Emir. J. Food Agric. 2014. 26 (8): 706-715
doi: 10.9755/ejfa.v26i8.17794
http://www.ejfa.info/

Plant Science

REGULAR ARTICLE

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

Tahira Hussain[1], Allah Bakhsh[2, 3*], Bushra Munir[1], Samina Hassan[1], Abdul Qayyum Rao[2], Ahmad Ali Shahid[2], Bushra Rashid[2] and Tayyab Husnain[2]

[1]*Department of Botany, Kinnaird College for Women, Lahore, Pakistan*
[2]*National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan*
[3]*Department of Agricultural Genetic Engineering, Faculty of Agricultural Sciences and Technologies, Niğde University, 51240 Niğde, Turkey*

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, Earius 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

Received 09 February 2014; Revised 03 April 2014; Accepted 05 April 2014; Published Online 15 June 2014

*Corresponding Author

Allah Bakhsh
Department of Agricultural Genetic Engineering, Faculty of Agricultural Sciences and Technologies, Niğde University, 51240 Niğde, Turkey

Emails: abthebest@gmail.com; allah.bakhsh@nigde.edu.tr

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 reseachers 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. thuriengenesis* 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 *crylAc* 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 (H_2SO_4) @ 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 c*ry1Ac* 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 $MgCl_2$, 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 94ºC for 4 minutes, with 35 cycles of denaturation at 94ºC for 1 minute, annealing at 54ºC for 40 seconds while extension at 72ºC for 40 seconds. The final extension was adjusted at 72ºC 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 used were provided 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 charactistics 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 (Canming 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.

Transgenic line	Status (*cry1Ac*)	Observed (O)	Expected (E)	$(O-E)^2/E$	$\chi^2 = \sum (O-E)^2/E$	Tabulate value (*df*=1) at p = 5%
Bt-14	Detected	28	37.5	2.40	9.62	3.84
	Not Detected	22	12.5	7.22		
Bt-17	Detected	35	37.5	0.16	0.66	3.84
	Not Detected	15	12.5	0.50		

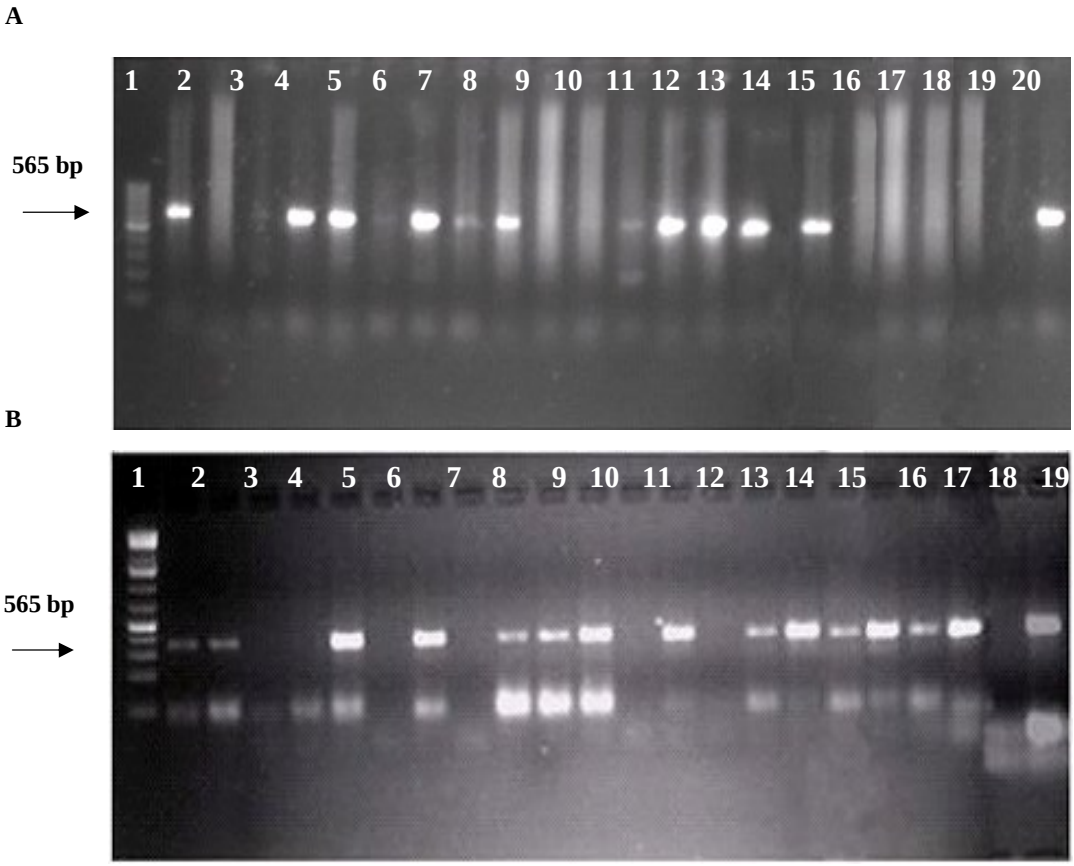

Figure 1. PCR assay showed the amplification of required *cry1Ac* band (565 bp) in progeny of *Bt*-14 transgenic line (A). Lane I: 100 bp ladder (Fermentas), Lane 2-21: Transgenic plants of line *Bt*-14, Lane 22: Negative control (DNA from untransformed plant), Lane 23: Positive control (plasmid DNA); PCR amplification of required *cry1Ac* band (565 bp) in progeny of *Bt*-17 transgenic line (B). Lane I: 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 *Bt*-14 transgenic line which had comparatively low expression. It was found that *Bt*-17 line showed 91% expression of *cry1Ac* in tested plants while *Bt*-14 line showed 75% expression of *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 c*ry1A* 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).

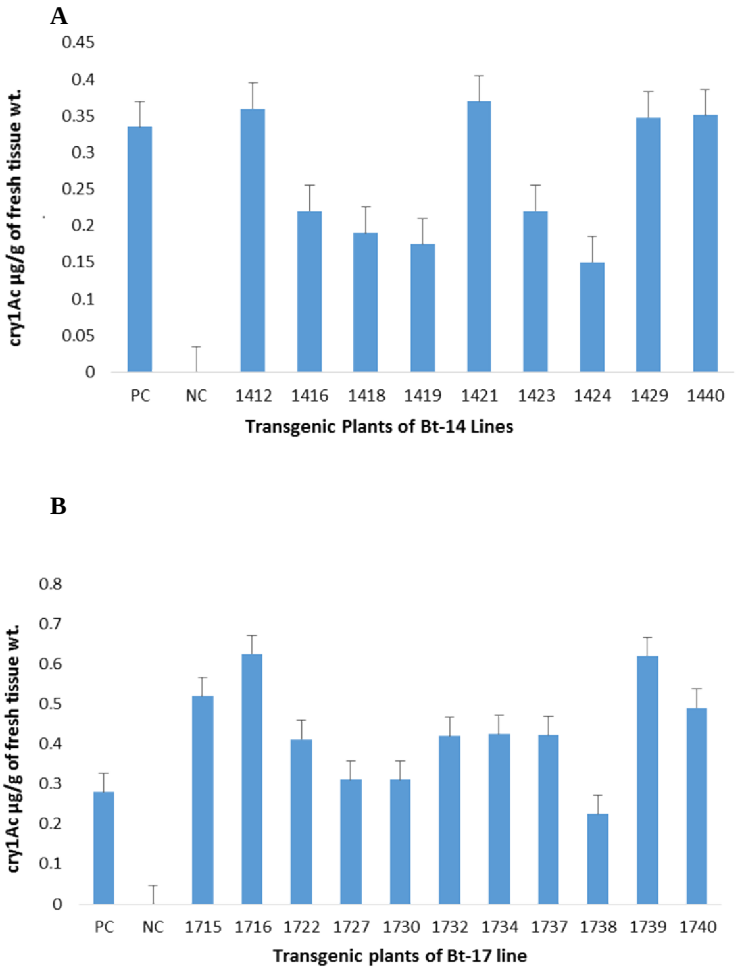

Figure 2. Quantitative expression of *cry1Ac* protein in progeny of *Bt*-14 (a) and *Bt*-17 (b) transgenic lines by ELISA. PC is the positive control provided in the kit while NC is the negative control.

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 lepidopteran. The transgenic lines under study conferred full protection against targeted insect pests.

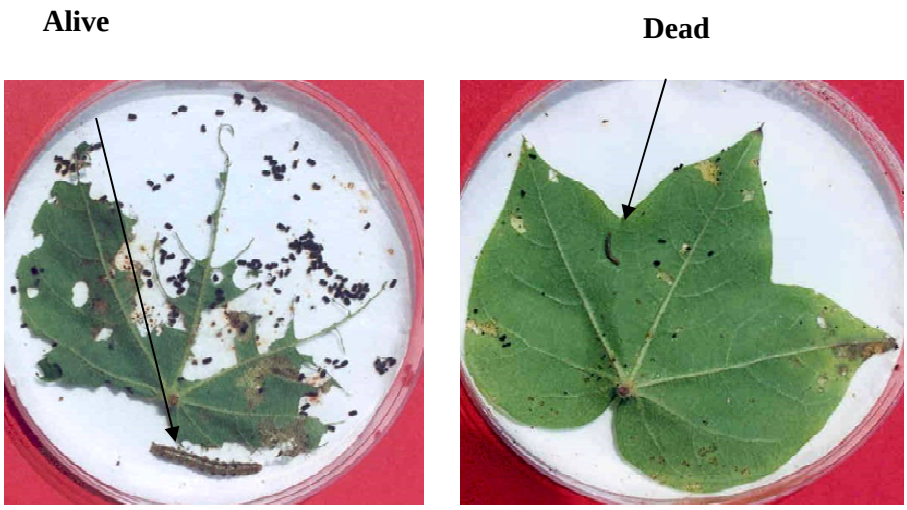

Figure 3. The efficacy of insecticidal genes in transgenic cotton evaluated by leaf biotoxicity assay. The fresh leaves from transgenic and control plants are taken and placed on moist filter paper in petri plates accommodating *Heliothis* larvae, pre-fasted for 4-6 hours. Mortality rates of larvae are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

Table 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.

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

Figure 4. A view of transgenic lines (*Bt*-17) grown in field along with parental line (CIM-482); a non-transgenic control variety. The transgenic lines were surrounded by rows of non transgenic cotton, further surrounded by refugia of *Sorghum bolor* L.

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 reseachers 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.

Acknowledgments

The authors (T. Hussain and B. Munir) acknowledge Director CEMB for providing opportunity to work at institute and giving free access to laboratory chemical and reagents. Many thanks to CEMB insect rearing laboratory (Raza Ali Zaidi and Abdul Kareem) for providing *Helicoverpa* larvae and help in conducting leaf biotoxicity assay. Authors acknowledge the assistance of Mr. Muhammad Ilays during field visits and data collections.

References

Adamczyk, J. J., J. O. Perera and W. R. Meredith. 2009. Production of mRNA from the *Cry1Ac* transgene differs among Bollgard lines which correlate to the level of subsequent protein. Transgenic Res. 18:143-149.

Altman, D. W., J. H. Benedict and E. S. Sachs. 1996. Transgenic plants for the development of durable insect resistance, a case study for

cotton and *Bacillus thuringiensis*. Ann. N.Y. Acad. Sci. 792:106-114.

Bakhsh, A, A. Q. Rao, A. A. Shahid, T. Husnain and S. Riazuddin. 2009. Insect resistance and risk assessment studies in advance lines of Bt cotton harboring *Cry1Ac* and *Cry2A* genes. Am. Eur. J. Agric. Environ. Sci. 6:1-11.

Bakhsh, A. 2010. Expression of two insecticidal genes in Cotton. Ph.D. Thesis, University of Punjab, Lahore, Pakistan.

Bakhsh, A., A. Q. Rao., A. A. Shahid., T. Husnain and S. Riazuddin. 2010. CaMV35S is a developmental promoter being temporal and spatial in expression pattern of insecticidal genes (*cry1Ac* & *cry2A)* in cotton. Aust. J. Basic Appl. Sci. 4:37-44.

Bakhsh, A., K. Shahzad and T. Husnain. 2011. The variation in spatio temporal expression of insecticidal genes in transgenic Cotton. Czech. J. Genet. Plant Breed. 47:1–9.

Bakhsh, A., S. Siddiq and T. Husnain. 2012. A molecular approach to combat spatio-temporal variation in insecticidal gene (Cry1Ac) expression in cotton. Euphytica 183:65-74.

Barwale, R. B., V.R. Gadwa, U. Zehr and B. Zehr. 2004. Prospects for Bt cotton technology in India. AgbioForum. 7:23-26.

Brookes, G. and P. Barfoot. 2010. GM Crops: Global Socio-economic and Environmental Impacts 1996-2008. P.G. Economics Ltd, Dorchester, UK

Canming, T., S. Jing, Z. Xiefi, G. Wangzhen, Z. Tianzhen, S. Jinlian, G. Congfen, Z. Weijun, C. Zhiian and G. Sandui. 2000. Inheritance of resistance to *Helicoverpa armigera* of 3 kinds of transgenic *Bt* strains available in upland cotton in china. Chin. Sci. Bull. 45:363-367.

Chen, S., J. Wu, B. Zhou, J. Huang and R. Zhang. 2000. On the temporal and spatial expression of Bt toxin protein in Bt transgenic cotton. Acta Gossypii Sin. 12:189-193.

Cheng, X., R. Sardana, H. Kaplan and I. Altosaar. 1998. *Agrobacterium* transformed rice plants expressing synthetic *crylAb* and *crylAc* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA 95:2767–2772.

Dong, H. Z., W.J. Li, W. Tang and D.M. Zhang. 2005. Increased yield and revenue with a seedling transplanting system for hybrid seed production in Bt cotton. J. Agron. Crop Sci. 191:116-124.

Dhaliwal, H. S., M. Kawai and U. Hirofumi. 1998. Genetic Engineering for abitoic stress tolerance in plants. Plant Biotechol. 15:1-10.

Duan, X., X. Li, Q. Xue, M. Abo-El-Saad, D. Xu and R. Wu. 1996. Transgenic rice plants harbouring an introduced potato proteinase inhibitor II gene are insect resistant. Nature Biotechnol. 14:494-498.

Economic survey of Pakistan 2010-11. Agriculture Chapter #2. Ministry of Finance, Govt. of Pakistan.

Fearing, P. L., D. Brown, D. Vlachos, D. Vlachos, M. Meghji and L. Privalle. 1997. Quantitative analysis of CryIA(b) protein expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. Mol. Breed. 3:169-176.

Finnegan, J and D. McElroy. 1994. Transgene inactivation: plants fight back! Bio/Technol. 12:883-888.

Fitt, G. P., J. C. Daly, C. L. Mares and K. Olsen. 1998. Changing Efficacy of Transgenic Cotton Plant Patterns and Consequences. Sixth Australian Entomological Research Conference, Brisbane, Australia.

Gahakwa, D., S. B. Maqbool, X. Fu, D. Sudhakar, P. Christou and A. Koli. 2000. Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes Show similar behaviour in diverse genetic backgrounds. Theor. Appl. Genet. 101:388–399.

Gatehouse, A. M. R, V. A. Hilder and D. Boulter. 1992. Potential of plant-derived genes in the genetic manipulation of the crops for insect resistance, In: Biotechnology in Agriculture N° 7: Plant genetic manipulation for crop protection, CAB International, pp.155-181.

Greenplate, J. T., G. P. Head, S. Penn and V.T. Kabuye. 1998. Factors potentially influencing the survival of Helicoverpa zea on Bollgard cotton. In: P. Dugger, and D. A. Richter (Eds.), pp. 1030-1033. Proceedings, 1998 Beltwide Cotton Conference, National Cotton Council of America, Memphis, TN.

Guo, W. Z., J. Sun, Y. F. Guo and T. Z. Zhang. 2001. Investigation of different dosage of inserted Bt genes and their insect-resistance in transgenic Bt cotton. Acta Genet. Sin. 28:668-676.

Hobbs, S. L. A., T. D. Warkentin and C. M. O. Delong. 1993. Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21:17-26.

Hussain, S. 2002. Genetic Transformation of Cotton with *Galanthus Nivalis Agglutinin* (GNA) gene. Ph.D. Thesis. The University of Punjab, Lahore, Pakistan.

Ivo, N. L., C. P. Nascimento, L. S. Vieira, F. A. P. Campos and F. J. A. Aragão. 2008. Biolistic-mediated genetic transformation of cowpea (*Vigna unguiculata*) and stable Mendelian inheritance of transgenes. Plant Cell Rep. 27:1475-1483.

James, C. 2002. Global Review of Commercialized Transgenic Crops: 2001 Feature: Bt Cotton. ISAAA Briefs No. 26. ISAAA, Ithaca, NY.

James, C. 2012. Global status of commercialized biotech/GM crops: 2011. ISAAA Brief No. 44, ISAAA, Ithaca, NY.

James, C. 2013. Global status of commercialized biotech/GM crops: 2011. ISAAA Brief No. 46, ISAAA, Ithaca, NY.

Jiang, J., S. D. Linscombe, J. Wang and J. H. Oard. 2000. Field evaluation of transgenic rice (*Oryza sativa* L.) produced by Agrobacterium and particle bombardment methods. In: Plant and Animal Genome VIII Conference, San Diego, CA, USA.

Khan, G. A., A. Bakhsh, M. Ghazanffar, S. Riazuddin and T. Husnain. 2013. Development of transgenic cotton pure lines harboring a pesticidal gene (*cry1Ab*). Emir. J. Food Agric. 25:434-442.

Khan, G. A., A. Bakhsh., S. Riazuddin and T. Husnain. 2011. Introduction of cry1Ab gene into cotton (*Gossypium hirsutum*) enhances resistance against Lepidopteran pest (*Helicoverpa armigera*). Span. J. Agr. Res. 9:296–300.

Li, H., L. Jinhua, J. K. Hemphill, J. T. Wang and J. Gould. 2001. A rapid and high yielding DNA miniprep for cotton (*Gossypium* spp.). Plant Mol. Biol. Rep. 19:183-183.

Mahon, R., J. Finnergan and K. Olsen andL. Lawrence. 2002. Environmental stress and the efficacy of Bt cotton. Aust. Cotton Grower 22:18-21.

Matzke, M and A. J. M. Matzke. 1995. How and why do plants inactivate homologous (trans) genes? Plant Physiol. 107:679-685.

Matzke, M. A., M. F. Mette and A. J. M. Matzke, 2000. Transgene silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Mol. Biol. 43:401-415.

Meyer, P. 1998. Stabilities and instabilities in transgene expression, In: Transgenic Plant Research, K. Lindsey (Ed.), pp. 263-275. Harwood Academic Publishers, Switzerland.

Micallef, M. C., S. Austin and E. T. Bingham, 1995. Improvement of transgenic alfalfa by backcrossing. In Vitro. Cell Dev. Biol. Plant. 31:187-192.

Qaim, M. 2009. The economics of genetically modified crops. Annu. Rev. Resour. Econ. 1:665–693.

Rahman, M., H. Rashid., A. A. Shahid., K. Bashir., T. Husnain and S. Riazuddin. 2007. Insect resistance and risk assessment studies of advanced generations of basmati rice expressing two genes of *Bacillus thuringiensis*. Electron. J. Biotechnol. 10:240-251.

Rahman. M., A. Q. Rao, F. Batool, S. Azam, A. A. Shahid and T. Husnain. 2012. Transgene copy number is responsible for phenotypic variations in transgenic basmati rice. J. Anim. Plant Sci. 22:1004-1013.

Rao, A. Q. K. S. Bajwa, A. N. Puspito, M. A. U. Khan, M. A. Abbas., M. Rehman, A. Bakhsh., A. A. Shahid, 1. A. Nasir and T. Husnain. 2013. Variation in expression of phytochrome B gene in cotton (*Gossypium hirsutum* L.). J. Agric. Sci. Technol. 15:1033-1042.

Rao, C. K. 2005. Transgenic Bt Technology: 3. Expression of Transgenes. http://www. monsanto. co.uk/news/ ukshowlib.phtml? uid¼9304.

Rashid, B., S. Zafar, T. Husnain and S. Riazuddin. 2008. Transformation and inheritance of Bt genes in *Gossypium hirsutum*. J. Plant Biol. 51:248-254.

Sachs, S. E., J. J. Benedict, F. J. Taylor, M. D. Stelly, W. D. Altman, A. S. Berberich and K. S. Davis. 1998. Expression and segregation of genes encoding Cry1A insecticidal proteins in cotton. Crop Sci. 38:1-11.

Scott, A., D. Woodfield and D. W. R. White. 1998. Allelic composition and genetic background effects on transgene expression and inheritance in white clover. Mol. Breed. 4:479–490.

Shu, Q., H. Cui, G. Ye, D. Wu, Y. Xia, M. Goa and I. Altosaar. 2002. Agronomic and Morphological Characterization of *Agrobacterium*-Transformed Bt Rice Plants. Euphytica 126:345-52.

Sohail, M. N., S. M. Karimi, S. Asad, S. Mansoor, Y. Zafar and Z. Mukhtar. 2012. Development of broad-spectrum insect-resistant tobacco by expression of synthetic cry1Ac and cry2Ab genes. Biotechnol. Lett. 34:1553–1560.

Somers, D. A., K. A Torber, W. P. Pawlowski and H. W. Rines. 1994. Genetic engineering of oat. In: R. J. Enry and J. A. Ronald (Eds.), pp. 37-46. improvement of cereal quality by genetic engineering. Plenum Press, New York.

Spencer, T. M., J. V. O'Brien, W. G. Start and T. R. Adams. 1992. Segregation of transgene in maize. Plant Mol. Biol. 18:201–210.

Srivastava, V., V. Vasil and K. Vasil. 1996. Molecular characterization of the fate of transgenes in transformed wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 92:1031-1037.

Victor, I. K., B. J. Carroll, S. C. M. Thama and J. D. G. Jones. 1993. Alkali treatment for rapid preparation of plant material for reliable PCR analysis, Plant J. 3:493-494.

Wan, Y and P. G. Lemaux. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104:37–48.

Wu, J., X. Zhang, Y. Nie and X. Luo. 2005. *Agrobacterium* tumefaciens and regeneration of insect-resistant plants. Plant Breed. 124:142–146.

Wu, G., H. Cui, G. Ye, Y. Xia, R. Sardana, X. Cheng, Y. Li, I. Altosaar and Q. Shu. 2002. Inheritance and expression of the *cry1Ab* gene in Bt (*Bacillus thuringiensis*) transgenic rice. Theor. Appl. Genet. 104:727-734.

Xia, L., Q. Xu and S. Guo. 2005. Bt insecticidal gene and its temporal expression in transgenic cotton plants. Acta Agron. Sin. 31:197-202.

Xia, L.H., Y.R. He, L.U. Chun, Z.Y. Yang and Z.J. Hong. 2007. Inheritance and resistance to insects in *CryIA*(c) transgenic cabbage. Chin. J. Agric. Biol. 4:57-61.

Zhang, B. H., T. L. Guo and Q. L. Wang. 2000. Inheritance and segregation of exogenous genes in transgenic cotton. J. Genet. 79:71-75.

Zhang, S., D. Warkentin, B. Sun, H. Zhong and M. Sticklen. 1996. Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected (bar) transgenes in maize (*Zea mays* L.). Theor. Appl. Genet. 92:752-761.