Transformation of Insect and Herbicide Resistance Genes in Cotton (Gossypium hirsutum L.)

M. F. Awan¹, M. A. Abbas¹, A. Muzaffar¹, A. Ali¹, B. Tabassum¹, A. Q. Rao¹, I. Ahmad Nasir¹, and T. Husnain¹

ABSTRACT

Manipulation of different genes in crop plants to get desirable characters has become an important tool of plant biotechnology. In the current study, cotton variety MNH-786 was modified for its characteristics to show resistance against lepidopteran insects and herbicide by transformation of Cry1Ac+Cry2A and GTGene cloned in a different cassette under 35S Promoter. Mature embryos of cotton MNH-786 were injured by a sharp blade at the shoot apex and infected with the Agrobacterium tumefaciens harboring transgene constructs. Transformed cotton plants were successfully acclimatized in pots and later the greenhouse. Gene specific PCR and ELISA confirmed the transgene presence and its protein expression which was considerably higher in transformed plants. Overall transformation efficiency was 1.05%. All larvae of Helicoverpa armigera feeding on transgenic cotton leaves of T₀ were found dead as compared to the control ones feeding on leaves from non-transgenic cotton. Transgenic plants also survived a glyphosate spray dose of 1,900 ml acre⁻¹ as compared to herbs/weeds growing along with them, which burned completely five days post glyphosate application.

Keywords: Bt cotton, Glyphosate, Transgenic, Weedicide resistance.

INTRODUCTION

Cotton is the essential non-food cash crop and a major source of foreign exchange remunerations in Pakistan. It accounts for 8.2 percent of the value added in agriculture and about 2 percent to GDP (Khan and Khan, 2003; Batool et al., 2010). In Pakistan, cotton crop provides source of revenue to millions of people, but its production is gradually becoming expensive due to high water consumption, use of expensive pesticides, insecticides, and fertilizer and yield losses due to weeds. It has also been estimated that 14% losses in total agriculture occurs due to insect pests, of which 84% is in cotton (Shahid et al., 2012), 37% yield losses occur due to weeds while remaining 34% losses occur due to insects 11% due to micro-organisms and virus while 16-18% due to pathogens (Farooq et al., 2011; Oerke, 2006 and Orke et al. 2004). In the past, insects of cotton were controlled by spraying expensive pesticides, while weeds were controlled through manual hoeing. The heavy use of pesticide may result in import of pesticide of 81015498/USD (Khooharo et.al 2008), and manual hoeing may result in labor intensive work along with yield losses up to 25% (Khan and Khan, 2003) due to delayed weed removal. Manual weeding is also expensive as it is time consuming and labour intensive (Cheema et al., 2005). Also, total weed seed numbers in the soil seed bank have been shown to increase significantly after changing from conventional chemical weed control to non-chemical means (Bond and Grundy, 2001). Hence, the levels of input required using non-chemical methods are

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not sustainable or economically viable. Combinations of mechanical weed control techniques fail to provide complete weed control (Bale et al., 2008), and also risk inflicting damage to the crop because the crop plants are usually small at the time when weeds are most vulnerable to these methods.

The advent of biotechnology offers an alternative to the weed control methods presently used in cotton. Glyphosate (N-phosphonomethylglycine) is a broad-spectrum systemic herbicide used to kill weeds, especially annual broadleaf weeds and grasses known to compete with commercial crops grown around the Globe. Glyphosate interferes in the shikimate metabolic pathway by inhibiting the synthesis of 5-enolpyrovyl3-phosphoshikimate (EPSPS). It prevents the synthesis of three aromatic amino acids including tryptophan, phenylalanine, and tyrosine (Yamada et al., 2009). It has been reported that glyphosate causes the fruit (boll) abscission in cotton which ultimately reduces the yield of cotton (Monks et al., 2007). In these days, many ways have been devised to develop resistance in high yielding varieties of cotton against insect pests and herbicides. Conventional plant breeding has become an old discipline which takes plenty of time to produce resistant varieties. Genetic engineering is a breeding strategy that assures to avoid the problems associated with the transfer of large blocks of genetic materials between two parents (Bajaj, 1998). The present study aimed to transform genes that had resistance against the lepidopteran insects’ pests (Cry1Ac+Cry2A) and Glyphosate (GTG) in the cotton variety MNH-786 (due to its susceptibility to insect pests) through A. tumifaciens. A similar type of study was done by Nasir et al. (2014) by transformation of double Bt genes along with GTGene in sugarcane and they concluded that expression of these three exogenous genes result in development of insect- as well as weedicide-resistance in sugarcane.

MATERIALS AND METHODS

Plant Material

Cotton seeds (G. hirsutum var.MNH-786) were collected from the Cotton Research Institute (CCRI) Purana Shujabad Rd, Multan 60000. Seeds were delinted with 100% H₂SO₄, sterilized with 5% (W/V) HgCl₂ and 10% (w/v) SDS followed by 5-6 washings with autoclaved distilled water (Rao et al., 2013). Subsequently, seeds were allowed to germinate by incubating them at 30°C for 48 hours. The germination index of cotton variety MNH-786 was calculated by counting germinated and un-germinated seeds of each experiment and dividing the number of germinated seed by total number of seeds and multiplied by hundred.

Agrobacterium-Mediated Transformation of Cotton with Cry1Ac+Cry2A and CP4 EPSPS gene Constructs

Glyphosate-tolerant lines of cotton have been produced using Agrobacterium-mediated transformation of mature embryos with Cry1Ac+Cry2A and CP4 EPSPS gene as done by Rao et al. (2009). Two constructs were used, all containing CaMV 35S constitutive promoter NOS terminator as shown in Figure 1. After two months on MS medium with kanamycin selection, putative transgenic plants were shifted in shoot regeneration media (kinetin) and root regeneration media [1-Naphthaleneacetic acid (NAA) and Indole-3-butryic acid] without kanamycin, as described before (Rao et al., 2009).

Genomic DNA Isolation and Polymerase Chain Reaction (PCR)

Genomic DNA was extracted from putative transgenic plants and purified from the immature leaves of putative transgenic
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**Figure 1.** (A) Constructs map showing GTGene cloned by using NCol and Bgl-II restriction sties while (B) Cry1Ac was cloned by using hind-III and Cry2A gene was cloned by using XbaI restriction enzyme.

Cotton plants using the protocol described by Lenin et al. (2001). PCR was done with the reaction mixture of 20 µL containing 1X PCR Buffer with 2.5 mM MgCl2, 300 ng DNA template, 1 mM dNTPs, 1 pico mole primers (forward and reverse; sequence given as Table 1), 2.5 U Taq DNA Polymerase. PCR was carried out in a thermo cycler according to following conditions, initial denaturation at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 51°C for (Cry1Ac) for 1 minute followed by extension at 72°C for 3 minutes. Final extension was done at 72°C for 5 minutes. PCR amplified products of Cry2A and GTGene having sizes of 565 and 600 bp respectively were resolved onto 1% (w/v) agarose gel, stained with ethidium bromide and visualized by gel doc system.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry2A-F</td>
<td>AGATTAACCCAGTCCAGAT</td>
<td>600 bp</td>
</tr>
<tr>
<td>Cry2A-R</td>
<td>GTTCGCAAGGACCTTTCTTAT</td>
<td></td>
</tr>
<tr>
<td>GTG-F</td>
<td>CCCCTGGTGACAAGTCCATCT</td>
<td>358 bp</td>
</tr>
<tr>
<td>GTG-R</td>
<td>CTGCGACCCCATCTCTCCTGA</td>
<td></td>
</tr>
<tr>
<td>Cry1Ac-F</td>
<td>ACAGAAGACCTTCAATATC</td>
<td>565 bp</td>
</tr>
<tr>
<td>Cry1Ac-R</td>
<td>GTTACCAGGTAAGATGTAAGA</td>
<td></td>
</tr>
</tbody>
</table>

**Protein Expression Analysis**

Expression analysis of Cry1Ac, Cry2A and GTGene was done by ELISA assay using Envirologix Kit (Cat# 051) 500 Riverside Industrial Parkway Portland, Maine, 04103-1486 USA. Plant samples were ground to fine powder in liquid nitrogen and 600 µL of protein extraction buffer (0.5M EDTA, 10% Glycerol, 5M NaCl, 2M Tris-Cl, 150 mM NH4Cl, 1.0 mM PMSF, 0.05% DTT) was added followed by 1 hour incubation on ice, centrifuged at 13,000 rpm for 25 minutes. Supernatant was used for further analysis. ELISA was performed according to the instruction provided in kit manual as done by Kiani et al. (2013) and.
Transgenic Cotton Plants Resistance against Insect Resistance/Bio-toxicity Assay

To check the efficacy of Cry1Ac and Cry2A endotoxins against targeted insect pests, laboratory bio-toxicity assays of cotton leaves with H. armigera larvae (2nd instar) were conducted. Five leaves from upper, middle, and lower portion of the cotton plants (transformed and control, non-transgenic) were detached in three times i.e. after 30, 60, and 90 days of crop age. The leaves were placed in petriplates having moist filter paper pieces. Subsequently, the second instars larvae of H. armigera were fed on them. After 3 days, leaves were observed for leaf damage and insect mortality.

Glyphosate Spray Assay

To check the effectiveness of GTG transgene in cotton, Glyphosate spray was applied on acclimatized transgenic cotton plants (in three repeats) in a greenhouse at 35±5°C. Herbicide Glyphosate is commercially available as Roundup™. It was prepared in water at a final concentration of 1,900 ml 80 L⁻¹. Prepared solution was applied (1,900 ml ha⁻¹) to field grown transgenic cotton plants along with the control non-transgenic cotton plants. The plants with production of aromatic amino acid would survive while others which failed to produce aromatic amino acid would die.

RESULTS

Transformation of G. hirsutum MNH-786 and Confirmation of Putative Transgenic Plants

Germination index of MNH-786 cotton variety was found to be 66.6% as shown in Figure 2. Total of 50-plantlets were obtained after two months selection of putative transgenic plants on kanamycin and the transformation efficiency was calculated to be 1.05% (Table 2). Amplification of CEMB-GTGene at 358 bp as shown in Figure 3 in transformed cotton plants confirmed the presence of Glyphosate gene in them. Amplification results of Cry1Ac

Table 2. Efficiency of transformation. Total no. of 4,745 embryos were isolated for transformation out of which 50 developed into positive plants transformation efficiency was calculated by dividing positive plants to total no of isolated embryos and multiplied by 100.

<table>
<thead>
<tr>
<th>No. of embryos isolated</th>
<th>Plants obtained after 8 weeks</th>
<th>Transformation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control experimental</td>
<td>Control experimental</td>
<td>Control Experimental</td>
</tr>
<tr>
<td>100</td>
<td>4745</td>
<td>86</td>
</tr>
</tbody>
</table>
and Cry2A at 565 and 600 bp for Cry1Ac and Cry2A genes, respectively, was revealed as shown in Figures 4 and 5. It was also found that some transformed plants (V4, V5, V7, V8, V9, V10 and V11) were PCR positive not only for GTGene but also for CEMB-Bt genes, as both constructs were separately electroporated in Agrobacterium as shown in Table 3. No amplification was detected in negative control. PCR positive transformed cotton plants were further confirmed for transgene specific protein expression. ELISA with GTG and Bt proteins with specific antibodies was done whose results demonstrated and confirmed PCR results. Quantification of GTGene and Bt proteins was done and represented Figure 6. These results counter confirmed PCR data, where similar transformed cotton plantlets showed PCR amplification for all three transgenes. One cotton plant (V6) was observed to be non-transgenic while all other experimental plants were transgenic and were successfully acclimatized in the field.

**Insect Bioassays**

Larvae of American cotton bollworm (H. armigera) were fed on transgenic and non-transgenic cotton leaves. All H. armigera larvae feeding transgenic cotton leaves that have Bt/Cry toxin proteins expressed in them were found dead, whereas larvae feeding on non-transgenic control cotton leaves were alive and remained healthy as shown in Figure 7. Mortality rate of feeding larvae was 100%, concluding that all tested transgenic cotton plants in T₀ generation exhibited strong anti-pest activity.

**Evaluation of Herbicide Resistance in Transgenic Cotton Plants**

Field grown transgenic cotton plants in T₀ generation were evaluated for their resistance against glyphosate, a commonly used weedicide in cotton field. Three-month-old transgenic cotton plants were
Figure 4. PCR Amplification of Cry1Ac genes in putative transformed plants. (Lane 1) 100 bp molecular weight marker; (Lane 2) Non-transgenic plant was used as negative control; (Lane 3) Plasmid was used as positive control, and (Lane 4-11) Putative transformed plants.

Figure 5. Amplification of Cry2A gene by PCR in putative transformed plants. (Lane 1) Non transgenic plant as negative control; (Lane 2) Plasmid as positive control; (Lane 3-10) Putative transformed plants, and (Lane 11) 1 kb molecular weight marker.

Figure 6. Graphical representation of Quantification of GTG and Bt Proteins. Blue bars shows the concentration of Cry1Ac, Red shows the concentration of GTGene and Green bars shows the concentration of Cry2A protein in µg g⁻¹ of the leaves.

Table 3. Summary of PCR results. Putative transgenic plants were confirmed by using PCR amplification plants named as P₇₈₆(V4), P₇₈₆(V5), P₇₈₆(V7), P₇₈₆(V8), P₇₈₆(V9), P₇₈₆(V10) and P₇₈₆(V11) were found positive for GTGene, Cry1Ac and Cry2A while plant P₇₈₆(V6) was PCR negative.

<table>
<thead>
<tr>
<th>Plant no.</th>
<th>Plant name</th>
<th>GTG</th>
<th>Cry1Ac</th>
<th>Cry2A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P₇₈₆(V4)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>P₇₈₆(V5)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>P₇₈₆(V6)</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>P₇₈₆(V7)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>P₇₈₆(V8)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>P₇₈₆(V9)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>P₇₈₆(V10)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>P₇₈₆(V11)</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
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</tbody>
</table>
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Figure 7. Bioassay of transgenic and control plant leaves. *Helicoverpa* was fed on both the control and non-transgenic cotton leaves and were observed after 3 days. A. In case of transgenic plant leaf and larva died after eating a small portion of leaf, while in non-transgenic control larva remained alive. (A) A transgenic plant leaf and larva is killed after eating a small portion of leaf, (B) A control non-transgenic plant. Larva ate it and is alive.

sprayed with Glyphosate at a final concentration of 1,900 ml acre\(^{-1}\). At the time of glyphosate assay, transgenic cotton field was shown to be full of weeds/herbs as shown in Figure 8-A. Toxic effects of glyphosate became visible on weeds three days post herbicide application, as can be clearly seen in Figure 8-B. These symptoms include plant wilting, drooping and slight burning spot appearance. Also, weeds were dead completely five days post glyphosate application as shown in Figure 8-C as compared to transgenic cotton plants which remained healthy and no visible symptoms of any kind of toxicity were observed. Conclusively, developed transgenic cotton plants were weedicide/herbicide and insect resistant while control plants along with weeds could not withstand glyphosate and were dead five days after spray.

DISCUSSION

Cotton is not only an important cash crop of Pakistan but also a significant segment of agricultural profile. Cotton generates 30% of foreign exchange earnings for Pakistan by contributing approximately 80% raw material to textile industry. In this particular study, an effort was made to improve the agronomic characteristics of this high input crop through the development of insect and herbicide resistant cotton plants.

In the present study, transformation of double *Bt* genes and *GTGene* was done by shoot apex cut method as done by Rao et al. (2011). Total of 4745 embryos were inoculated with the *Agrobacterium* strain LBA4404 containing gene of interest. Out of these 4,745 inoculated embryos, 560 transformed plants were obtained, but only 50 plants survived in the field after
acclimatization. The transformation efficiency was recorded to be 1.05% which was similar as obtained by Rao et al. (2011). Insects including bollworms are major pests of cotton causing significant yield losses. Insects not only decrease crop productivity but also their extensive uses have deleterious consequences/impacts towards human health.

Figure 8. Glyphosate spray on transgenic plants (a) Glyphosate (1,900 ml \text{acre}^{-1}) spray in the field of GTG positive transgenic plant (b) Field 3 days after spray (c) After five days weeds and control plants were dead while transgenic plants were healthy. It shows the presence of \textit{GTGene} in cotton plants. Presence of weeds in the field along with the control and transgenic plants. Glyphosate was being sprayed.
health, environmental quality, and biodiversity. For the sake of sustainable agriculture, environmentally friendly approaches to manage insects have to be developed through the use of biotechnology. The most effective and the cheapest method is to develop ‘insect resistance’ in transgenic crops. CEMB has isolated genes that code potential Cry toxin proteins from a soil bacterium ‘Bacillus thuringensis’ (Bt) that proved to be lethal for targeting insects. Transformation of double Bt genes, namely, Cry1Ac and Cry2A in G. hirsutum was done as done by Bakhsh et al. (2012) and developed insect resistant transgenic crop of cotton. The transgenic plants were confirmed by using PCR analysis (Khan et al., 2013b, Akhtar et al. 2014) and ELISA as done by Khan et al. (2013a). Both transformed genes differ in their sequence of amino acid and insecticidal activity.

Modern agriculture is supported by the use of herbicide treatment that enhances the crop production cost effectively. Weeds compete with cultivated crop for water, light and available nutrients (Tauseef et al., 2012). Although herbicide applications improve crop yield by breaking the competition, their extensive use destabilizes the soil ecosystem and nitrification cycles. A transgenic cotton resistant to herbicide Glyphosate, which inhibited the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSPS) synthase, a key catalyst in the production of aromatic amino acids, was developed. Glyphosate gene was expressed in transgenic cotton plants under the control of CaMV 35S, a strong constitutive promoter as done by Nasir et al. (2014) The results of ELISA of Bt and GTGene in transgenic cotton plants confirmed their successful expression. The principal motivation in developing glyphosate resistance in cotton is related to the broad spectrum activity of the herbicide and it is relatively low level of environmental contamination. In the present study, the transgenic cotton plants harboring CEMB GTGene have shown survival after the spray of Glyphosate at the rate of 1,900 ml acre⁻¹, which was more than the previously reported rate of 1,600 ml acre⁻¹ as recommended by Monsanto, 2011.

REFERENCES


(Gossypium hirsutum L.)

تزاريختي زن مقاومت به حشرات و علف كش ها در پنه (Gossypium hirsutum L.)

م. ف. اوان, م. م. عباس, ا. مظفر, ا. علي, ب. تيميم, ع. راوی, ا. حامد, نصر, و. ت.

حسنی

چکیده

برای به دست آوردن صفات مناسب در گیاهان دستگاهی زن از آرازی مهم در فناوری گیاهان به شمار می‌رود. در این پژوهش رقم پنه 86-786 با ترا بیشینی GTGene و Cry1Ac+Cry2A بنا بر سه توصیه شده در یک کست منفی می‌باشد. هنگامی که 355 برای وزن‌گی های مقاومت در رای به حشرات بال پولکدار (پرومی) و
علف کش‌ها نیز داده شد. به این منظور، جنین‌های بالغ به نام MNH 786 با تیغه‌های ساقه بپیده شد و با آگروبکتریوم Agrobacterium tumefaciens حامل ساختمان زن تراریخت تلقیح شد. گیاهان به تراریخت شده با موافقت در گلدان به سبب محتوای غیرقابل پیش‌بینی به سی‌آرآ و الیزه‌ای زن ویژه حضور زن تراریخت و بیان پروتئین آن را که در گیاهان تراریخت بسیار بیشتر بود تایید کرد. کارایی کلی فرآیند تراریختی برای 0/10/1% H. armigera بود. بر خلاف لاروهای شاهد که از یک پرگ به‌دست تراریختی تغذیه می‌کردند، لاروهاي T₀ غلیساته که روزی پرگ به‌دست تراریخت شده که روزی پرگ به‌دست تراریخت شده، در مقدار 1,900 ml acre⁻¹، بیشتر می‌چسبند و در ادامه دادن در حالی که علف‌ها و دیگر گیاهانی که کنار آنها بودند نه روز بعد از پاشش این ماده به کلی نابود شدند. به این منظور، جنین‌های بالغ به نام MNH 786 با تیغه‌های ساقه بپیده شد و با آگروبکتریوم Agrobacterium tumefaciens حامل ساختمان زن تراریخت تلقیح شد. گیاهان به تراریخت شده با موافقت در گلدان به سبب محتوای غیرقابل پیش‌بینی به سی‌آرآ و الیزه‌ای زن ویژه حضور زن تراریخت و بیان پروتئین آن را که در گیاهان تراریخت بسیار بیشتر بود تایید کرد. کارایی کلی فرآیند تراریختی برای 0/10/1% H. armigera بود. بر خلاف لاروهای شاهد که از یک پرگ به‌دست تراریختی تغذیه می‌کردند، لاروهاي T₀ غلیساته که روزی پرگ به‌دست تراریخت شده، در مقدار 1,900 ml acre⁻¹، بیشتر می‌چسبند و در ادامه دادن در حالی که علف‌ها و دیگر گیاهانی که کنار آنها بودند نه روز بعد از پاشش این ماده به کلی نابود شدند.