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Camv 35S is a Developmental Promoter Being Temporal and Spatial in Expression Pattern of Insecticidal Genes (Cry1ac & Cry2a) in Cotton.

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Abstract: Stable integration and spatio -temporal expression of two insecticidal genes (Cry1Ac and Cry2A) in transgenic cotton was studied. Firstly, integration of both genes was confirmed in these advance lines by different molecular analysis. The quantitative levels of both Cry1Ac and Cry2A genes were found variable among these cotton lines and also varied between different plant parts. These lines showed a stable integration of insecticidal genes in T5 and T6 Progeny. The expression of both genes declined gradually with the age of plant as well as in different plant parts through out the season. The leaves of Bt Cotton were found to have maximum expression of Cry1Ac and Cry2A genes followed by squares, bolls, anthers and petals. Toxin level in fruiting part was less as compared to other parts showing the in consistency in toxin level inspite of using 35S CaMV promoter which is constitutively expressing promoter. The study conducted suggested that CaMV 35S promoter is a developmental promoter and also it triggered research to find out new possible promoters to make expression of insecticidal genes consistent throughout life of cotton plant.

Key Words: 35S CaMV promoter, temporal and spatial expression.

INTRODUCTION

Cotton is the most important cash crop and backbone of textile industry of the world. Likewise Pakistan is the fifth largest producer of cotton in the world, the third largest exporter of raw cotton, the fourth largest consumer of cotton. Cotton is susceptible to attack by more than 15 economically important insects, the major Lepidopteron being, American Boll worm (*Heliothis armigera*), Pink Boll worm (*Pectinophora gossypiella*), Spotted Boll worm (*Earius insulana/vitella*), Army Bollworm (*spodoptera lithura*). At present, crop protection in agricultural system relies almost exclusively on the use of broad spectrum highly toxic agrochemical that has led to serious environmental problems and human health concerns, leading to make efforts towards developing its biological control measures. To combat losses from insect pests, insecticides are used excessively every year in developing countries like Pakistan.. In 2006-07, 20394 tons of pesticides were consumed which cost 61 Millions US Dollars (Economic Survery, 2006-07).

The most significant breakthrough in plant biotechnology is the development of the techniques to transform genes from unrelated sources into commercially important crop plants to develop resistance against insect pests (Dhaliwal *et al.,* 1998; Lycett and Grierson, 1990). *Bacillus thuringiensis* (*Bt*) is perhaps, the most important source of insect resistant genes. Genes from *B. thuringiensis* encode for crystal proteins, which are toxic against larvae of different insects, *e.g.* Lepidopterans (Cohen *etal.,* 2000; Hofte and Whitely, 1989; Schnepf *et al.,* 1998) Coleopterans (Herrnstadt *et al.,* 1986; Krieg *et al.,* 1983) and Dipteran insects (Andrewa *et al.,* 1987). These genes are generally safe for human consumption (BANR, 2000).

Mechanism of Endotoxins to kill the targeted insect is actually the action of the Bt Cry proteins involving solubilization of the crystal in the insect midgut, when ingested by larvae, toxin proteins bind to specific receptors in the midgut region, and toxin binding in susceptible insects disrupts midgut epithelium, thereby causing overall toxic effects and ultimately resulting in death of the larvae (Kranthi *et al.,* 2005). This strategy proved to be beneficial in many cotton growing countries of the world in order to get rid of heavy pest pressure which was really costly and laborious in terms of spraying schedule and was not environmental friendly as well.

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Transgenic cotton expressing Bt (Bacillus thuringiensis) toxins is currently cultivated on a large commercial scale in many countries, but observations have shown that it behaves variably in toxin efficacy against target insects under field conditions. Understanding of the temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production.

However, for the transgenic technology to be sustainable, it is very important that the endotoxins should be produced in adequate amount and at the proper time to protect the cotton crop against targeted insect pests mainly American Boll worm (*Heliothis armigera*), Pink Boll worm (*Pectinophora gossypiella*), Spotted Boll worm (*Earius insulana/vitella*).

A Locally Approved Cultivar CIM-482 was transformed with two insecticidal genes Cry1Ac and Cry2A using Agro bacterium mediated transformation, a construct pk2Ac harbouring two Bt genes was developed by prior to transformation by isolating Cry1Ac and Cry2A genes from locally soil dwelling bacterium (Rashid *et al.,* 2008). Both genes driven by constitutively expressing CaMV 35S Promoter that enables the expression of both endotoxins in almost all plant parts.

Present study was conducted in twelve advance transgenic cotton lines transformed with the two insecticidal genes Cry1Ac and Cry2A using Agrobecterium mediated transformation method. This study was carried out at campus of National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan with the purpose to determine stable integration of insecticidal genes in advance cotton lines and the expression of endotoxins temporally as well as spatially to estimate a toxin titer of both genes and its effect of the survival of targeted insect pests.

MATERIAL AND METHODS

Plant Material:

Twelve advance transgenic lines of cultivar CIM-482 transformed with two insecticidal genes Cry1Ac and Cry 2A were selected after multiplication in its subsequent generations and grown in campus of National Centre of Excellence in Molecular Biology (CEMB) in Year 2006-07 for this study.

Confirmation of Stable Gene Integration:

For the purpose to confirm the stable integration of insecticidal genes Cry1Ac and Cry2A in advance cotton lines (T6 Progeny), Molecular analysis like PCR and Southern Blot were performed.

Polymerase Chain Reaction (PCR):

Genomic DNA was isolated from fresh cotton leaves using the method described by Dellaporta *et al.,* (1983). PCR was run for the detection of integrated *Cry1Ac and Cry2A gene* to amplify internal fragments of 565bp and 600bp respectively by a modification of the method by Saiki *et a.l.,* (1988) as shown in Figure 1. DNA extracted from untransformed plants was used as negative control and that of plasmid pk2Ac as positive control. Data of few representative transgenic plants are shown in Figure 1. The PCR was performed at 94ºc for 4 minutes 94ºc for 1 minutes 52ºc for 1and 72ºc for 1 minutes followed by 35 times. The amplified PCR fragments were resolved on 1% agarose gel and observed under UV light For amplification of genes , following primer sequences were used.

5’-ACAGAAGACCCTTCAATATC-3’ (*Cry1Ac* Forward Primer)
5’-GTTACCGAGTGAAGATGTAA-3’ (*Cry1Ac* Reverse Primer)
5’-AGATTACCCCAGTTCCAGAT-3’ (*Cry2A* Forward Primer)
5’-GTTCCCGAAGGACTTTCTAT-3’ (*Cry2A* Reverse Primer)

Southern Blot Analysis:

Southern Blot Analysis was performed to confirm the integration of 3kb Fragment of Cry1Ac gene. PCR positive plants were further analyzed for southern blot. Genomic DNA was digested with HindIII enzyme and rest of the procedure was followed as described by Southcrn, (1975) as shown in Figure-3. Gene specific Probe of Cry1Ac was labeled using Fermentas Biotin DecaLabel™ DNA Labeling Kit (Cat #K0651). Detection procedure was followed as provided in Fermentas Biotin Chromogenic Detection Kit (Cat# K0661).

Biotoxicity Assay:

To check the efficacy of endotoxins against targeted insect pests, laboratory biotoxicity assays of cotton leaves with *Heliothis* larvae (2nd instar) were conducted. Five leaves from upper, middle and lower portion of each lines were detached in perti plate after 30, 60 and 90 days of crop age ,placed on moist filter papers, taken to laboratory and 2^{nd} instar larvae of *Heliothis* was fed to them. After 2-3 days mortality rate was noted which was variable having a mortality range of 60-100% while it was 0% in control CIM-482. Mortality rates was calculated as follows

$$\%\text{Mortality} = \frac{\text{No.of dead larvae} * 100}{\text{Total no. of larvae}}$$

Quantification of Cry1Ac and Cry2A Endotoxins:

Expression of both Cry1Ac and Cry2A genes was quantified by Enzyme Linked Immunosorbent Assay using Envirologix Kit (Cat # 051) temporally as well as spatially. After 15 days intervals plant samples were brought to Laboratory, ground in Liquid nitrogen and took one third of powdered leaves and other plant parts samples and added 600 ul protein extraction buffer (0.5M EDTA, Glycerol, 5M NaCl, 2M Tris-Cl, NH_4Cl, PMSF, DTT(Dithiotheritol). After 1hr of ice incubation, centrifuged at 13000 rpm for 25 min, Supernatant was used for further analysis.

ELISA was performed according to procedure given in the kit and quantification of endotoxins was done by plotting absorbance values of Cry1Ac and Cry2A test samples on the standard curve generated with purified Cry1Ac standards on each of ELISA plates and expresses as nano gram per gram of fresh tissue weight. Different plant parts were studied for spatial expression of toxin levels.

RESULTS AND DISCUSSION

Amplification of Insecticidal genes by PCR:

Polymerase chain reaction (PCR) of both genes Cry1Ac and Cry2A confirmed the stable inheritance of these genes to subsequent generations. 565 bp and 600 bp internal fragments for Cry1Ac and Cry2A respectively were amplified (Figure-1). No amplification was detected in negative control.

Integration of Cry1Ac gene in Cotton Plants:

Few of the PCR positive plants were further proceeded and the presence and stable integration of Cry1Ac gene in plant genome was confirmed by Southern blot analysis. Gene integration was detected by gene specific probe after the plasmid pk2Ac. DNA was digested with HindIII restriction enzyme. Plant genomic DNA digested with the same restriction enzyme and hybridized with Cry1Ac specific probe showed the integration of cry1Ac gene in Plant genome. Non Transformed CIM-482 plant DNA was used as negative control while that of plasmid DNA pk2Ac was used as Positive control (Figure-2).

Biotoxicity Assay:

Laboratory Biotoxicity assays with 2^{nd} Instar *Heliothis* larvae showed that expression of both genes is varying along with increasing age of plant. In Laboratory Biotoxicity assays, mortality %age of insect larvae was variable after 30, 60 and 90 days indicating the decline in insecticidal proteins levels. Larvae dead or alive were counted in each petri plate as shown in the Figure-3. Transgenic lines showed a mortality rate of larvae that ranged between 60-90 % as shown in Figure-4. The larvae which survived in few cases were too inactive or sluggish to be harmful for the plant. While in case of non transformed control CIM-482, no any morality of larvae was noted.

Temporal and Spatial Expression of Cry1Ac Gene:

Expression of Cry1Ac gene was quantified in advance cotton lines temporally as well as spatially after 15 days interval. Result showed that the toxin level declined along with the age of plant (Figure. 5) and also it varied among different plant parts being high in leaf ,then in square buds, bolls and anthers respectively (Figure. 6). Petals were showing less toxin expression in every line as compared to other plant parts being 8-10 ng per gram of fresh tissues weight.

Temporal and Spatial Expression of Cry2A gene:

Similarly expression of Cry2A gene was quantified in advance cotton lines temporally as well as spatially after 15 days interval. Result showed that the Cry2A toxin level also declined along with the age of plant (Figure- 5) and also it varied among different plant parts being high in leaf ,then in square buds, bolls and anthers respectively (Figure. 6). Petals were showing less toxin expression in every line as compared to other plant parts being 8-12 ng per gram of fresh tissues weight.

Discussion:

This study was undertaken to determine the integration of insecticidal genes in advance cotton lines (T5 progeny) and expression of these insecticidal genes with the age of plant as well as in different plant parts that showed that genes remained stable in subsequent generations as it was confirmed by PCR and Southern blot analysis of the transgenic plants (Figure-1&2) while expression level of these genes varied temporally and spatially.

Biotoxicity assays were conducted to determine the mortality %age of *Heliothis* larvae (2^{nd} instar) at different time interval of crop age. Cotton leaves were collected for biotoxicity assay after 30, 60 and 90 days of crop age. The results showed that there was gradual decline in efficacy of insecticidal genes expression against targeted insects after 30 days time intervals as some of larvae survived after 90 days assay which were killed in 30 days assay. Similar results were obtained by Kranthi *et al.,* (2005).

Study of toxin titer in cotton plant is very crucial as it must be in sufficient quantity at any time to protect the crop against lepidopterans especially boll worms. A gradual decline in endotoxins expression was found along the passage of time of plant growth and most importantly, the expression level was lower in reproductive parts of plants i.e. petal and another, the susceptible site for attack of boll worms that is an alarming sign for the cotton scientist to combat against these insect pests.

Expression level of Cry1Ac and Cry2A genes declines progressively over the crop growth with toxin level falling to 15-20 ng/g of fresh tissue weight. Expression was found highly variable in different plant parts. The leaves of Bt cotton were having maximum expression of both genes followed by square, bolls and petals. Lowest expression was found to be in petal of plants.

These results are in contrary with Adamczyk *et al.* (2001). who reported that there were no significant differences in Cry1Ac expression among the 11 varieties tested by them. Same results were found by Karanthi *et al.* (2005), Chen *et al* (2000), Xia *et el.* (2005), Olsen *et al.* (2005), Mahon *et al.* (2002), Fitt *et al.* (1998) and Greenplate *et al.* (1998). who found a gradual decline in endotoxins expression with the passage of time.

Gene expression varies with the nucleotide sequence of the gene, promoter, and the insertion point of the gene in the DNA of the transgenic variety, transgene copy number, the internal cell environment, as well as several external factors in the environment (Guo *et al.,* 2001; Hobbs *et al.,* 1993; Rao, 2005). Therefore, investigation at molecular, genetic, as well as physiological levels should help in understanding the differential expression of transgenes and the quantitative changes in insecticidal proteins in Bt cotton plants.

Nearly all transgenic crops around the world utilize the CaMV 35S promoter (Odell *et al.,* 1985). (or similar promoters from closely-related viruses) to drive transgenes. It is only now becoming clear that this promoter is not as robust as laboratory and glasshouse studies have suggested and its function is influenced by as yet undefined physiological and perhaps environmental factors (Sunilkumar *et al.,* 2002).

The need of the hour is to make the expression of insecticidal genes consistent through out the life of transgenic plant. For this purpose, there is need to use the type of promoter that are constitutive in nature. This study suggests that 35S CaMV is the major player in being the temporal and spatial expression of insecticidal genes.

We must rely on conventional breeding and selection to solve these problems of variable efficacy of transgenic cotton, but it will be useful, in the longer term, to identify other gene promoters that can drive strong expression of transgenes throughout the season. It is also important to have such promoters available for the next generation of transgenic cotton so that different traits can be stacked without relying on the same promoter so as to avoid transcriptional gene silencing induced by multiple copies of a single promoter such as the CaMV 35S promoter (Fagard and Vaucheret, 2000).

Van Leeuwen Wessel *et al.,* (2001) reported the variation in expression patterns of three promoters (Cauliflower Mosaic Virus (CaMV) 35S, modified CaMV 35S and the promoter of an Arabidopsis thaliana Lipid Transfer Protein gene) using firefly luciferase reporter system. The expression of luciferase gene varied not only among independent transformatants but also between leaves on the same plant and within a leaf. Imaging of luciferase activity in the same leaves over 50 days period showed that individual transformatants show different types of temporal regulation and also this spatial and temporal pattern is inherited by the next generation.

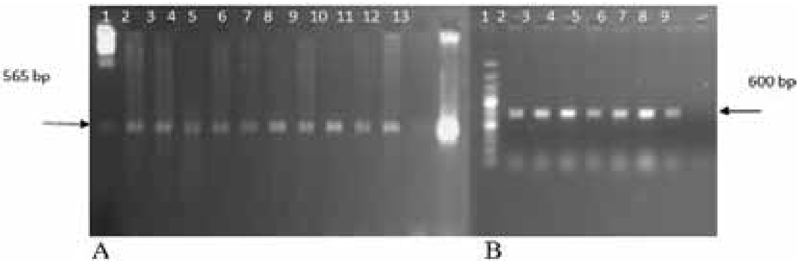

Fig. 1: A. PCR amplification of Cry1Ac gene
Lane 1 : Lamba HindIII Marker
Lane 2-11 : Transgenic plants
Lane 12 : -ve Control (CIM-482)
Lane 13 : +ve control (pk2Ac plasmid)

B. PCR amplification of Cry2A gene
Lane1 : 100 bp plus ladder
Lane 2- 7 : transgenic plants
Lane8 : +ve control (Pk2Ac plasmid)
Lane 9 : -ve control (CIM-482)

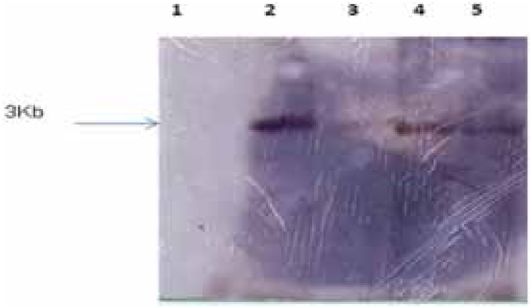

Fig. 2: Southern blot of Cry1Ac gene in transgenic plants

Lane 1 : negative Control (Nontranformed CIM-482 plant)
Lane 2 : Positive Control (Plasmid DNA Pk2Ac)
Lane 3-5 : Transgenic Plants

Fig. 3: Biotoxicity assay of transgenic plants along with control
A: Heliothis larvae feeding on the leaf of control plant.
B: A dead Heliothis larvae feeding after on the leaf of transgenic plants

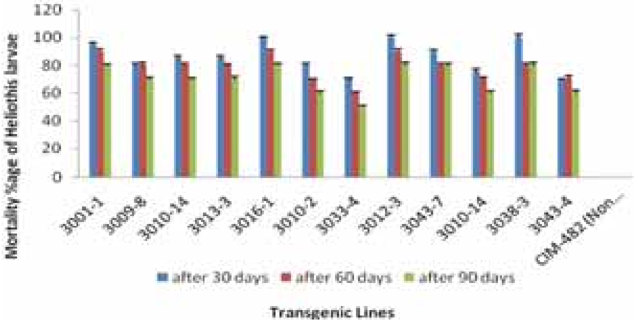

Fig. 4: Graph Showing Mortality rates of Heliothis Larvae in different transgenic lines after 30, 60 and 90 days

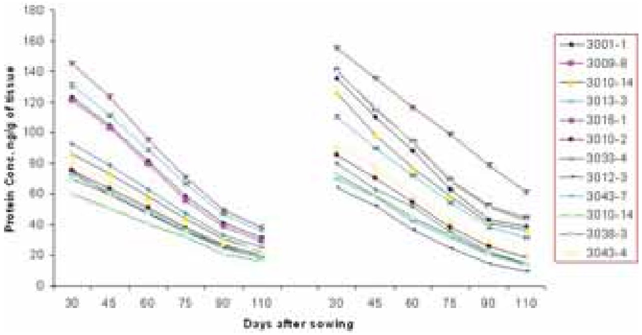
(1A) (2A)

Fig. 5: Temporal Expression of Cry1Ac and Cry2A genes in different cotton lines.

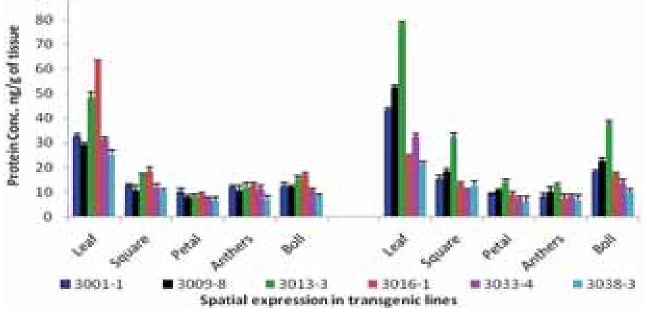
(1A) (2B)

Fig. 6: Spatial Expression of Cry1Ac and Cry2A genes in different plant parts of cotton lines

A number of independently derived transgenic soybean plants expressing a chimeric β-glucuronidase (GUS) gene under the control of the 355 CaMV promoter and a nopaline synthase polyadenylation signal were analyzed by Nong-Sung Yang *et al.,* (2005) and variable GUS expression was found in different plant parts of transgenic soyabean.

Transgene promoter activity can be characterized by the distribution of different expression levels with in a plant, each level occurring with its own frequency. Since every independent transformatant shows minor or major differences in spatial and temporal regulation of a transgene, apparently in every transformatant there is a different influence from flanking plant DNA sequences. This results show that 35CaMV promoter driving insecticidal genes regulates the expression with the age of plant as well as in different plant parts.

This study may trigger research into possible new promoters that will induce more consistent production of insecticidal genes throughout the life of the cotton plant. Efforts could be focused on developing transgenic cotton varieties with tissue-specific promoters to enhance the expression of toxin genes in fruiting parts that are more susceptible to attack.

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