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Spatio Temporal Expression Pattern of an Insecticidal Gene (*cry2A*) in Transgenic Cotton Lines

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Abstract

The production of transgenic plants with stable, high-level transgene expression is important for the success of crop improvement programs based on genetic engineering. The present study was conducted to evaluate genomic integration and spatio temporal expression of an insecticidal gene (*cry2A*) in pre-existing transgenic lines of cotton. Genomic integration of *cry2A* was evaluated using various molecular approaches. The expression levels of *cry2A* were determined at vegetative and reproductive stages of cotton at regular intervals. These lines showed a stable integration of insecticidal gene in advance lines of transgenic cotton whereas gene expression was found variable with at various growth stages as well as in different plant parts throughout the season. The leaves of transgenic cotton were found to have maximum expression of *cry2A* gene followed by squares, bolls, anthers and petals. The protein level in fruiting part was less as compared to other parts showing inconsistency in gene expression. It was concluded that for culturing of transgenic crops, strategies should be developed to ensure the foreign genes expression efficient, consistent and in a predictable manner.

Keywords: *cry2A* gene, insect resistance management, variation, efficient

Introduction

The application of biotechnology tools to agriculture has allowed scientists to transform plants without the need for sexual compatibility between species, thus establishing the possibility of rapidly producing new crop varieties with traits beneficial to human health and the environment. Plants have been transformed successfully to improve their pest and disease resistance, herbicide tolerance, nutritional qualities, and stress tolerance (Mackey and Santerre, 2000).

Pakistan is an important cotton and yarn producing country with the potential to become a key force in the global cotton and textile market place. Cotton and cotton products contribute about 10% to gross domestic product (GDP) and 55% to the foreign exchange earnings of the country (Bakhsh *et al.,* 2010).

Genetically modified (GM) crops were cultivated on 148 million hectares globally in 2010. In Pakistan, insect Resistant cotton was grown on 2.4 million hectares out of 2.8 million hectares allocated land (James, 2010). *Bacillus thuringiensis* (*Bt*) is perhaps, the most important source of insect resistant genes. Among them, transgenic cotton expressing insecticidal proteins from *B. thuringiensis* (*Bt*) has been one of the most rapidly adopted GM crops in the world (Barwale *et al.,* 2004; Dong *et al.,* 2005; James, 2002) containing *cry* gene(s) such as *cry1Ac*, *cry1Ac* + *cry2Ab* or *cry1Ac* + *cry1F.* Insect resistant cotton is considerably effective in controlling lepidopteran pests, and is highly beneficial to the grower and the environment by reducing chemical insecticide sprays and preserving population of beneficial arthropods (Gianessi and Carpenter, 1999; Tabashnik *et al.,* 2002).

Transgenic cotton expressing Bt (*Bacillus thuringiensis*) toxins is currently cultivated on a large commercial but observations have shown that it behaves variably in toxin efficacy against target insects under field condition. For the insect resistant cotton to be sustainable, it is important that the toxin protein be expressed in adequate quantities in appropriate plant parts at the requisite time of the season to afford protection against major target insect pests. However, a number of studies conducted have indicated that the levels of toxin protein in cotton tissues fluctuate during the whole growing season, and may logically cause variation in efficacy of Bt cotton against lepidopteron pests (Bakhsh *et al.,* 2010, 2011; Benedict *et al.,* 1996; Chen *et al.,* 2000; Greenplate *et al.,* 2001; Kranthi *et al.,* 2005; Mahon *et al.,* 2002). Understanding of the temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production.

The present study was conducted to evaluate the genomic integration and expression of insecticidal gene (*cry2A*) in pre-existing transgenic lines (T_5 Progeny) with the age of plants as well in different plant parts. The study was carried out at campus of National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan.

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Materials and methods

Twelve pre-existing transgenic cotton lines (3001-1, 3009-8, 3010-14, 3013-3, 3016-1, 3010-2, 3033-4, 3012-3, 3043-7, 3010-14, 3038-3 and 3043-4) developed out of genetic transformation of a local cotton cultivar (cv. 'CIM-482') harbouring insecticidal gene (Rashid *et al.,* 2008) were planted at CEMB campus experimental field along with non-transgenic control using randomized complete block design with three replications. Experimental fields were surrounded with 5 rows of untransformed 'CIM-482' and 'MNH-93' (another locally approved cotton cultivar). *Sorghum bicolor* L. was grown around the field to isolate field from surroundings following recommendation and biosafety guidelines.

Confirmation of Gene Integration

The various molecular approaches i.e. PCR and southern blot were performed to confirm the stable integration of insecticidal gene *cry2A* in transgenic lines. 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 c*ry2A* to amplify internal fragments of 600 bp by a modification of the method by Saiki *et al.* (1988) using forward 5'-AGATTACCCCAGTTCCAGAT-3', 5'-GTTCCCGAAGGACTTTCTAT-3' as reverse primers. DNA extracted from untransformed plants was used as negative control and that of plasmid *pk2Ac* as positive control. The PCR was performed at 94°C for 4 minutes 94°C for 1 minute 52°C for 1 minute and 72°C for 1 minute followed by 35 times.

Southern blot analysis was performed to confirm the integration of ~1.8 kb fragment of *cry2A* gene in representative plants of transgenic lines. Genomic DNA was digested with *pvuII* enzyme and rest of the procedure was followed as described by Southern (1975). Gene specific probe of *cry2A* 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).

Temporal and Spatial Expression of cry2A Endotoxin

For spatio temporal studies, a single structure was selected for quantification of c*ry2A* in transgenic lines. For each sample date and for all transgenic lines, a single mainstem terminal leaf was collected from five plants/transgenic line after every 15 days interval. For spatial expression, various plant parts i.e. leaf, anthers, pollens, square buds and bolls were selected at reproductive growth stage. The samples were transported to the laboratory and were processed the same day.

Expression of c*ry2A* 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 according to procedure given in the kit and quantification of *cry2A* endotoxins was done by plotting absorbance values of *cry2A* test samples on the standard curve generated with purified *cry2A* standards on each of ELISA plates and expressed as nanogram of *cry2A* per gram of fresh tissue weight. Protein expression data of *cry2A* was further subjected to statistical analysis using SPSS software (version 11.0, SPSS Inc) to evaluate the differences among transgenic lines, sampling dates, interaction of transgenic lines and sampling dates at 5% level of significance.

Leaf Biotoxicity Assay

Based on spatio-temporal results, leaf biotoxicity assays were performed to counter check the variation in efficacy of endotoxin *cry2A* against targeted insect pests after 30, 60 and 90 days of crop age. The five leaves from the upper, middle and the lower portion of each transgenic line along with the leaves from non-transgenic control line in triplicates were detached on moist filter papers in petri plates and 2nd instar *Heliothis* larvae were fed to them. After 2-3 days, mortality rates of *Heliothis* larvae were recorded. The mortality rates were calculated as follows:

%Mortality = No. of dead larvae/Total no. of larvae ×100

Results

Confirmation of Gene Integration

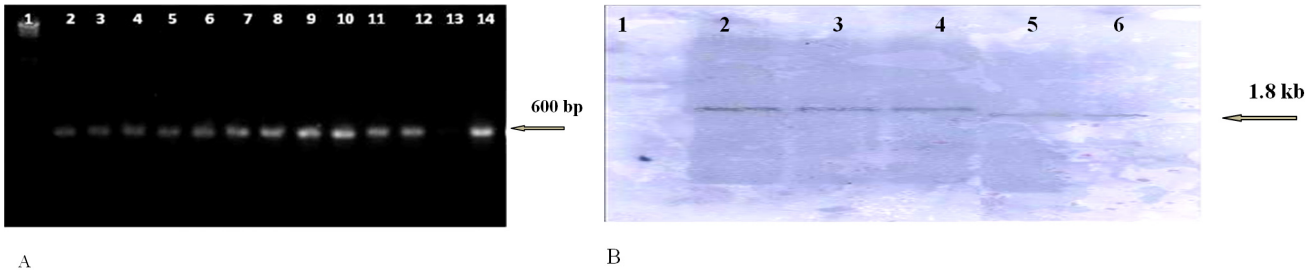
Polymerase chain reaction (PCR) assays of transgenic lines confirmed the stable integration of *cry2A* gene to subsequent generations; gene specific primers amplified 600 bp internal fragment of *cry2A* gene (Fig. 1A) while no amplification was observed in non-transgenic plant sample (Negative control).

Few PCR positive plants were further subjected to southern blot to verify integration of *cry2A* gene in plant genome. Gene integration was detected by gene specific probe. Results revealed the integration of *cry2A* gene (~1.8 kb) 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 (Fig. 1B).

Temporal and Spatial Expression of cry2A Endotoxin

Temporal expression of *cry2A* gene was quantified in transgenic lines after 15 days interval. The result showed that the toxin level declined with the age of plant (Fig. 2) being maximum at vegetative stage, 30 days crop. As the crop progressed towards the maturity, a decline in expression level of *cry2A* was observed. Similar expression pattern was found in all these transgenic lines. Statistical analysis revealed that *cry2A* differences among transgenic lines, sampling dates and their interaction were significant at 5% level of significance.

To evaluate spatial expression, different plant parts i.e. leaves, square buds, bolls, anthers, petals and ovary were sampled from the field and subjected to ELISA assay. The result revealed that the expression of *cry2A* was variable in different plant parts, being maximum in the leaves fol-



A B

Fig. 1. Confirmation of gene integration in transgenic plants. (A) PCR assay showed the amplification of internal fragment of *cry2A* band. Lane 1: Lamba *HindIII* Marker, Lane 2-12: Transgenic plants of various cotton lines under study, Lane 13: Negative Control (Non transformed CIM-482), Lane 14: Positive control (plasmid DNA), (B) Southern blot analysis of *cry2A* gene in transgenic lines. Lane 1: Negative control (Non transformed 'CIM-482'), Lane 2: Positive control (plasmid DNA *pk2Ac*), Lane 3-6: Transgenic cotton plants showing integration of *cry2A* gene

lowed by square buds, bolls and anthers (Fig. 3). Petals were showing less toxin expression as compared to other plant parts being only 8-10 nanograms per gram of fresh tissues weight while *cry2A* expression in ovary remained undetectable.

Leaf Biotoxicity Assay

Laboratory biotoxicity assays with 2nd Instar *Heliothis* larvae were conducted to confirm the variation in expression of the insecticidal gene at 30, 60 and 90 days of crop age. The results showed that transgenic lines had a varying mortality rate of *Heliothis* larvae that ranged between 60-90% at different growth stages. The varying mortality rates indicated a variation in *cry2A* protein levels. Transgenic lines that showed 100 mortality rate of *Heliothis* larvae at 30 days crop age, showed varying 60-80% mortality rate at 90 days of crop age (Fig. 4) while in case of non-transformed control CIM-482, no any larval mortality was recorded.

Discussion

The present study was undertaken to evaluate the genomic integration and expression of insecticidal gene (*cry2A*) in pre-existing transgenic lines with the age of plants as well in different plant parts. Result showed that gene integration remained stable in subsequent generations as it was confirmed by PCR and Southern blot analysis of the transgenic plants (Fig. 1). Spatio temporal studies revealed that expression level of *cry2A* declined during the crop growth with toxin level falling to 15-20 nanogram per gram of fresh tissue weight (Fig. 2). These results are in agreement with previous studies conducted by Fitt *et al.* (1998), Greenplate *et al.* (1998), Chen *et al.* (2000), Mahon *et al.* (2002), Xia *et al.* (2005), Olsen *et al.* (2005), Manjunatha *et al.* (2009) and Adamczyk *et al.* (2009), Bakhsh *et al.* (2010) and Bakhsh *et al.* (2011, 2012) who have reported inconsistency in insecticidal gene expression over the crop growth period. Expression levels of insecticidal gene also remained variable in different plant parts (Fig. 3). The leaves of transgenic cotton were having maximum expression as compared to fruiting parts (Adamczyk *et al.,* 2001; Bakhsh *et al.,* 2010, 2012; Gore *et al.,* 2001; Greenplate, 1999; Greenplate *et al.,* 2000). Furthermore, laboratory biotoxicity assays with 2nd Instar *Heliothis* larvae were conducted to confirm the variation in expression of the insecticidal gene at 30, 60 and 90 days of crop age. The results obtained from biotoxicity assays at different intervals also confirmed the variation in *cry2A* expression (Fig. 4). Study of toxin titer in cotton plant is very crucial as it must be in sufficient quantity 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

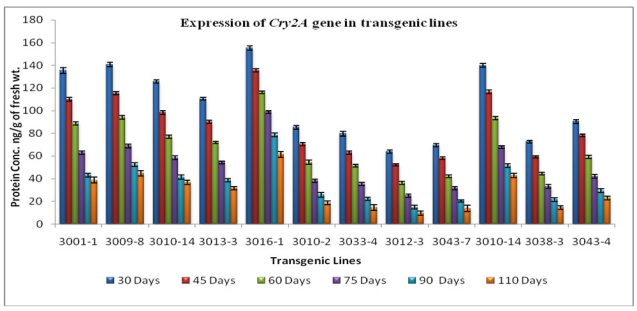


Fig. 2. Spatio temporal expression of *cry2A* in transgenic cotton lines. (A) Temporal expression of *cry2A* was quantified after an interval of 15 days showing the maximum expression at vegetative growth stage while a decline in expression at the later stages of crop plants was observed

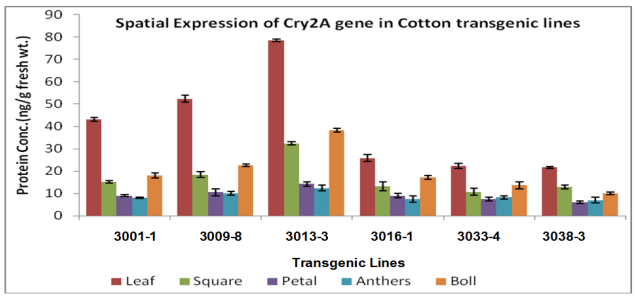


Fig. 3. Spatial expression of *cry2A* quantified in different plant parts showing maximum in leaf followed by followed by square buds, bolls and anthers

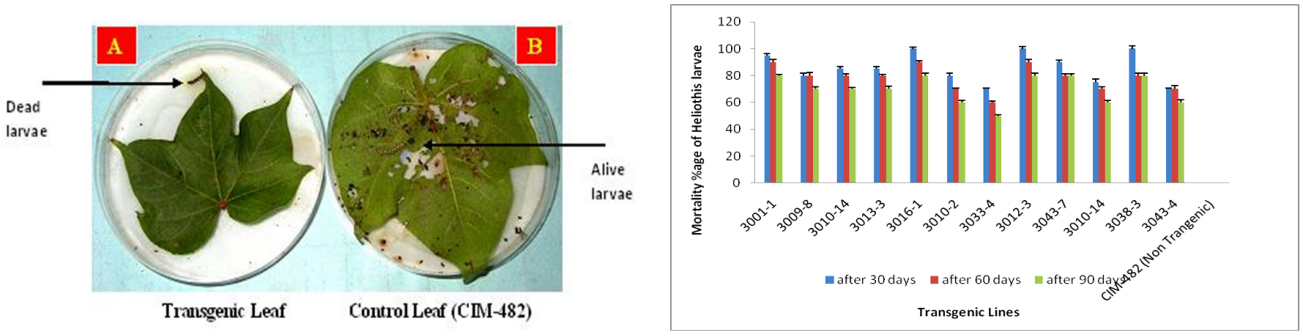


Fig. 4. Mortality % of 2nd *Heliothis* Larvae in different transgenic lines at 30, 60 and 90 days of crop plants. (A) *Heliothis* larvae feeding on the leaf of control plant while *Heliothis* larvae were dead after feeding on leaves of transgenic plants, (B) Graph shows the mortality percentage of *Heliothis* larvae. Lines showed 100% mortality of larvae at 30 days crop stage while this percentage varied at 90 days observation while no any larval mortality was recorded in non-transgenic control cotton cultivar 'CIM-482'

was lower in reproductive parts of plants i.e. petal and another, the susceptible site for attack of boll worms.

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 insect resistant cotton plants.

The mechanisms of variation in endotoxin protein content in plant tissues are rather complicated. The reduction in *Bt* protein contents in late-season cotton tissues could be attributed to the overexpression of the *Bt* gene at earlier stages, which leads to gene regulation at post-transcription levels and consequently results in gene silencing at a later stage. Methylation of the promoter may also play a role in the declined expression of endotoxin proteins. This has triggered research in finding possible new promoters that will induce more consistent production of insecticidal genes throughout the life of the cotton plant (Bakhsh *et al.,* 2012). Therefore, efforts should also focus on evolving new transgenic cotton varieties with tissue-specific promoters to enhance the expression of toxin genes in fruiting parts that are susceptible to attack.

Thus, the variations in the efficacy of insecticidal genes in transgenic cotton and the involved mechanisms need to be understood fully, so as to plan rational resistance management strategies to retard the rate of the development of resistance, and to control target pests effectively by enhancing endotoxin expression through genetic or agronomic management. It can be concluded that developing new cotton varieties with more powerful resistance, applying certain plant growth regulators and maintenance of general health of the transgenic crop are important in realizing the full transgenic potential in transgenic Bt cotton.

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