotton from 60 to 120 DAS were observed, it was decided to know the effects of temperature on transprotein accumulation in leaves. Toxin levels were increased from 25°C to 40°C at 60 DAS. An increasing trend in the accumulation of toxin was observed with increasing temperatures from 25°C to 40°C at 80, 100 and 120 DAS. Nevertheless, the levels were reduced at 50°C for all age plants (60 to 120 DAS), indicating that temperature affects the accumulation of toxin in leaves of the transgenic cotton. Interestingly, accumulation levels were less affected in MNH-886 compared to FH-142, confirming that expression of *cry1Ac* gene under 35S promoter allows genotype-dependent accumulation of toxin in plants.

Altogether data show a large accumulation of the expressed insecticidal protein in the leaves as well as in the seeds of transgenic clones. Near absence of insecticidal protein in transgenic cotton roots eliminates the possibility of transgenic plants being called into question for toxic effects on microorganisms in the plant root zone. However, presence of

highest toxin levels in the seed while considering the commercial feasibility of transgenic Bt δ-endotoxin-containing seedcake and oil demands for an alternative promoter to control the expression of *Bt* gene in edible parts of the plant. Further, minimum levels of toxin in flower parts and locules failed to control pink bollworm, again demanding expression of *Bt* genes under tissue specific promoter for effective control of bollworms, considering the egg laying and feeding behavior of insects.

***Conclusions*:** Bollgard cotton genotypes expressing *cry1Ac* gene were studies under variable temperature and at different growth stages. The accumulation of Cry1Ac protein was quantified in various plant tissues and variable expression level was found in all tested plant parts. Under heat stress conditions top third leaves were used to analyze the toxic protein and found that toxin level is very much influenced by temperature and age of the plant. The maximum protein accumulation was recorded at 40°C when the plant age was 100 days with minimum at 25°C. The protein level was decreased with the advancement in growth stage of the plant. At all growth stages and heat stresses, the MNH-886 performed well in comparison to FH-142 Bt cotton genotype. The variations in expression of *cry1Ac* were also verified at mRNA level and a direct relationship between mRNA and accumulated protein levels were observed. Variable toxin accumulation was also observed in different parts of the plant of Bt cotton, and hence it was concluded that the varying level of Cry protein in different plant tissues is due to the expression of *cry1Ac* gene under CAMV 35S promoter, which is developmentally regulated and could be responsible for the temporal and spatial accumulation of Cry1Ac protein in cotton.

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