

TRANSGENE COPY NUMBER AND PHENOTYPIC VARIATIONS IN TRANSGENIC BASMATI RICE

Mahmood-ur-Rahman, A. Q. Rao*, F. Batool*, S. Azam*, A. A. Shahid* and T. Husnain*

Department of Bioinformatics and Biotechnology, Government College University (GC University), Faisalabad.

*National Center of Excellence in Molecular Biology, University of the Punjab, Lahore-53700, Pakistan.

Corresponding Author e-mail: mahmood1233@yahoo.com

ABSTRACT

Bt genes were detected on metaphase chromosomes of transgenic Basmati rice in order to find out the effects of copy number and location of transgene (s) on important agronomic traits of transgenic Bt rice. Phenotypic traits of transgenic plants highly varied from control. The transgenic plants were shorter in height and internodal length and were early maturing. Awns were totally absent in transgenic plants containing two Bt genes. Integration of the Bt genes on the host genome and their copy number was determined through Fluorescence *in situ* hybridization. No correlation was found between presence of Bt genes on different chromosomes and phenotypic traits. However, transgene copy number was found negatively correlated with agronomic traits. With the increase in copy number of transgene (s), the plant height, internodal length and days to maturity were decreased. Positive correlation was found between the Bt protein concentration and transgene copy number. The concentration of Bt protein was higher in plants having more copies of transgene indicating that copy number and not the location of the transgene determined phenotypic variation in transgenic plants.

Keywords: Bt rice, FISH, Position Effect, Copy Number, Transgene

INTRODUCTION

Plant genetic engineering has moved in 1990s from laboratory to farm as a powerful technology to provide improved crops, resistant against various biotic and abiotic stresses. Most of the research addressed the effects of transgenes on targeted processes such as pathways that control quality and productivity. The technology has been developed to monitor the pathways that control the fundamental biological processes associated with plant growth (van Hal *et al.* 2000). Transgenes interfere with the natural biological processes in different ways. Sachuh *et al.* (1993) observed tremendous heritable variations in the transgenic populations and Bao *et al.* (1996) further documented a wide spectrum of alterations in the genome in transformed rice plants regenerated from protoplasts. The variations in transgenic populations varied with the transformation method. Wang *et al.* (1996) reported that morphological traits varied greatly in *Agrobacterium*-transformed poplar plants. A similar phenomenon was also observed in transgenic potatoes (Dale and McPartlan 1992).

Since the mid-1990s, transgenic plants with enhanced tolerance to herbicides, resistance to diseases and insect pests, and with improved quality have been commercially exploited (James 1999; Dhaliwal and Uchimiya 1999). Transgenic maize (corn), soybean, cotton, and potatoes are now grown on million-hectare scale, (Dunwell 2000; James 2008). Since the first

engineered fertile japonica and indica rice plants were independently developed by Shimamoto *et al.* (1989) and Datta *et al.* (1990), a large number of transgenic rice plants, though not yet commercialized, have been obtained (Tyagi *et al.* 1999; Tyagi and Monhanty 2000). Studies on inheritance and expression mode of transgenes have been widely conducted in transgenic plants, but research focusing on agronomic and morphological variations in transgenic plants is still limited.

The field trials of *Bt* rice also revealed morphological variations in transgenic lines as compared to control plants (Shu *et al.* 2002; Bashir *et al.* 2004 and 2005; Mahmood-ur-Rahman *et al.* 2007) and variations in *Bt* expression during the plant life cycle (Bashir *et al.* 2004 and 2005) or during several generations (Wu *et al.* 2002). Different transgenic tobacco lines that expressed high levels of the BC1 protein had phenotypes with severe stunting and leaf mottling (Duan *et al.* 1997). There was also significant variation between individual plants and between lines (Mahmood-ur-Rahman *et al.* 2007).

Fluorescence *in situ* hybridization (FISH) is the most direct method to physically locate transgenes within the genome (Jiang and Gill 1994; Leggett *et al.* 2000) in plants as well as in mammalian species. It is a method of localizing and detecting specific DNA sequences in morphologically preserved tissues sections or cells. In present study, the *Bt* gene was detected on rice mitotic chromosomes by Fluorescence *in situ* hybridization (FISH) in transgenic Basmati rice lines and correlation studies were carried out to find links between location

and copy number of the transgene (s) and agronomic and morphological traits of transgenic plants.

MATERIALS AND METHODS

Plant Material and Experimental Design: The seeds of *Oryza sativa* L. variety Basmati-370 were obtained from Rice Research Institute (RRI), Kala Shah Kaku, Lahore, Pakistan. The plants were transformed with *cry1Ac*, *cry2A* and *cy1Ac & cry2A* (Table-1). Three different plasmids were used in these studies as previously described (Bashir *et al.* 2004 and 2005; Riaz *et al.* 2006; Mahmood-ur-Rahman *et al.* 2007). The plasmid pIA1 having Bt gene *cry2A* under the control of *CaMV 35S*

promoter while *cry1Ac* was cloned in pIA2 plasmid driven by *ubiquitin* promoter were used. Plasmid pSM6 contained both the transgenes under the same promoters (Figure-1). The transformation was done through particle bombardment method as described previously (Riaz *et al.* 2006). Eight transgenic lines were selected on the basis of insect resistance, agronomic performance under field conditions. T4 to T8 generations of transgenic lines were sown in the field for three consecutive years according to randomized complete block design (RCBD) with four replications of 700 plants per block as described by Mahmood-ur-Rahman *et al.* (2007) following the biosafety guidelines (NBC 1999).

Table-1: Transgene Location and Copy Number in different transgenic rice lines

S. No.	Line	Gene (s)	Location of Bt gene*		Copy Number	
			<i>cry1Ac</i>	<i>Cry2A</i>	<i>cry1Ac</i>	<i>Cry2A</i>
1	L-5	<i>cry1Ac</i>	**Ch. 9, 10	---	4	---
2	L-6	<i>cry1Ac</i>	Ch. 2, 8	---	2	---
3	L-3-382-5	<i>cry1Ac</i>	Ch. 1	---	1	---
4	L-4	<i>cry2Ac</i>	---	Ch. 4	---	2
5	L-3-311-42	<i>cry2Ac</i>	---	Ch. 3	---	1
6	L-8-22-2	<i>cry1Ac & cry2A</i>	Ch. 1, 10, 11	Ch. 5	4	1
7	L-8-22-32	<i>cry1Ac & cry2A</i>	Ch. 1, 2, 9, 11	Ch. 4	4	1
8	L-8-22-35	<i>cry1Ac & cry2A</i>	Ch. 7	Ch. 2, 10	2	2

* Presence of transgene on which chromosome, **Ch. = Chromosome

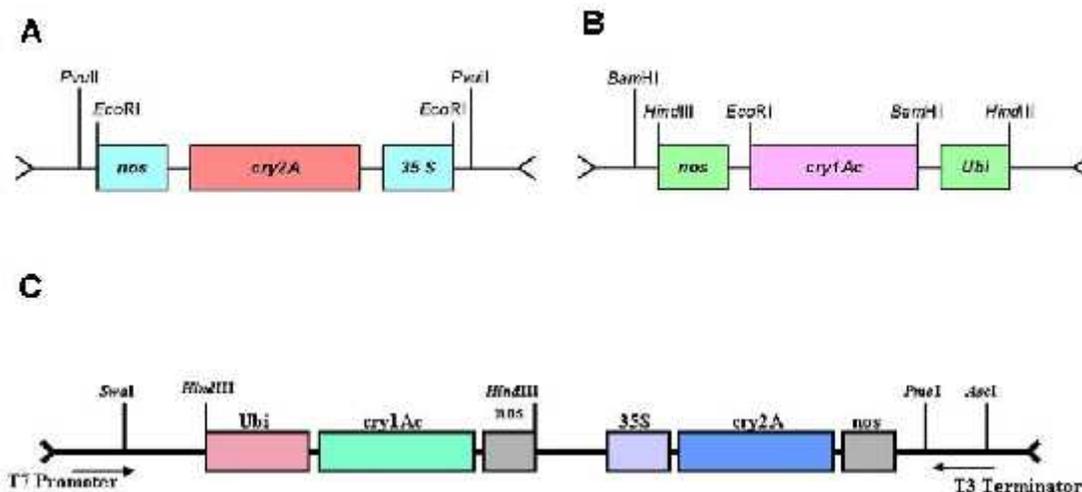


Figure-1: Plasmids used in this study; A) pIA1 having *cry2A*, B) pIA2 containing *cry1Ac* and C) pSM6 having both transgenes (*cry1Ac & cry2A*).

Damage of YSB and RLF: Transgenic as well as control plants were artificially infested with Yellow Stem Borer (YSB) according to the procedures described by Bashir *et al.* (2004). Data regarding insect damage were collected as described previously (Mahmood-ur-Rahman *et al.* 2007) and the data were processed through analysis of variance (ANOVA) for the percent damage and the

differences among different lines were determined by using Least Significant Difference (LSD) test at 5% level of significance.

Spatio-temporal gene expression: Protein was extracted with the procedure as described by Shan *et al.* (2005) and concentration of Cry1Ac and Cry2A were determined for spatial transgene (s) expression in leaves, stem, straw,

panicle, seed, seed coat and root by ELISA using Enviroligix kits AP 003 and AP 005 (Enviroligix, Maine, USA). For temporal expression, protein was extracted from leaves at 30, 60, 90, 120 days after transplanting. Protein was also extracted from dried leaves 15 days after harvesting. All these samples were processed for ELISA using Enviroligix kits AP 003 and AP 005 (Enviroligix, Maine, USA) according to the manufacturer's instructions.

Morphological Characters: Morphological traits like plant height, internodal length, days to maturity and presence of awns of transgenic rice as well as control were recorded according to the recommendations by INGER (1996) and methods adopted by Mahmood-ur-Rahman *et al.* (2007). The data regarding morphological traits were analysed through the analysis of variance (ANOVA) followed by LSD test at 5% level of significance to determine differences among the means.

Fluorescence *in situ* hybridization (FISH): Probe for transgenes was labeled by Fluorescein ULS[®] Labeling Kit (Fermentas K0641) according to the instructions by the manufacturer and *in situ* hybridization was carried out with the metaphase chromosomal spreads according to the protocol adopted by Mahmood-ur-Rahman *et al.* (2010).

Counterstaining: The hybridized slides were counterstained by Propidium Iodide (PI) diluted 2500 times on ice by adding 0.8 μ l PI and 2000 μ l 1X PBS (10X= 1.3M NaCl; 70mM Na₂HPO₄; 30mM NaH₂PO₄; pH 7.4). PI solution was added (500 μ l) on each slide and incubated for 2-5 minutes at room temperature. Then slides were washed with 3ml of 1X PBS from a pipette, covered with cover slip and stored at dark at 4 °C until further analysis.

Transgene Copy Number and Location: The fluorescent signals were detected by Fluorescent microscope (Carl Zeiss AXIO 100). Transgene copy number was estimated by directly visualizing of fluorescent signals. The captured images were subjected to image analysis tools of computer software "Genus 3.7" provided by Cytovision Applied Imaging Systems. The karyotypes were made by using the same software according to the instructions of manufacturer and location of the transgene (s) was estimated.

RESULTS AND DISCUSSION

Transgenic plants are resistant to target insects: The transgenic lines expressing *cry1Ac* gene showed variable level of resistance at vegetative and flowering stage against YSB and RLF. L-3-382-5 was 99% resistant against YSB. Among the lines containing *cry2A* gene, L-3-311-42 was found more resistant as compared to L-4. All the transgenic lines containing two *Bt* genes (*cry1Ac* & *cry2A*) were excellent in the field when YSB resistance was determined. They were more than 90% resistant against YSB. All the transgenic lines were significantly different from control (Figure-2). The transgenic lines were variable in insect resistance ranging from 81-98%, however, lines having two *Bt* genes were more than 90% and 98% resistant against YSB and RLF respectively. These lines provided built-in resistance against target insects at all stages of plant growth for several generations (Bashir *et al.* 2005; Mahmood-ur-Rahman *et al.* 2007). These transgenic rice lines expressing genes from *Bacillus thuringiensis* can reduce the use of conventional pesticides against insect pests.

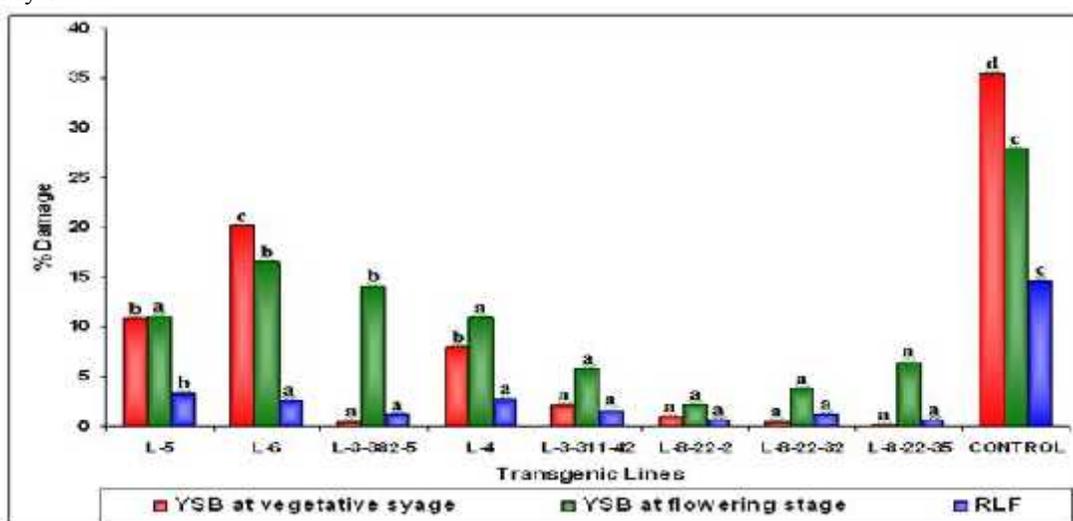


Figure-2: Damage of YSB and RLF to various lines of transgenic Basmati rice.

Note: Bars with the same letters are not different from each other according to ANOVA followed by LSD at 5% significance level.

Spatio-temporal Transgene Expression in Bt rice: Spatio-temporal gene expression is the activation of genes within specific tissues of the organism at specific times during development. Maximum expression was found in leaves of transgenic plants. *Bt* protein quantified in leaves of L-8-22-35 was 1.06µg/g followed by L-8-22-32 which produced 0.94µg/g of Cry1Ac protein. Comparatively less *Bt* protein was quantified in seed coat

of the plant ranging from 0.04-0.20µg/g of tissue (Figure-3A). The lines having two *Bt* genes produced more toxin as compared to the lines having only one gene. The concentration of Cry2A was less as compared to the Cry1Ac, however, lines with double gene produced more toxin. The expression of *cry2A* gene was more in leaves of the plants as compared to other tissues (Figure-3B).

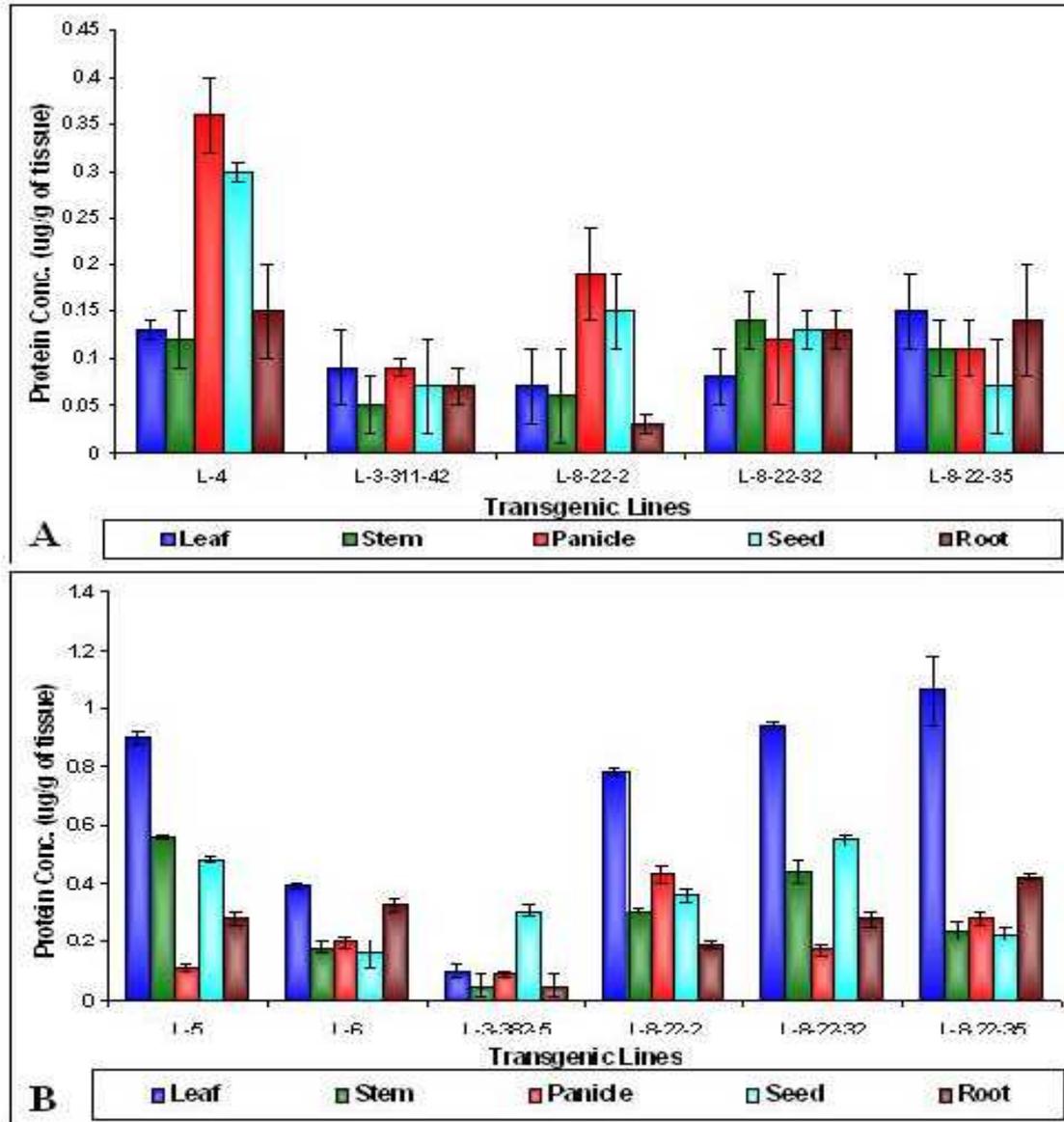


Figure-3: Spatial expression of transgenes. (A) *cry1Ac*, (B) *cry2A*.

Maximum level of the Cry1Ac was determined at 30 days after transplanting (DAT) which decreased gradually in subsequent days and after harvesting, negligible amount of Cry1Ac was detected in transgenic plants. Maximum Cry1Ac protein was detected in L-8-22-35 which was 1.04µg/g of the tissue at 30 DAT which

declined subsequently. All the lines containing two *Bt* genes produced more Cry1Ac protein as compared to the protein produced in lines having single gene (Figure-4A). The expression of *cry2A* gene was also declined with the increase in the age of plant. It was observed that the

expression of *cry2A* gene was less as compared to the *cry1Ac* gene (Figure-4B).

The expression of *Bt* genes declined with increase of the age of plants. Our results are in agreement with the studies performed by Bashir *et al.* (2004 & 2005). The reasons of declining expression in transgene expression are unknown. The toxin titer of *cry1Ac* was

found more than *cry2A* in Bt lines having both the genes. This may be due to unknown kind of antagonistic effect of genes or the promoter type may be the reason. *cry1Ac* is under *CaMV 35S* promoter while *cry2A* is driven by *ubiquitin*. *CaMV 35S* might be the stronger promoter than *ubiquitin*. Another explanation may be less number of copies of *cry2A* gene for this phenomenon.

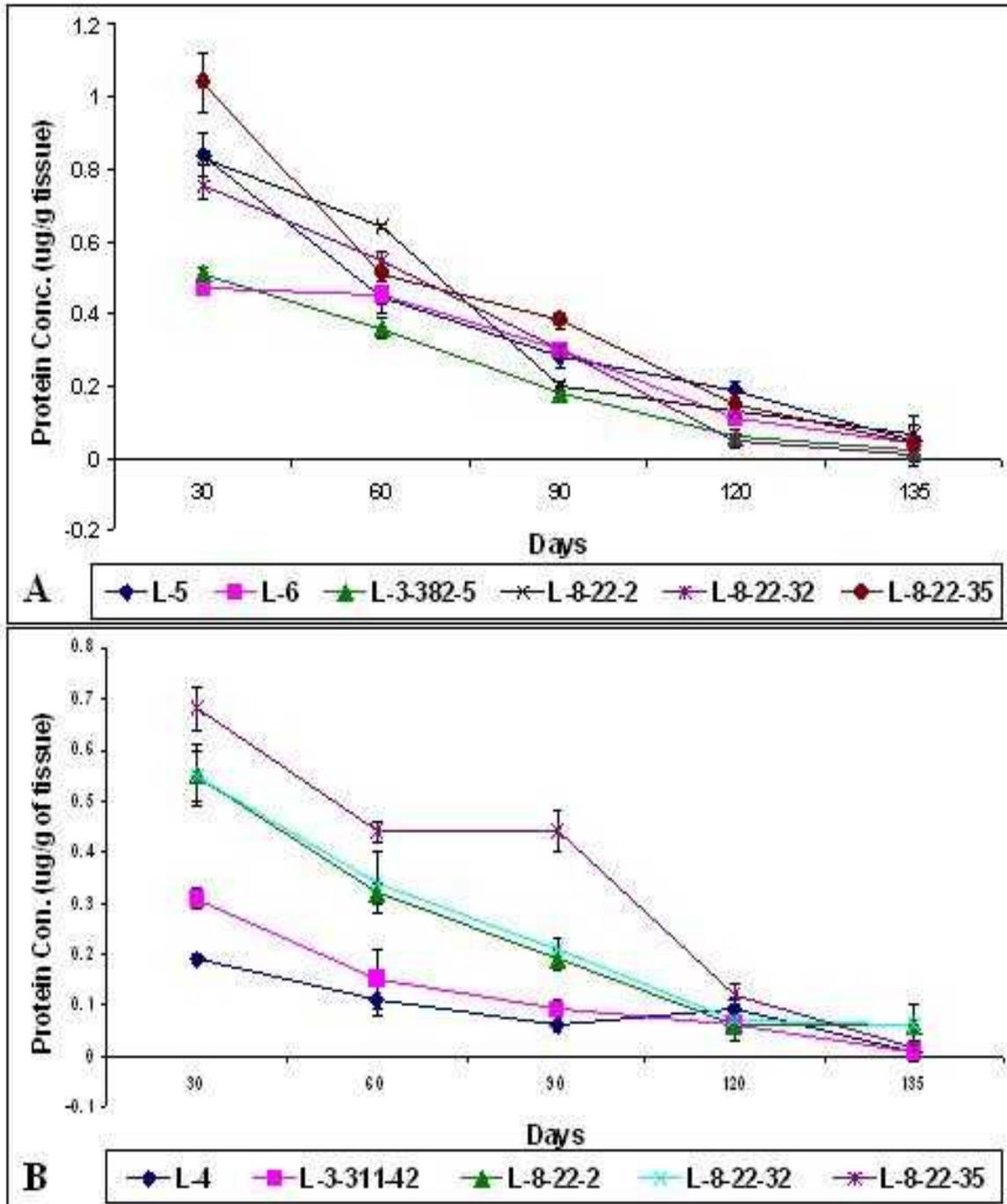


Figure-4: Temporal expression of transgenes (A) *cry1Ac*, (B) *cry2A*.

Transgenic plants are morphologically different from their parents: The introduction of a transgene into a recipient genome is a complex event depending on the transgene itself and the host genome interaction. The transgene expression level may vary, depending upon a number of factors (Kohli *et al.* 1999; Yin and Malepszy 2003) including "Position Effect" and copy number of transgene play a major role. Bhattacharya *et al.* (1994) determined the levels of transgene expression for individual inserts that were integrated into different locations in tobacco genome and concluded that the variability observed in a transgenic population is due to the "Position Effect" of transgene and transgene copy number may also be another factor.

Significant differences in agronomic and morphological traits were observed between lines containing one and two *Bt* genes with control. Transgenic lines having two *Bt* genes were significantly different from the lines containing one *Bt* gene (either *cry1Ac* or *cry2A*) as well as from control. Transgenic lines with two *Bt* genes were shorter compared to lines containing *cry1Ac* or *cry2A* alone. They were also significantly shorter than untransformed control plants. Transgenic lines were found 108-138 cm in height compared to control which was 156 cm in height. The height of the plant decreased with the increase in copy number of transgene (Sachuh *et al.* 1993) (Table-2).

Our results revealed that the copy number of *Bt* genes is a source of phenotypic variations in transgenic plants. Several other factors such as developmental and/or physiological states of tissues, transformation method, nature of promoter, may also play a significant

role in transgene expression variability (Jones *et al.* 1987).

The average internodal length was found significantly different between transgenic lines and controls. Transgenic plants having one gene (either *cry1Ac* or *cry2A*) are same as control. The plants having two genes were short in stature. Transgenic plants having less copy number of *Bt* gene had more internodal length (Table-2). They were approximately 25 days early in maturity as compared to the control. The awns were totally absent in lines having two *Bt* genes while they were present in control and other transgenic lines with the exception of L-5 and L-3-311-42 which did not contain awns (Table-2). Possible reasons for the phenomenon may be the somaclonal variations (Larkin and Scowcroft 1981), breakdown of plant genes caused by transgene insertion or insertion mutagenesis (van Lijsebetens *et al.* 1991), pleiotropy or transgene induced endogenous silencing (Matzke *et al.* 2000).

Transgene Location and Copy Number: The integration of transgene was random. No correlation was found between transgene location and phenotypic traits of the transgenic plants, however, copy number of the transgene was found positively correlated with morphological characters. With increasing transgene copy number smaller plant height, internodal length and days to maturity and vice versa were recorded. (Table-2 & 3). The figures 5 & 6 show that the integration of transgene is independent of the chromosome number and is widely distributed throughout the genome. However, the copy number varied from one to four.

Table-2: Gene location correlated with plant characteristics

Plant Character	Location of transgene on chromosome number							Control
	Ch.1	Ch.2	Ch.7	Ch.8	Ch.9	Ch.10	Ch.11	
<i>cry1Ac</i>								
YSB Damage	3.68 ^a	10.2 ^b	3.38 ^a	18.4 ^c	6.53 ^a	6.26 ^a	1.87 ^a	35.45 ^d
RLF Damage	1.08 ^a	1.91 ^a	0.75 ^a	2.58 ^a	2.29 ^a	2.04 ^a	1.00 ^a	14.66 ^b
Plant Height	111 ^a	120 ^b	102 ^a	144 ^c	112 ^a	115 ^a	98 ^a	156.1 ^d
Internodal Length	16.6 ^a	16.8 ^a	15.0 ^a	18.8 ^b	17.1 ^b	17.1 ^b	14.83 ^a	20.41 ^b
Days to Maturity	111 ^a	118 ^b	108 ^a	130 ^b	117 ^b	118 ^b	107 ^a	130 ^b
Protein Conc.*	0.47±0.02	0.46±0.04	0.64±0.01	0.40±0.01	0.52±0.03	0.53±0.00	0.54±0.01	----
<i>cry2A</i>								
YSB Damage	5.71 ^a	3.98 ^a	5.81 ^a	4.46 ^a	6.33 ^a	---	---	35.45 ^b
RLF Damage	1.05 ^a	1.58 ^a	2.00 ^a	1.41 ^a	0.875 ^a	---	---	14.66 ^b
Plant Height	106 ^a	100 ^a	111 ^a	120 ^b	100 ^a	---	---	156.1 ^c
Internodal Length	14.33 ^a	14.6 ^a	16.49 ^a	14.83 ^a	14.08 ^a	---	---	20.41 ^b
Days to Maturity	109 ^a	110 ^a	118 ^b	111 ^a	108 ^a	---	---	130 ^c
Protein Conc.	0.51±0.03	0.15±0.03	0.24±0.04	0.49±0.03	0.53±0.02	---	---	---

YSB Damage= %, RLF Damage= %, Plant height= cm, Internodal length= cm, Protein Concentration= µg/g of the fresh tissue

* Average temporal expression of transgenes at 30, 60 90 days after transplanting

Note: Numbers with the same letters are not different from each other according to ANOVA followed by LSD at 5% significance level and the numbers followed by ± shows standard deviation of the means (n=5).

It was also observed that less copy number of *cry2A* gene was integrated into the host genome as compared to *cry1Ac* gene. In case of *cry1Ac*, there were 1 – 4 copies while most of the transgenic lines with *cry2A* had only one copy (Figure-5 & 6). Only L-4 and L-8-22-35 varieties contained two copies of *cry2A* gene and there was no transgenic line having more than two copies of *cry2A*. Our studies observed positive correlation between transgene copy number while no correlation between location of the transgene on chromosomes. The reduced variability revealed a strong correlation between copy

number and level of *GUS* gene expression for plants carrying up to two copies of the T-DNA (Mlynarova *et al.* 1995). All plants carrying the same number of gene copies give the same level of expression (Reitman *et al.* 1990) while Mlynarova *et al.* (1995) observed that higher copy number yielded significantly lower *GUS* activities. This inverse correlation may be due to one or combination of the various gene silencing phenomenon described for transgenes in plants (Flavell 1994; Mlynarova *et al.* 1995).

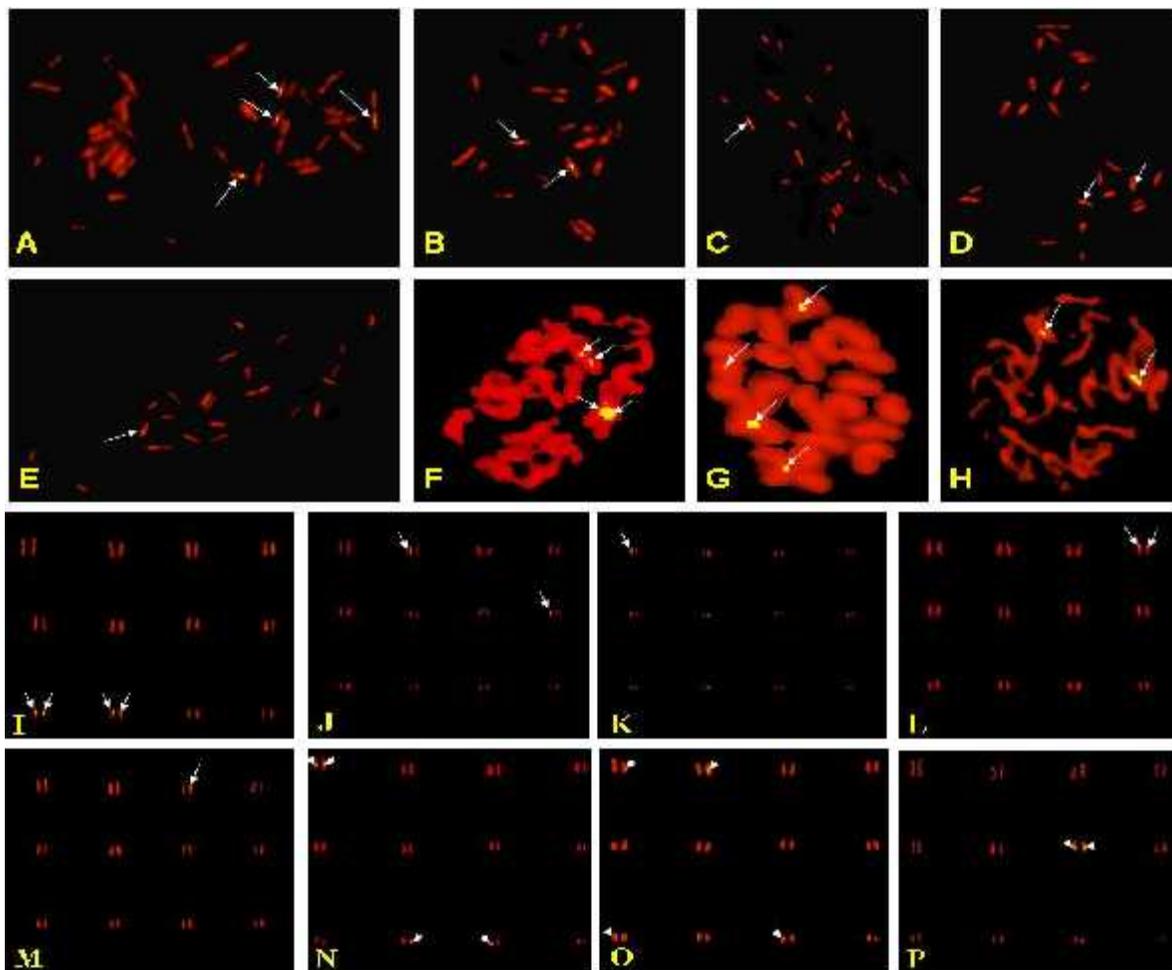


Figure-5: Fluorescence *in situ* hybridization analysis of transgenic Basmati rice lines having *cry1Ac* gene on its different chromosomes: (A) line L-5, (B) line L-6, (C) line L-3-382-5, (D) line L-4, (E) line L-3-311-42, (F) line L-8-22-2, (G) line L-8-22-32 and (H) line L-8-22-35. Karyotype analysis of transgenic Basmati rice lines having *cry1Ac* gene on its different chromosomes: (I) line L-5 has Bt genes on homologous chromosomes numbers 9 & 10, (J) line L-6 has Bt genes on chromosomes 2 & 8, (K) line L-3-382-5 has only one copy of Bt gene on chromosome 1, (L) line L-4 has two copies of Bt gene on homologous chromosome number 4, (M) line L-3-311-42 has Bt gene on chromosome number 3, (N) Bt gene was integrated on chromosome numbers 1, 10 & 11 in line L-8-22-2, (O) line L-8-22-32 has four copies of Bt gene on chromosome numbers 1, 2, 9 & 11 and (P) line L-8-22-35 has two copies of Bt genes on homologous chromosome number 7. Yellow; *cry1Ac* signal, Red; propidium iodide counterstain. Figures A-E; images were taken using 40X objective lens, Figures F-H; images were taken using 100X oil immersion objective lens.

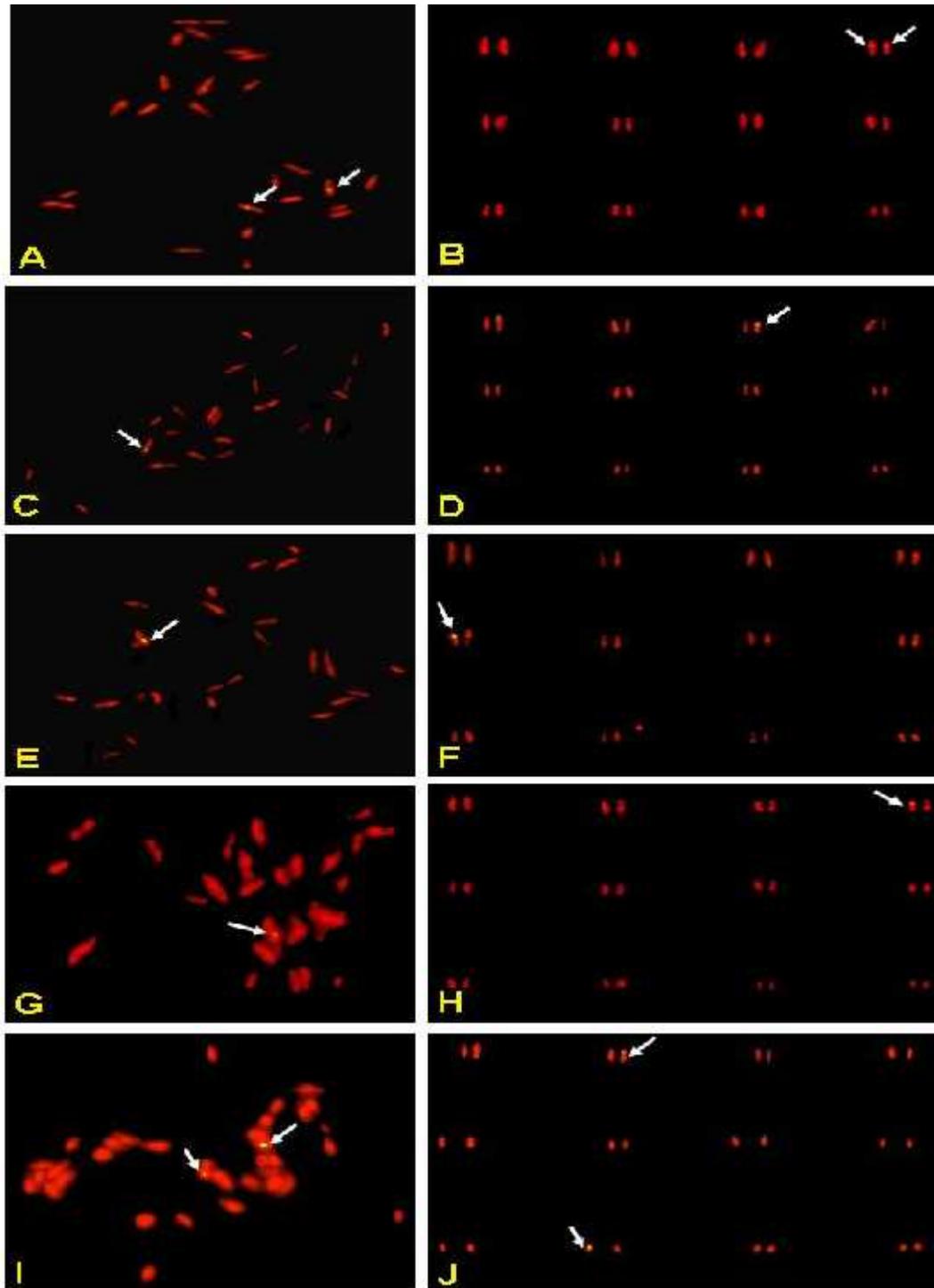


Figure-6: Fluorescence *in situ* hybridization analysis of transgenic Basmati rice lines having *cry2A* gene on its different chromosomes: (A) line L-4, (B) karyotype of line L-4 has two copies of Bt gene on homologous chromosome number 4, (C) line L-3-311-42, (D) karyotype of line L-3-311-42 has one copy of Bt gene on chromosome number 3, (E) line L-8-22-2, (F) karyotype of line L-8-22-2 has one copy of Bt gene on chromosome number 5, (G) line L-8-22-32, (H) karyotype of line L-8-22-32 has only one copy of Bt gene on chromosome number 4, (I) line L-8-22-35 and (M) karyotype of line L-8-22-35 has two copies Bt gene on chromosome numbers 2 & 10. Yellow; *cry2A* signal, Red; propidium iodide counterstain. Images were taken using 40X objective lens.

Transgene Copy Number makes the plants different from their parents: The plants having more copies of transgenes integrated into their genome are more resistant to YSB and RLF. It is obvious that more copy number of Bt genes produce more toxic that kill the target insects more efficiently, thus conferring more resistance. Interestingly, as the number of copies of the transgene in host plant increases, the height of the plants decreases significantly. The same is true for days to maturity of the plants. Protein concentration is more in plants having more copy number (Table-3).

Table-3: Copy number of transgene correlated with plant characteristics

Plant Character	Copy number of transgene			Control
<i>cryIAc</i>				
YSB Damage	7.31 ^b	10.89 ^c	4.88 ^a	35.45 ^d
RLF Damage	1.25 ^a	1.66 ^a	1.77 ^a	14.66 ^b
Plant Height	138 ^c	124 ^b	108 ^a	156.13 ^d
Internodal Length	20.33 ^b	16.9 ^a	16.38 ^a	20.41 ^c
Days to Maturity	121 ^c	117 ^b	114 ^{ab}	130 ^d
Protein Conc.*	0.35±0.03	0.52±0.01	0.53±0.03	----
<i>cry2A</i>				
YSB Damage	2.57 ^a	6.43 ^a		35.45 ^b
RLF Damage	1.17 ^a	1.50 ^a		14.66 ^b
Plant Height	99 ^a	114 ^a		156.13 ^b
Internodal Length	14.72 ^a	16.58 ^a		20.41 ^b
Days to Maturity	109 ^a	119 ^b		130 ^c
Protein Conc.	0.29±0.03	0.32±0.02		----

YSB Damage= %, RLF Damage= %, Plant height= cm, Internodal length= cm, Protein Concentration= µg/g of the fresh tissue

* Average temporal expression of transgenes at 30, 60 90 days after transplanting

Note: Numbers with the same letters are not different from each other according to ANOVA followed by LSD at 5% significance level and the numbers followed by ± shows standard deviation of the means (n=5).

Conclusions: The transgenic plants having Bt genes are the most effective tools to control insect pests of important crops. But with the integration of transgene, the plant faces major morphological and agronomic changes alongwith the desired trait. These changes occur due to various factors as discussed above including transgene copy number. More Bt gene copy number results in higher Bt protein production which in turn induces more resistance in the plants against target insects. It was also concluded that with the increased Bt gene copy number various phenotypic characters like plant height, internodal length and days to maturity were also decreased. No effect was found whether on which chromosome the transgene was located.

Acknowledgements: The authors gratefully acknowledge the financial support from the Higher Education Commission, Government of Pakistan. The

authors are also thankful to Dr. Fida Muhammad Abbasi, Senior Scientific Officer, National Agriculture Research Centre, Islamabad, Pakistan for his valuable suggestions and critical evaluation of the *in situ* hybridization protocol.

REFERENCES

- Bao, P. H., S. Granata, S. Castiglione, G. Wang, C. Giordani, E. Cuzzoni, G. Damiani, C. Bandi, S.K. Datta, K. Datta, I. Potrykus, A. Callegarin, and F. Sala (1996). Evidence for genomic changes in transgenic rice (*Oryza sativa* L.) from protoplast. *Transgenic Res.* 5: 97-103.
- Bashir, K., T. Husnain, T. Fatima, Z. Latif, S. A. Mehdi, and S. Riazuddin (2004). Field evaluation and risk assessment of transgenic indica basmati rice. *Mol. Breed.* 13: 301-312.
- Bashir, K., T. Husnain, T. Fatima, Z. Latif, N. Riaz, and S. Riazuddin (2005). Novel Indica Basmati Line (B-370) expressing two unrelated genes of *Bacillus thuringiensis* is highly resistant to two lepidopteran insects in the field. *Crop Protection.* 24(10): 870-879.
- Bhattacharyya, M. K., B. A. Stermer, and R.A. Dixon (1994). Reduced variation in transgene expression from a binary vector with selectable markers at the right and left T-DNA borders. *Plant J.* 6(6): 957-968.
- Dale, P. J. and H. C. McPartlan (1992). Field performance of transgenic potato plants compared with controls regenerated from tuber discs and shoot cuttings. *Theor. Appl. Genet.* 84: 585-591.
- Datta, S. K., K. Datta, and I. Potrykus (1990). Fertile indica rice plants regenerated from protoplasts isolated from microspore cell suspensions. *Plant Cell Rep.* 9: 253-256.
- Dhaliwal, H. S. and H. Uchimiya (1999). Genetic engineering for disease and pest resistance in plants. *Plant Biotechnol.* 16: 255-261.
- Duan, Y., C. A. Powell, D.E. Purcifull, P. Broglio, and E. Hiebert (1997). Phenotypic variations in transgenic tobacco expressing mutated Geminivirus Movement/Pathogenicity (BC1) proteins. *MPMI.* 10(9): 1065-1074.
- Dunwell, J. M. (2000). Transgenic approaches to crop improvement. *J. Exp. Bot.* 51: 487-496.
- Flavell, R. B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Nat. Acad. Sci. USA.* 91: 3490-3496.
- INGER (1996). Standard evaluation system for rice. International Rice Research Institute, Los Banos, Philippines.

- James, C (1999) Global review of commercialized transgenic crops: ISAAA Briefs No. 12: Preview. ISAAA: Ithaca NY.
- James, C (2008) Global status of commercialized biotech/GM crops: 2008, ISAAA Briefs No. 39. ISAAA: Ithaca NY.
- Jiang, J. and Gill, B. S. (1994). Nonisotopic *in situ* hybridization and plant genome mapping: The first 10 years. *Genome*. 37(5): 717-725.
- Jones, J. D. G., D. E. Gilbert, K. L. Grady, and R. A. Jorgensen (1987). T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. *Mol. Gen. Genet.* 207: 478-485.
- Kohli, A., D. Gahakwa, P. Vain, D.A. Laurie, and P. Christou (1999). Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. *Planta*. 208: 88-97.
- Larkin, P. J. and W. R. Scowcroft (1981). Somaclonal variation: a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* 60: 197-214.
- Leggett, J. M., S. J. Perret, J. Harper, and P. Morris (2000). Chromosome localization of cotransformed transgenes in the hexaploid cultivated oat *Avena sativa* L. using fluorescence *in situ* hybridization. *Heredity*. 84: 46–53.
- Mahmood-ur-Rahman, 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*. *Elec. J. Biotechnol.* 10(2): 241-251.
- Mahmood-ur-Rahman, S. Noreen, T. Husnain, S. Riazuddin (2010). Fast and efficient method to determine the position of alien genes in transgenic plants. *Emir. J. Food Agric.* 22(3): 223-231.
- 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 mechanism in plants and vertebrates. *Plant Mol. Biol.* 43: 401-415.
- Mlynarova, L., R. C. Jansen, A. J. Conner, W. J. Stiekema, and J.P. Nap (1995). The NAR-Mediated Reduction in Position Effect can be uncoupled from Copy Number-Dependent Expression in Transgenic Plants. *The Plant Cell*. 7: 599-609.
- NBC (National Biosafety Committee Pakistan) (1999). Biosafety guidelines in genetic engineering and biotechnology. Ministry of Environment Local Government, Rural Development, Government of Pakistan.
- Reitman, M., E. Lee, H. Westphal, and G. Felsenfeld (1990). Site-independent expression of the chicken β^A -globin gene in transgenic mice. *Nature*. 348: 749-752.
- Riaz, N., T. Husnain, T. Fatima, R. Makhdoom, K. Bashir, L. Masson, I. Altosaar, and S. Riazuddin (2006). Development of Indica Basmati rice harboring two insecticidal genes for sustainable resistance against Lepidopteran Insects. *S. Afr. J. Bot.* 72(1): 217-223.
- Sachuh, W., M. R. Nelson, D. M. Bigelow, T. V. Orum, C. E. Orthj, P. T. Lynch, P. S. Eyles, N. W. Blackhay, J. Jones, E. C. Cocking, and M. R. Davey (1993). The phenotypic characterization of R₂ generation transgenic rice plants under field conditions. *Plant Sci.* 89: 69-79.
- Shan, G., S. K. Embrey, R. A. Herman, J. D. Wolt, D. Weston, and L. M. Mayer (2005). Biomimetic extraction of *Bacillus thuringiensis* insecticidal crystal proteins from soil based on invertebrate gut fluid chemistry. *J. Agric. Food. Chem.* 53: 6630-6634.
- Shimamoto, K., R. Terada, T. Izawa, and H. Fujimoto (1989). Fertile transgenic rice plants regenerated from transformed protoplasts. *Nature*. 338: 274-276.
- Shu, Q., H. Cui, G. Ye, D. Wu, Y. Xia, M. Gao, and I. Altosaar (2002). Agronomic and morphological characterization of *Agrobacterium*-transformed Bt rice plants. *Euphytica*. 127: 345–352.
- Tyagi, A. K., M. S. Bajaj, A. Chaudhury, and S. C. Maheshwari (1999). Transgenic rice: A valuable monocot system for crop improvement and gene research. *Crit. Rev. Biotechnol.* 19(1): 47–79.
- Tyagi, A. K. and A. Mohanty (2000). Rice transformation for crop improvement and functional genomics. *Plant Sci.* 158: 1–18.
- van Hal, N. L. W., O. Vorst, A. M. M. L. van Houwelingen, E. J. Kok, A. Peijnenburg, A. Aharoni, A. J. van Tunen, and J. Keijer (2000). The application of DNA microarrays in gene expression analysis. *J. Biotechnol.* 78: 271-280.
- van Lijsebetens, M., R. Vanderhaeghen, and M. van Montagu (1991). Insertional mutagenesis in *Arabopsis thaliana* Isolation of a TDNA-linked mutation that alters leaf morphology. *Theor. Appl. Genet.* 81: 277–284.
- Wang, G., S. Castiglione, Y. Chen, L. Li, Y. Han, Y. Tian, D. W. Gabriel, Y. Han, K. Mang, and F. Sala (1996). Poplar (*Populus nigra* L.) plants transformed with a *Bacillus thuringiensis* toxin gene: insecticidal activity and genomic analysis. *Transgenic Res.* 5: 289-301.
- Wu, D. X., Q. Y. Shu, Z. H. Wang, H. R. Cui, and Y. W. Xia (2002). Quality variations in transgenic rice with a synthetic cry1Ab gene from *Bacillus thuringiensis*. *Plant Breed.* 121: 198–202.
- Yin, Z. and S. Malepszy (2003). The transgenes are expressed with different level in plants. *Biotechnologia.* 2(61): 236-260.