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Comparative Account of *Bt* Gene Expression in Cotton under Normal and Salt Affected Soil

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Abstract

Expression of *Bt* gene in cotton is affected by various environmental factors. The objective of the current study was the detection and quantification of *Bt* endo-toxins in local Pakistani cotton genotypes in various plants under different salt stress levels in various plant parts. Detection of *Bt* gene type was performed by immune strip analysis while, quantification of *Bt* toxin was carried out by commercially available ELISA kits. Ten transgenic cotton genotypes of local origin were assessed for the detection of *Cry1Ac*, *Cry1F* and *Cry2Ab* genes. Immunostrip analysis for all *Bt* cotton genotypes revealed the presence of only *Cry1Ac* gene. PCR result confirmed the presence of only Mon531 (non-patented) event of 346 base pair amplicon size and ruled out the presence of any other patented event in Pakistani *Bt* cotton varieties. The toxin value show highly significant differences under different salt stress levels. *Bt* toxin values were inversely affected by the increase in salinity levels. *Bt* toxin values tend to decrease after 90 days of sowing in all treatments. Moreover, higher values on fresh weight basis were noted in leaf tissues followed by square tissues.© 2013 Friends Science Publishers

Keywords: ELISA; *Bt*cotton; Salt stress; *Cry1Ac;* Immunostrip; *Bt*toxin

Introduction

Cotton is an important cash crop and main pillar of textile industry of Pakistan (Bakhsh *et al*., 2010). Globally Pakistan occupies fourth position in cotton production by contributing 55% of the foreign exchange earnings of the country (Alam *et al.,* 2000; Ali *et al.,* 2010). Cotton is susceptible to more than fifteen economically key pests, mainly Lepidoptera insects. To achieve the goal, genetically modified cotton (*Bt* cotton) is engineered by insecticidal genes (*Bt* gene) taken from gram positive, soil bacterium *Bacillus thuringiensis* (Chen *et al*., 2003). *Bt* cotton is not only effective against many cotton pests, but also human and environment friendly as minimize the agrochemicals use (Mehmood *et al.,* 2012).

Insect control is correlated with the synthesis of insecticidal endotoxin by the expression of *Cry* genes (Gutierrez *et al*., 2006). Reduction in toxin level may cause inadequate targeted pest control and provoke resistance in cotton insects against *Cry* protein (Tabashnik *et al*., 2008). The usefulness of GM cotton is allied to the expression level of endotoxin (Gutierrez *et al*., 2006) that remains variable throughout the plant life cycle (Olsen *et al.,* 2005). The efficacy of *Bt* gene against pest varies among cotton genotypes (Adamczyk and Sumerford, 2001), plant tissues (Abel and Adamczyk, 2004), various environmental features, types of gene (Gore *et al.,* 2001; Gore and Adamczyk, 2004; Jackson *et al.,* 2004),and plant age (Wan *et al.,* 2005). The production of endotoxin level decreased with the aging of crop. Therefore, at advanced stages the GM plants may offer chances for cross resistance to targeted insects (Kranthi *et al*., 2005).

Abiotic stresses like salinity, water logging, drought, temperature and nutrients deficiency have influenced *Bt* protein content in genetically modified cotton (Mahon *et al*., 2002; Barrett-Lennard, 2003; Benedict *et al*., 2006; Chen *et al*., 2005). Salt stress reduces plant development and yield through nutritional imbalance (Dar and Anwar, 2005), toxicity of Na^+ and Cl^- ions and osmotic effects on metabolism (Sairam and Tyagi, 2004). Salt stress significantly reduced the *Bt* protein in fully expanded leaves of cotton (Jiang *et al*., 2006). Salinity alone reduced *Cry* gene expression from 11 to 22 % and total protein (soluble protein) contents up to 7.2% within 7-21 days of growth period (Gehlot and Shekhawat, 2005). Similarly in combination of both water logging and salinity minimize *Bt*

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protein from 50–72% (Barrett-Lennard, 2003). About 6.67 million hectares (40%) of cultivated land of Pakistan is affected to various degrees of soil salinity (Khan, 1998), and salinity level is increasing at high rate of 40,000 hectares annually (Alam *et al.*, 2000).

This work deliver a clear picture concerning the effect of salinity on *Cry1Ac* gene expression and will help to adopt proper agronomic practices for applicable insect pest management in local genetically modified cotton cultivars under salt stress conditions in Pakistan.

Materials and Methods

Plant Material

Ten *Bt* cotton (local origin) varieties were analyzed in the present study to evaluate *Cry1Ac* gene expression under various salt stress levels at different growth stages. These varieties were *FH-113, MNH-886, Bt-121, FH-114, Bt-703, Bt-802, Bt-3701, MG-06, Sitara-2008* and *IR-1524*. Their seeds were obtained from National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad.

Pot Experiments

The triplicate experiments were conducted under glass house condition where temperature was maintained at 35 ± 5^{o}C. Each pot (38 cm in diameter and 40 cm in length) was filled with 15 kg of treated soil. Five seeds were sown in each pot (Fig. 1A).

Soil Preparation and Salt Treatment

Soil was prepared by mixing soil, farm yard manure and sand by 2:1:1. The soil mixture was fully grounded and sieved. Apart from control, three saline regimes (10 dS m^{-1}, 17.5 dS m^{-1}, 25 dS m^{-1}) were created by adding calculated amounts of sodium chloride (NaCl) within soil and mixed for homogenization of salt. Each pot was filled with 15kg of soil within all regimes. The electrical conductivity (ECe) of each salinity level was calculated after 35 (DAS) using the following formula.

Amount of salt = Saturation percentage × TSS × molecular weight of the salt × weight of soil/100×1000. Calculated ECs are given in Table 1.

Where, TSS (total soluble salts) = (required ECe – existing ECe) × 10

Electrical conductivity of soil paste extract (ECe) was measured by EC meter.

Polymerase Chain Reaction (PCR)

DNA was extracted from the fresh leaves of each entry through CTAB method (Sambrook and Russel, 2001). For confirmation of transgene at DNA level, event specific primers of Monsanto (Mon531) both reverse and forward were used (Yang *et al.*, 2005).

F (Forward sequence): 5'AAGAGAAACCCCAATCATAAAA3'.

R (Reverse sequence): 5'GAGAATGCGGTAAAGATACGTC3'.

The PCR profile was optimized at 94^{o}C in denaturation step followed by annealing (50^{o}C) and finally extension (72^{o}C) in 35 cycles. The amplified product was separated on 1% agarose gel and visualized by UV illuminator. An amplicon size of 346 bp was obtained.

Immuno strip Analysis

For immunostrip analysis 100 mg fresh leaf samples were taken according to manufacturer's instructions (Agdia Inc. USA) and tested for the detection of *Cry1Ac, Cry1F* and *Cry2Ab*. Care was taken to avoid the entrance of strips more than 0.5 cm or ¼ inch during the reaction time. The reaction was considered valid when control line was appeared within 3 min. Two types of immunostrip specific for "*Cry1F*" (Cata. #: STX010300) "*Cry1Ac and Cry2Ab*" (Cata. #: STX06800) were used. Reaction results were recorded as positive (+) and negative (-) on the basis of test line appearance on the strip within due time.

Sandwich-ELISA

After 90,120 and 150 days of sowing, quantification of *Cry1Ac* encoded toxin was carried out through sandwich-ELISA. Third fully expanding leaves, squares and bolls tissues of each plant were used for analysis. Twenty milligram (20 mg) fresh plant tissues were sampled, grinded manually in extraction buffer provided by manufacturer of kit (Envirologix Inc. USA).

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) and means were separated using Least Significant Differences (LSD). Statistix v. 8.1 (Analytical Software, 2005) package was used for this purpose.

Results

Immunostrip Analysis for *Bt* Genes Detection and PCR Confirmation

Ten local genetically modified *Bt* cotton genotypes were tested by immunostrip for the detection of three commercial *Bt* genes viz., *Cry1Ac, Cry2Ab* and *Cry1F*. Result showed that all the genotypes carried *Cry1Ac* gene (Table2) as shown in (Fig. 1B),while immunostrip analysis showed 100% negative reactions in all genotypes for *Cry1F* (Wide Strike event) and *Cry2Ab* (Bollgard-II event) genes. PCR product of 346 bp was amplified for all Mon531 positive genotypes (Fig. 2).

Table 1: ECe measurement of all soil treatment after 35 DAS

Soil Sample (developed ECe)	ECe
Random selection from control sample	0.96-1.4 dS m^{-1}
Random selection from 10 dS m^{-1}	11.2-12.6 dS m^{-1}
Random selection from 17.5 dS m^{-1}	15.7-19.3 dS m^{-1}
Random selection from 25 dS m^{-1}	22.9-27.5 dS m^{-1}

Table 2: Immunostrip analysis of ten local *Bt* cotton genotypes

Genotypes	FH-113	FH-114	MG-06	Bt-121	Bt-3701	Bt-803	Bt-703	Sittara 2008	MNH-886
Cry1Ac	+	+	+	+	+	+	+	+	+
Cry2Ac/1F	-	-	-	-	-	-	-	-	-

+ = *Bt* gene presence, - = *Bt* gene absence

Fig.1A: Local origin cotton genotypes in glass house **B:** Strips showing +ve results

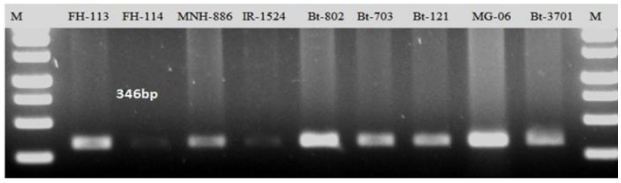

Fig. 2: Confirmation of *Bt* gene event in ten *Bt*-varieties through PCR (1kb ladder)

ANOVA Results for Toxin Accumulation

Analysis of variance showed significant ($P \leq 0.05$) differences in *Bt* toxins accumulation due to salts, varieties and tissues. However, significant differences among tissues for *Bt* toxin accumulation appeared only after 90 days of sowing (Table 3). Interactions i.e., Tissues × Salts were significant ($P \leq 0.05$) at 120 and 150 days after sowing showing relative ranking of tissues for toxin accumulation changed due to salinity regimes. Interaction of Varieties × Salts was significant ($P \leq 0.001$) only at 150 days after sowing showing that varieties ranking changed across salinity regimes at later growth stages for toxin accumulation. This may be due to differential salinity tolerance among the cotton genotypes and salt tolerant genotypes may have shown higher values for toxin accumulation (Jiang *et al*., 2006). However, magnitude of the mean sum square of interactions was smaller than individual main effects of factors such as salts, tissues and varieties. Therefore, varieties and tissues means were averaged over the salts regimes (Table 4).

Quantification of *Cry1Ac*Toxin in Leaf and Square Tissues at 90 DAS

Individual mean of treatments, tissues and varieties at all growth stages are shown in Table 4.Plants of all transgenic *Bt* genotypes that showed positive reactions in immunostrip analysis for *Cry1Ac* gene, were further exposed to sandwich ELISA for quantification of *Cry1Ac*toxin (Fig.3). Leaves and squares were taken from individual plants of control, 10 dSm^{-1} salt stress level (SSL), 17.5dSm^{-1} SSL and 25dSm^{-1} SSL. In leaves and squares, levels of *Cry1Ac* toxin were quantified by ELISA assay. Toxin concentration tends to decrease with salt regimes (control, 10 dsm^{-1}, 17 dsm^{-1}, 25 dS m^{-1}). Control showed the highest concentration of toxin while its concentration was lowest at salinity regime of 25 dsm^{-1}. However, average mean was statistically similar at 10 $dsm^{-1}$and 17 dsm^{-1}. Average concentration of toxin in tissues i.e., leaves and square was also non-significant ($P \geq 0.05$).

The quantity of *Cry1Ac* toxins in leaf tissues on fresh weight basis of control plants ranged from 0.7516 to 0.4429 µg/g. While the toxin values under different salt stress varied from 0.7247 to 0.3401 µg/g at 10 dS m^{-1} SSL, 0.7038 to 0.2859 µg/g at 17.5 dS m^{-1} SSL and 0.5099 to 0.2228 µg/g at 25 dSm^{-1} SSL for ten genotypes. Among the varieties, *Bt-802* showed the highest concentration of toxin followed by varieties *Bt-121*, *Sittar 08*, *Bt-3701* and *Bt-703*. These varieties showed non-significant ($P \geq 0.05$) differences for *Bt-toxin* (Table 4).

Quantification of *Cry1Ac*Toxin in Leaf, Square and Boll Tissues at 120 DAS

Leaf, square and boll tissue samples were taken from individual plants of ten *Bt* cotton genotypes under control and different salt stress. Results showed that the quantity of *Cry1Ac* toxin in leaf tissues on fresh weight basis before the salt stress ranged from 0.8626 to 0.4413 µg/g in ten *Bt* genotypes. While after salt stress, toxin levels in leaf tissues ranged from 0.9290 to 0.6368 µg/g at 10 dSm^{-1} SSL, 0.8252 to 0.4347 µg/g at 17.5 dS m^{-1} SSL and 0.5591 to 0.3226 µg/g at 25 dS m^{-1} SSL for different genotypes. Average concentration was the highest in control. However it showed non-significant ($P \geq 0.05$) difference with toxin concentration level at 10 dS m^{-1} which was followed by the concentrations at 17.5 dS m^{-1} and 25 dS m^{-1}(Table 4).

Significant differences also existed in plants tissues at this growth stage (120DAS) (Table 4). The highest concentration was observed for leaf tissues followed by square tissues while boll showed the least concentration of toxin. Results showed that all studied *Bt* genotypes vary from one another with respect to *Bt* toxin levels, the quantity of *Bt* toxins in square tissues on fresh weight basis before the salt stress ranged from 0.6067 to 0.1423 µg/g. While the *Bt* toxin values under salt stress varied from 0.5651 to 0.1250 µg/g at 10 dS m^{-1} SSL, 0.5031 to 0.1184 µg/g at 17.5 dS m^{-1} SSL and 0.4480 to 0.0505 µg/g at 25 dS m^{-1} SSL for

Table 3: Percentage of total sum of square for ANOVA

Source of variation (SOV)	90 DAS (% SS)	120 DAS (% SS)	150 DAS (% SS)
Salt	28.96***	19.10***	19.85***
Tissue	33.42	55.39***	45.61***
Variety	12.33*	5.18***	7.22***
Salt × Tissue	2.75	0.91*	3.34***
Salt × Variety	4.22	1.66	3.00**
Tissue × Variety	10.96**	5.32***	9.11***
Salt × Tissue × Variety	3.08	5.29*	4.44

% SS followed by ***, ** and * within the column are significantly different at (p<0.001), (p<0.01) and (p<0.05), respectively. DAS= days after sowing

Table 4: *Bt* toxin level (µg g^{-1}) at different salinity levels, tissues and cotton varieties

Salt treatment	90 DAS	120 DAS	150 DAS
Control	0.5714± 0.026a	0.5044± 0.02a	0.3167± 0.013a
10 dS m^{-1}	0.4936± 0.026b	0.4740± 0.02a	0.2630± 0.013b
17.5 dS m^{-1}	0.4452± 0.026b	0.3297± 0.02b	0.1999± 0.013c
25 dS m^{-1}	0.3551± 0.026c	0.2572± 0.02c	0.1608± 0.013d
Tissue			
Leaf	0.4840±0.019a	0.6197±0.017a	0.3540±0.011a
Square	0.4486±0.019a	0.3540±0.017b	0.2161±0.011b
Boll		0.2003±0.017c	0.1352±0.011c
Variety			
FH113	0.4619±0.04 b	0.4832±0.03ab	0.2928±0.02ab
FH114	0.4444±0.04b	0.4030±0.03bc	0.2434±0.02bc
Bt121	0.4792±0.04ab	0.3742±0.03cd	0.2385±0.02c
MNH886	0.3925±0.04b	0.3878±0.03cd	0.2389±0.02bc
IR1524	0.3930±0.04b	0.3572±0.03cd	0.2114±0.02cd
Sittar08	0.4910±0.04ab	0.3332±0.03cd	0.2156±0.02cd
Bt3701	0.4871±0.04ab	0.3193±0.03d	0.1959±0.02cd
MG 06	0.4483±0.04b	0.3781±0.03cd	0.2329±0.02cd
Bt802	0.5800±0.04a	0.4876±0.03a	0.2996±0.02a
Bt703	0.4857±0.04ab	0.3898±0.03cd	0.1817±0.02d

Means ± SE followed by the same letters within the column are not significantly different (p>0.01; LSD)
DAS= days after sowing

Fig. 3: Quantification of *Bt* toxins by sandwich ELISA

different genotypes, respectively. The quantity of *Bt* toxin in boll tissues on fresh weight basis before the salt stress ranged from 0.7992 to 0.4973 µg/g. Under stress condition *Bt* toxin varied from 0.7871 to 0.4872 µg/g at 10 dS m^{-1} SSL, 0.6721 to 0.3411 µg/g at 17.5 dS m^{-1} SSL and 0.5411 to 0.232 µg/g at 25 dS m^{-1} SSL for different genotypes, respectively.

As noted earlier at 90 DAS, *Bt-802* again showed the highest concentration of toxin followed by variety FH-113.

Quantification of *Cry1Ac*Toxin in Leaf, Square and Boll Tissues at 150 DAS

Leaf, square and boll tissues (green immature tissue) were taken from control and salt stress treated plants at 10 dS m^{-1}, 17.5 dS m^{-1} and 25 dS m^{-1} SSL. Significant difference (P≤0.05) existed due to salinity regimes (Table 4). The highest average concentration was noted for control followed by salt regimes of 10 dS m^{-1}, 17.5 dSm^{-1} and 25 dS m^{-1} respectively. Moreover significant differences also noted for plant tissues with leaves were the highest accumulator of *Bt* toxin, while bolls accumulated the lowest level of toxin concentration (Table 4).

Results revealed that the quantity of *Cry1Ac* toxin in leaf tissues on fresh weight basis before the salt stress ranged from 0.6067 to 0.1423 µg/g in ten *Bt* genotypes. While after salt stress, toxin levels in leaf tissues ranged from 0.5651 to 0.1250 µg/g at 10 dS m^{-1} SSL, 0.5031 to 0.1095 µg/g at 17.5 dS m^{-1} SSL and 0.4480 to 0.0505 µg/g at 25 dS m^{-1} SSL for different genotypes, respectively. The quantity of *Bt* toxins in square tissues on fresh weight basis before the salt stress ranged from 0.3484 to 0.2174 µg/g. While under salt stress toxin varied from 0.2838 to 0.1996 µg/g at 10 dSm^{-1} SSL, 0.2393 to 0.1228 µg/g at 17.5 dSm^{-1} SSL and 0.1963 to 0.1247 µg/g at 25 dSm^{-1} SSL for different genotypes, respectively. The quantity of *Bt* toxin in boll tissues on fresh weight basis before the salt stress ranged from 0.2032 to 0.1291 µg/g. While the *Bt* toxin values under salt stress were varied from 0.1697 to 0.1351 µg/g at 10 dS m^{-1} SSL, 0.2032 to 0.0932 µg/g at 17.5 dS m^{-1} SSL and 0.1412 to 0.0553 µg/g at 25 dSm^{-1} SSL for different genotypes.

The genotypes *Bt-802* showed the highest concentration of *B*t toxin followed by genotype FH-113. Genotype *Bt-703* showed the lowest concentration of *Bt* toxin in this growth stage (Table 4).

Discussion

Before quantification of endo-toxin level confirmation of number and type of *Bt* gene was important. Results confirmed the presence of *Cry1Ac* gene only using immunostrip analysis and ruled out the presence of any other *Cry* gene (*Cry2Ab*or *Cry1F*). Ali *et al*. (2010) also reported the same results that only *Cry1Ac* gene exist in all 36 *Bt* genotypes cultivated in two main cotton growing districts (Sindh and Punjab) of Pakistan. They also confirmed the presence of only *Cry1Ac*gene through molecular analysis. Molecular analysis revealed the presence of Mon 531 (non-patented event) of Monsanto in Pakistani genotypes and insure the absence of any other patented events (Mon1445, Mon15875). Mon531 event is specific for *Cry1Ac*gene only (Yang *et al.*, 2005).

Various factors affect the expression of *Cry1Ac* gene, in which plant genotype is an important factor that affects the *Cry* gene expression. In present study plant genetic

backgrounds have been found to affect the *Bt* gene expression with high expression level of *Bt*-802. Mainly due to the interaction of *Bt* gene with different host genetic backgrounds. Similar results were also reported by Adamczyk and Sumerford (2001) who examined 13 commercial varieties *Bt* cotton across two sites in 2000 for endotoxin expression with high expression level in two varieties (DP 458B/RR and NuCOTN 33B).Data support the impact of parental background in the term of *Cry1Ac* gene expression in cotton.

For durable pest resistance United state Department of Agriculture recommended *Bt* cotton with an average expression level of 1.5 µg/g fresh weight basis .Monsanto USA providing *Bt* cotton seed whose expression level high than the recommended level. In Pakistan, the *Cry1Ac* expression level is lower than recommended level. This low level of expression is alarming as it may provoke cross resistance in insect against *Cry1Ac*, and thus depriving the farmers to the benefit of *Bt* technology.

Appropriate level of *Bt* toxin in plant part play an important role in plant protection against target pest (Greenplate *et al.,* 2001). The *Cry* protein concentration in all tissue of the plant does not remain constant throughout the plant life span. Due to this variation in *Bt* protein levels the efficacy of *Bt* cotton to control target worms may decrease (Mahon *et al.,* 2002). Results of the current study showed that higher level of *Cry1Ac* protein was recorded in leaves followed by squares and minimum level in boll tissues. Similar study was also conducted by Kranthi *et al.* (2005) and results showed the same expression trend of *Cry1Ac.* Chen *et al.* (2000) also conducted same study with finding that *Bt* toxin concentration in fully expanded leaves of cotton plant was significantly higher than other parts.

Bt protein concentration at 90 and 120 DAS was higher at different salt stress levels, showed that *Bt* protein concentration at early stage of plant growth remain high, this high level of toxin concentration at early stage of plant is mainly due to participation of complex set of genes (Seki *et al.,*2001). Complex changes occur in the mRNA binding protein which may result in high level of *Cry1Ac* gene expression (Galau *et al.,* 1987).

From results it is clear that increasing salt concentration i.e., control, 10 $dSm^{-1,}$ 17.5 dSm^{-1} and 25 dSm^{-1} *Bt* toxin level decreased in leaves. Results are in agreement with the finding of Jiang *et al.* (2006) who reported that high salinity may decrease the *Cryo* protein level, because normal gene expression is disrupted by high salt concentration. Plant hormone signals affected negatively by increased salt stress level, which retorted to the decreased water potential. Various biochemical and physiological processes were affected adversely by severe NaCl concentration (Munns, 2002).

Significant difference in *Bt* toxin level was observed in different plant parts (leaf, squares, bolls) at (120 and 150) days after sowing (DAS) under different salt stress levels (SSL). Data from the square analysis showed that 3 genotypes FH-113, *Bt*-802 and MNH-886 express maximum *Cry1Ac* toxin. The minimum expression of *Cry1Ac* toxin level in squares was quantified under severe salt stress in *FH-114*, *Bt*-121 and *Bt*-3701. Severe salt stress not only affects the expression level of *Bt* gene in leaf but also in other parts. *Cry1Ac* protein concentration was higher in control plants and decreased after each salt stress significantly due to NaCl toxicity (Sairam and Tyagi, 2004). Similarly, reduction in *Bt* protein contents were also confirmed by Luo *et al.*(2008). Salinity changed nitrogen metabolisms such as, protein synthesis, nitrogen uptake and protein decomposition (Hocking *et al*., 1985; Lin *et al*., 1995; Millar and Dennis, 1996; Jiang *et al*., 2006).

Bolls are the plant part that is directly attacked by worms. It is important to monitor the *Bt* gene expression in these sensitive parts of cotton before planning appropriate agronomic practices. Data of the current work showed that significant difference in *Bt* protein was found in the boll tissue of cotton genotypes at normal, 10, 17.5 and 25 dSm^{-1} salt stress levels. According to Luo *et al*. (2008) under salt stress, the expression of *Cry1Ac* decreases in boll tissues of transgenic cotton. Toxin concentration in bolls might also be affected by other factors.

Comparison of toxin level at 120 and 150 DAS showed that, *Bt* toxin level decreased regularly as the crop shifted toward maturity. Different factors are responsible for the fluctuating levels of *Cry1Ac*protein that is mainly due to variation in *Cry1Ac*gene expression. This variation in the expression of insecticidal gene might occur due to variation in nucleotide sequence, gene copies number, used promoters, insertion site in the host genomic DNA (Guo *et al.,* 2001; Rao, 2005). At lateral stage the production and expression of mRNA declined that at result *Cry1Ac* toxin level also declined (Mahon *et al.,* 2002), and also due to disturbance of used promoter methylation (Xia *et al.,* 2005). For applicable protection against specific insect, adequate level of insecticidal protein are important at proper time and stage.

In conclusion , the usefulness of *Bt* cotton application is correlated with *Bt* gene expression that varies with plant parts and also with age. Severe salt stress may cause fluctuation in *Cry1Ac* gene expression, which will not only reduce the cotton output but also increase the risk of cross resistance development in target worms. This is mainly due to low toxin production in plant parts. It is concluded from the result of the current study that severe salt stress adversely affects the *Bt* toxin level in genetically modified cotton genotypes, Salt stress condition may increase the insect pest attack. In local cotton genotypes lower *Cry1Ac* toxin level exist than USAD recommended level. To delay the development of cross resistance in pest, genotypes having low *Cry1Ac* toxin level should not be recommended. Pakistani cotton genotypes harbor only *Cry1Ac* gene. Packages of *Bt* genes should be retained to delay and avoid the development of cross resistance in target cotton insect/pests.

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