Mendelian segregation pattern and expression studies of insecticidal gene (cry1Ac) in insect resistant cotton progeny

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Abstract

The progenies of transgenic lines Bt-14 and Bt-17 developed as an independent transformation event from a local cotton variety CIM-482 harboring two insecticidal genes (cry1Ac & cry2a) were evaluated to determine resistance against lepidopterans, mainly Helicoverpa armigera L. under field conditions. The standard molecular techniques, i.e. polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and western dot blot were used to confirm gene presence and expression level of transformed Bt gene, and its transfer pattern to further progeny. PCR confirmed integration of insecticidal gene in most of the plants in transgenic progeny, while expression of Bt gene quantified by ELISA and western dot blot showed variation in cry1Ac expression levels but interestingly, it conferred full protection against targeted insect pests. The leaf bioassays were conducted to determine the effectiveness of Bt genes against Helicoverpa armigera by calculating the mortality percentage of larvae. Most of the transgenic lines showed 70-100% mortality age of Helicoverpa armigera. The agronomic characteristics of the transgenic lines were also recorded along with non transgenic control variety CIM-482. Morphological, agronomic and fibre data of these transgenic lines was recorded and analyzed statistically. Our results show that these transgenic lines (especially Bt-17 line) are promising cotton germplasm to be used in an efficient breeding programme.

Key words: Commercialization, Genetic approaches, Resistant, Transformation

Introduction

Cotton (Gossypium hirsutum L.) is a soft, fluffy, staple fiber that grows in a boll around the seeds of the plant. It is an important fibre crop and backbone of textile industry worldwide. Cotton and cotton products contribute significantly in Pakistan’s economy as major share in GDP (Economic Survey of Pakistan 2010-11). Cotton plant has been subjected to extensive research aimed at improving its genetic architecture to obtain greater benefits. Cotton breeders have developed some outstanding cultivars using conventional breeding approaches in the past, however, the pace of developing promising cultivars cotton has been delayed and limited due to unavailability of wider genetic variation among existing germplasm (Bakhsh et al., 2009; Khan et al., 2013)

The insect pests are a major threat to cotton production worldwide and are considered main constrain in cotton productivity. The losses from insect pests and disease have been estimated as 37% to agricultural production world-wide, while being 13% only due to insect pests (Gatehouse et al., 1992). More than fifteen economically important insect pests attack cotton crop, sucking as well as chewing pests, however lepidopterans are most devastating i.e. Helicoverpa armigera, Pectinophora gossypiella, Earias insulana/vitella and spodoptera litura. The widely used approach to protect cotton has been the use of broad spectrum synthetic insecticides. The deployment of these agrochemicals has resulted in serious environmental and human health concerns (Bakhsh...
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et al., 2009). The non-judicious and continuous use of these insecticides also led to the development of resistance in insect populations against these insecticides. Moreover these have harmful effects on non-target/beneficial insects and potential to damage natural environment. Conventional plant breeding methodologies have helped cotton breeders to improve crop, however resistance to insect pests and diseases is not available in germplasm. The less availability of new genetic information in plants has delayed developing plant varieties with novel characters through plant breeding techniques (Hussain et al., 2002). The advent of genetic engineering technologies has helped plant researchers to transfer genes from irrelevant origin into significantly important crop plants to induce insect resistance (Dhaliwal et al., 1998). Insect resistant Bt plants have emerged as potential alternative to the synthetic insecticides (Khan et al., 2011; Sohail et al., 2012).

Transgenic cotton expressing Bt genes from B. thurigengesis is rapidly planted GM crop in the world (James, 2002; Barwale et al., 2004; Dong et al., 2005). The genetically modified crops have been widely accepted and adopted reaching to 175 million hectares (James, 2013). Bt cotton was grown on 2.8 million hectares out of 3.4 million hectares in Pakistan (James, 2012). The adoption of transgenic insect resistant cotton has resulted in the reduction of use of insecticides worldwide, Mexico (77%), China (65%), Argentina (47%), India (41%), and South Africa (33%) respectively (Qaim et al., 2009) which ultimately led to significant increase in farm yield. In China, the genetically modified cotton produced economic benefit that valued $15 billion between 1996 and 2012, while $2.2 billion gain was recorded during the past year. India enhanced farm income by the use of Bt cotton by US$5.1 billion in the period 2002 to 2008 and US$1.8 billion in 2008 alone (Brookes and Barfoot 2010) while US$1.7 billion was reported in Pakistan (James, 2012).

The inheritance and expression of transgenes in subsequent progenies is highly significant in order to implement transgenic technology successfully for crop improvement. Some researchers have shown that once foreign genes are incorporated into the host plant cells; are truly transferred to further progenies through sexual generations while retaining high meiotic and expression stability (Duan et al., 1996; Fearing et al., 1997; Scott et al., 1998). However, a few researchers have also reported the loss, inactivation or silencing of the introduced gene in the progenies of transgenic plants (Finnegan and McElroy 1994; Matzke and Matzke 1995; Srivastava et al., 1996; Zhang et al., 1996). The primary transformants showing strong transgene expression may not give rise to progeny with the same characteristics (Rao et al., 2013). The production of transgenic plants with stable, high-level transgene expression is important for the success of crop improvement programs based on genetic engineering (Meyer, 1998). The present study was carried out to evaluate transgene stability and resistance level of transgenic cotton lines Bt-14 and Bt-17 against targeted insect pest (Helicoverpa armigera) under field conditions. To determine whether the introduced insecticidal gene (cry1Ac) is being stably inherited in fifth transgene progeny, Mendelian segregation pattern of (cry1Ac) has also been studied.

Material and Methods

Plant material

The present research work was conducted at Plant Biotechnology Laboratory, CEMB, University of the Punjab, Pakistan. Earlier, Agrobacterium mediated transformation of a local cotton cultivar CIM-482 was achieved successfully by Rashid et al., (2008). The transgenic cotton plants expressed cry1Ac and cry2A endotoxins under the constitutive promoter 35S CaMV. Initial screening of the progeny was conducted in the greenhouse and later on in field conditions. Single plant progenies were developed out of these primary transformants as described previously by Bakhsh et al., (2009) based on morphological, agronomical and molecular data collected from field and laboratory. Seeds of the homozygous plants with similar agronomic characteristics were bulked and named as Bt-14 and Bt-17. For this study, seeds were taken from cotton seed store and delinted with commercial sulfuric acid (H2SO4) @ 100 mL per kg of seed cotton. After removing the fuzz, seeds were washed with tape water 4-5 times to make seeds free of acid. These lines were sown along with their parent variety (untransformed CIM-482) according to randomized complete block design (RCBD). Experimental field was surrounded with five rows of untransformed CIM-482 and another non transgenic cotton variety (MNH-93) that served as refugia to delay pest resistance against Bt crop. The field was further isolated from surrounding by planting Sorghum bicolor L. (Bakhsh et al., 2009).

Molecular analysis for gene integration and expression

Polymerase chain reaction (PCR) was carried out using gene specific primer to amplify cry1Ac gene from transgenic cotton plants. For this
purpose, genomic DNA was isolated and purified from leaves using protocol established by Li et al. (2001). PCR was carried out in a 20 µL reaction volume with 50 ng of DNA template, 1X reaction buffer, 1.5 mM MgCl₂, 10 ng of each primer, 1 mM of dNTPs mix and one unit of Taq DNA polymerase. PCR conditions followed were as initial denaturation at 94°C for 4 minutes, with 35 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 40 seconds while extension at 72°C for 40 seconds. The final extension was adjusted at 72°C for 10 min. The binary vector pk2Ac DNA was used as positive control whereas as DNA isolated from untransformed plants was used as negative control.

In an order to quantify the expression level of cry1Ac protein, double-antibody sandwich ELISA was performed using Envirologix kit. Approximately 500mg of fresh terminal leaves was ground in liquid nitrogen using mortar and pestle, protein extraction buffer was added to it and quantification procedure was followed according to the instructions provided in kit. Positive and negative controls were used in kit and were used according to the instructions. The OD values at 430 nm were used to calculate the amount of cry1Ac protein by comparing it with the standard cry1Ac protein.

To quantify the level of cry1Ac protein, western dot blot was also performed because of its ease and quick in results. An estimated quantity protein (10ng) was loaded on Hybond-C membranes. The membrane was completely air-dried and incubated in blocking buffer/reagent for 30 min at room temperature or overnight at 4°C. The membrane was washed thrice with 1X PBS. The membrane was probed with the diluted primary antibodies (1:5000) for 1 hour and washed three times with 1X PBS. Afterwards, the membrane was probed with the diluted secondary antibodies (1:5000) for 1 hour and washed again three times with 1X PBS. The color was developed in AP Buffer (NBT/BCIP). Bt contents were quantified after scanning the blots by using software Lab works 4.0 (UVP Co.).

**Leaf biotoxicity assay**

The efficacy of Bt gene(s) against Helicoverpa armigera was evaluated by leaf biotoxicity assays. The second instar larvae of Helicoverpa armigera was used for this purpose. Five fresh leaves from each transgenic and control plant were placed in petri plates and one day pre-fasted 2nd instar larva was released in each plate to feed the leaf. The data on insect mortality were recorded on daily basis up to fifth day.

**Agronomic characteristics of transgenic lines**

Different morphological and agronomic characteristics including seed cotton yield (g), plant height (cm), number of bolls per plant, seed cotton yield per plant and average boll weight was also recorded. The data collected of the above-mentioned characters were subjected to analysis of variance and mean comparisons.

**Results and Discussion**

Transgenic technology has resulted in development of crop plants expressing desirable traits with resistance to pests, herbicides, pathogens, and environmental stress (Wu et al., 2005). The present study was undertaken to evaluate the performance of transgenic progenies of Bt-14 and Bt-17 lines which were developed from independent transformation events of a local cotton cultivar CIM-482 transformed with two insecticidal genes (cry1Ac and cry2a) via Agrobacterium-mediated transformation as described previously by Rashid et al., (2008).

PCR method is routinely used to check the integration and stable inheritance of any gene in plant progenies (Victor et al., 1993). It requires small amount of genomic DNA either in ultra-purified form or crude form. In our study, 50 individual progeny plants from Bt-14 and Bt-17 transgenic lines were analyzed for gene integration and expression studies along with non-transgenic control plants (Figure 1a, b). Out of 50 tested plants of Bt-14 line, 28 plants showed required band of cry1Ac gene showing distorted Mendelian ratio in the progeny while in case of Bt-17 transgenic line, 35 showed required cry1Ac band (Table 1). The line Bt-17 followed Mendelian fashion when results were subjected to Chi square test. There are many reports regarding the inheritance and expression stability of foreign genes in transgenic crops. Mendelian inheritance of foreign genes in transgenic plants has been observed in alfalfa (Micallef et al., 1995), rice (Duan et al., 1996), maize (Fearing et al., 1997), cotton (Canning et al., 2000; Zhang et al., 2000; Xia et al., 2007; Zhang et al., 2007; Daud et al., 2009; Bakhsh, 2010) and cowpea (Ivo et al., 2008). However in some cases, incorporated gene exhibited distorted Mendelian segregation pattern (Spencer et al., 1992; Wan and Lemaux, 1994; Somers et al., 1994; Altman et al., 1996; Wu et al., 2002; Rashid et al., 2008; Bakhsh, 2010).
Table 1. Chi Square test to evaluate goodness of fit test of cry1Ac gene in Bt-14 and Bt-17 transgenic lines.

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Status (cry1Ac)</th>
<th>Observed (O)</th>
<th>Expected (E)</th>
<th>(O-E)/E</th>
<th>Tabulate value (df=1) at p = 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bt-14</td>
<td>Detected</td>
<td>28</td>
<td>37.5</td>
<td>2.40</td>
<td>9.62</td>
</tr>
<tr>
<td></td>
<td>Not Detected</td>
<td>22</td>
<td>12.5</td>
<td>7.22</td>
<td></td>
</tr>
<tr>
<td>Bt-17</td>
<td>Detected</td>
<td>35</td>
<td>37.5</td>
<td>0.16</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Not Detected</td>
<td>15</td>
<td>12.5</td>
<td>0.50</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. PCR assay showed the amplification of required cry1Ac band (565 bp) in progeny of Bt-14 transgenic line (A). Lane 1: 100 bp ladder (Fermentas), Lane 2-21: Transgenic plants of line Bt-14, Lane 22: Negative control (DNA from untransformed plant), Lane 23: Positive control (plasmid DNA); PCR amplification of required cry1Ac band (565 bp) in progeny of Bt-17 transgenic line (B). Lane 1: 1 Kb plus ladder (Fermentas), Lane 2-21: Transgenic plants of Bt-17 line, Lane 22: Negative control (DNA from untransformed plant), Lane 23: Positive control (plasmid DNA)

The expression level of cry1Ac was quantified by ELISA. Transgenic plants were selected randomly from each transgenic cotton line and subjected to assay. Transgenic cotton line Bt-14 showed varying level of Bt toxin from 0.10 to 0.365 µg/g of fresh tissue (Figure 2a) while in the case of Bt-17 line, transgenic plants had expression level of cry1Ac from 0.255-0.630 µg/g of fresh tissue (Figure 2b). Similarly, in western blot results, Bt-17 line had more expression levels of cry1Ac as
compared to \textit{Bt}-14 transgenic line which had comparatively low expression. It was found that \textit{Bt}-17 line showed 91% expression of \textit{cry1Ac} in tested plants while \textit{Bt}-14 line showed 75% expression of \textit{cry1Ac}. The variation in insecticidal gene expression among transgenic lines was found variable; these results are in agreement with the previous findings of Sachs et al. (1998) who reported variation in \textit{cry1A} expression strongly influenced by environmental factors. The temporal or spatial variation in expression level of insecticidal genes have also been reported by Wu et al. (2002), Mahon et al. (2002), Xia et al. (2005), Adamczyk et al. (2009), Bakhsh et al. (2010, 2011, 2012). There are many worth considering factors that can contribute variation in gene expression. Among these factors, gene sequence, type of promoter, gene integration site in plant genome, transgene copy number, internal cell environment and several external factors have been reported (Hobbs et al., 1993; Guo et al., 2001; Rao, 2005; Bakhsh et al., 2012).

![Figure 2.](image)

Figure 2. Quantitative expression of \textit{cry1Ac} protein in progeny of \textit{Bt}-14 (a) and \textit{Bt}-17 (b) transgenic lines by ELISA. PC is the positive control provided in the kit while NC is the negative control.
To determine the efficacy of transgenic lines against targeted insect pests, leaf biotoxicity were performed (Figure 3). The laboratory biotoxicity assays with 2nd Instar *Helicoverpa armigera* larvae showed that expression of the introduced genes (*cry1Ac*) transgenic lines is sufficient to kill the targeted insect. In laboratory biotoxicity assay, most of the transgenic plants in the progeny were showing 70-100% larval mortality while no any larval mortality was observed in non-transformed control plants (Table 2). Difference in the mortality percentage in leaf biotoxicity assay can be attributed to the variation in expression of insecticidal gene in transgenic progenies which could be due to multiple factors like length of the gene used, promoter type, number of copies integrated in plant genome, internal or external cell environments (Guo et al., 2001; Hobbs et al., 1993; Rao, 2005). The results of leaf bioassays assays were in agreement with the previous studies conducted by various researchers (Fitt et al., 1998; Greenplate et al., 1998; Chen et al., 2000; Mahon et al., 2002; Xia et al., 2005; Bakhsh et al., 2012). The differences in *cry* protein levels can also be due to various factors as described earlier here. Therefore, the study of toxin titer in cotton plant is very important as it should be in adequate quantity to protect the crop against lepidopeteran. The transgenic lines under study conferred full protection against targeted insect pests.

### Alive

![Non Transgenic Leaf](image1)

![Transgenic Leaf](image2)

### Dead

Figure 3. The efficacy of insecticidal genes in transgenic cotton evaluated by leaf biotoxicity assay. The fresh leaves from transgenic and control plants are taken and placed on moist filter paper in petri plates accommodating *Heliothis* larvae, pre-fasted for 4-6 hours. Mortality rates of larvae are recorded. Alive larvae feeding on non-transgenic leaf on leaf, dead larvae after chewing transgenic leaf on right.

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>Plant height (cm)</th>
<th>No. of bolls/plant</th>
<th>Days to mature</th>
<th>Leaf bioassay (% mortality)</th>
<th>Seed yield/plant (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bt</em>-14</td>
<td>90.5a</td>
<td>43.1a</td>
<td>109.3a</td>
<td>85.9a</td>
<td>139.5a</td>
</tr>
<tr>
<td><em>Bt</em>-17</td>
<td>92.2a</td>
<td>42.3a</td>
<td>110.0a</td>
<td>90.5a</td>
<td>141.1a</td>
</tr>
<tr>
<td>CIM-482 (control)</td>
<td>110b</td>
<td>27b</td>
<td>150.7b</td>
<td>0.00b</td>
<td>95.6b</td>
</tr>
</tbody>
</table>

Table 2. The data of various traits was recorded between transgenic lines and untransformed CIM-482. Each data figure is an average of 10 plants per line. Numbers with same letters within column are not significantly different from each other according to LSD test at 5% level of significance.
The agronomic characteristics of cotton cultivars are very crucial for the conventional breeders to incorporate these cultivars as a parent in varietal developmental process. The transgenic lines harboring two insecticidal genes have shown much better performance as compared to untransformed control variety. The transgenic lines under study showed significant statistical differences for plant height, number of bolls, days to mature and average yield per plant when compared with untransformed control (Figure 4), although most of the characters between transgenic lines were non-significant (Table 2). One of the appealing characters of these transgenic lines was days to maturity as compared to control variety. A difference of approximately 35 days was recorded in early maturity which is a desirable character for farming community in cotton growing zone in Pakistan as farmers can cultivate wheat crop one month earlier in September/October as crop rotation. Transgenic lines have been reported early as well as late in maturity (Jiang et al., 2000). Rahman et al. (2007, 2012) and Bakhsh et al. (2009) reported development of short statured transgenic lines of rice and cotton when compared to the control variety. Shu et al. (2002) concluded that this variation could be caused by a disruptive effect associated with transgene insertion, a mutation located near the transgene on the chromosome (linked traits), pleiotropy, or endogenous gene silencing (Matzke et al., 2000). Some researchers reported that such variation may also be due to overexpression Bt gene in transgenic plants, exceeding 1% of the total soluble protein (Cheng et al., 1998; Gahakwa et al., 2000); contrary to data obtained in the present study. The complete understanding of actual genetic and physiological basis of this variation requires further investigation.

**Conclusion**

Based on molecular and agronomic data recorded, we conclude that this transgenic line Bt-17 is a promising germplasm source that can be utilized in conventional breeding programme which would be much helpful to the plant breeders while making selections and planning future experiments for cotton improvement.

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