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**Short Communication**

# A fast and efficient method to determine the position of alien genes in transgenic plants

**Mahmood-ur-Rahman, S. Noreen, T. Husnain* and S. Riazuddin**

**National Centre of Excellence in Molecular Biology (CEMB), 87-West Canal Bank Road, Thokar Niaz Baig, Lahore-53700, Pakistan**

**Abstract**: Phenotypic variations are commonly observed in transgenic plants. Basmati rice (*Oryza sativa* L.) and cotton (*Gossypium hirsutum*), locally transformed with *Bt* genes, also exhibited such variations in their morphology. One possible cause of these variations might be the position of transgene on chromosome (s). An experiment was conducted to determine the position of the alien genes. It was determined by Fluorescence *in situ* hybridization (FISH) by hybridizing fluorescien labeled DNA probe on mitotic chromosomes of transgenic rice and cotton. An easy and simple protocol was developed as compared to available lengthy and difficult methods. The method is quick and efficient.

**Keywords**: *Bt* genes, Fluorescence *in situ* hybridization, transgene, DNA probe.

# طرق سريعة وفعالة تزيد لتحديد اماكن الجينات الغريبة فى النباتات المعدلة وراثيا

**محمود – ار- رحمان, س. نورين، ت. حسنين* و س. ريازودين**

**المركز الوطنى للتفوق فى البيلوجيا الجزيئة CEMB، 87، شارع الضفة الغربية لشارع القناة، سوكار نايزبيق، لاهور، 35700، باكستان**

**الملخص**: نلاحظ عادة وجود الاختلافات المظهرية في النباتات المعدلة وراثيا وخاصة في نبات الأرز البسمتى والقطن والذي تحول محليا بواسطة تقنية جينات بي تي مما اظهر اختلافات وتنوع في شكلها الخارجي ومن أهم الأسباب لهذه الاختلافات والتغيرات هو موقع الجينات المحورة علي الكروموسوم وقد تم إجراء هذه التجربة لتحديد موقع هذه الجينات الغريبة وقد تم تحديدها بواسطة تقنية Fluorescence *in situ* hybridization (FISH) ومن خلال تهجين الحمض النووي المعلم بالفلوروسين والمحقق بواسطة الكروموسومات من نبات الأرز والقطن المعدل وراثيا وقد تم الحصول في نهاية التجربة على بروتوكول سهل وبسيط ومتقدم مقارنة مع البروتوكولات المستخدمة حاليا والتي تتميز بالطول والصعوبة وعليه فإننا طورنا هذه الطريقة المستخدمة في هذه التجربة وكانت سريعة وفعالة

* Corresponding Author, *Email*: tayyabhusnain@gmail.com

## Introduction

Transgenic plants are the most urgent demand to combat with various biotic and abiotic stresses. A large number of transgenic rice plants with different kind of transgenes have been obtained (Bajaj and Mohanty, 2005). Rice and cotton were transformed with two *Bt* genes *cry1Ac* and *cry2A* (Bashir et al., 2004; Majeed et al., 2000; Riaz et al., 2006; Rashid et al., 2008) and evaluated under field conditions (Bashir et al., 2005; Mahmood-ur-Rahman et al., 2007; Bakhsh et al., 2009) in Pakistan. Transgenic plants showed great morphological variations and were different in phenotype as compared to control plants (Bashir et al., 2004, 2005; Mahmood-ur-Rahman et al., 2007).

Bhattacharrya et al., (1994) observed the variability in transgenic tobacco plants having alien genes at different locations of the genome. These variations may be due to different positions of transgene, so it is important to study the position of the transgenes on chromosomes in order to answer the causes of these variations. In the present work, easy, direct and simple procedure was designed to study the integration of *Bt* gene (*cry1Ac*) in transgenic rice and cotton by Fluorescence *in situ* Hybridization (FISH) and its position was determined.

## 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* and *cry2A*. Three different plasmids containing Bt genes were used in these studies as previously described (Bashir et al., 2004, 2005; Riaz et al., 2006; Mahmood-ur-Rahman et al., 2007). The transformation was done through particle bombardment method as described previously (Husnain et al., 1995, 1997; Riaz et al., 2006). Transgenic plants were sown under field conditions according to randomized complete block design (RCBD) with four replications as described by Mahmood-ur-Rahman et al., (2007) following the biosafety guidelines (NBC, 1999).

### Polymerase Chain Reaction (PCR)

DNA from transgenic and Control plants was isolated as described by Dellaporta et al., (1983) and PCR analysis was carried out by using specific primers (Forward primer: 5'-ACAGAAGACCCTTCAATATC-3' and Reverse primer: 5'-GTTACCGAGTGAAGATGTAA-3') for *cry1Ac* gene under the following PCR conditions: 95°C for 4 minutes, 52°C for 1 minute and 72°C for 1 minute (one cycle) followed by 95°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute (30 cycles) in Thermal Cycler (Model MJ Research, PTC100). DNA extracted from non-transgenic plants was used as negative control while plasmid DNA carrying *cry1Ac* gene was used as positive control.

### Probe Preparation

PCR product was run on 1% agarose gel, DNA bands were cut under UV light and eluted by using DNA Extraction Kit (Fermentas K0513) following standard protocol given by manufacturer. The eluted PCR product was labeled by Fluorescien ULS® Labeling Kit (Fermentas K0641) according to the instructions provided by the manufacturer.

### Chromosome Preparation

Growing root tips were collected (1-2 cm long) and washed in distilled water. They were fixed in fixative (3 vols. Ethanol and 1 vol. Glaciel Acetic Acid) for at least overnight. Fixative was removed and roots were again washed in distilled water. Meristematic portion of roots was cut (1-2 mm) and incubated in enzyme solution: 2% Pectolyase (Sigma cat# P 3026) and 3% Cellulase (Sigma cat# C

1184) at 37ºC for 60-90 minutes for rice and 4-5 hours for cotton roots followed by washing gently with distilled water. Chromosomes were spread on microscopic glass slide with a drop of fixative and air-dried. Slides were observed under phase contrast microscope (Carl Zeiss AXIO 100) and selected for FISH. The slides were dehydrated in 70%, 95% and 100% ethanol for 5 minutes in each solution respectively and stored at room temperature.

**RNase Treatment**

1% RNase A solution was diluted 100 times (10µl of 1% RNase A, 10µl 1M Tris HCl pH 8.0, 5µl 15mM NaCl and 925µl $dH_2O$). 100µl of RNase was added to each slide, covered with cover slip and incubated in wet chamber for 45-60 minutes at 37ºC. The slides were washed in 2X SSC at room temperature followed by dehydration in 70%, 95% and 100% ethanol respectively.

***In situ* Hybridization**

Hybridization solution (60% dFA, 20X SSC, 50% Dextran Sulphate, 10µg carrier DNA, 20-30ng probe DNA) denatured at 80-90ºC for 10 minutes followed by quick chilling on ice. Then, 35µl of it was added to each slide, covered with cover slip and air dried. The chromosomes were denatured at 80ºC for 10 min in water bath in 2X SSC. The slides were incubated at 37ºC for 18 hours in wet chamber. Next day, the slides were washed with 2X SSC at 42°C and then with 4X SSC at 42°C.

**Counterstaining with Propidium Iodide (PI)**

Propidium iodide (1mg/ml) was diluted 2000 times on ice by adding 1.0µl PI and 1999µl 1X PBS (10X= 1.3M NaCl; 70mM $Na_2HPO_4$; 30mM $NaH_2PO_4$; pH 7.4). It was added (500µl) on each slide and incubated for 5 minutes at room temperature. Then slides were washed with 3ml of 1X PBS, covered with cover slip and stored at dark at 4ºC.

**Fluorescent Signal Detection**

The fluorescent signals were detected by Fluorescent microscope (Carl Zeiss AXIO 100) using appropriate filter set. The picture of fluorescence signal was taken by CCD camera attached with microscope and analyzed by using software Genus 3.7 provided by Cytovision Applied Imaging Systems. The karyotyping was done using the same software package and the position of the *Bt* gene was determined.

**Results**

The transgenic lines were confirmed through PCR for the integration of transgene. The Bt gene *cry1Ac* was amplified in rice (Figure 1) and cotton (Figure 2). The amplification of the 565bp fragment of Bt gene indicated the successful transformation of alien gene into the host genome of rice (*Oryza sativa* L.) and cotton (*Gossypium hirsutum* L).

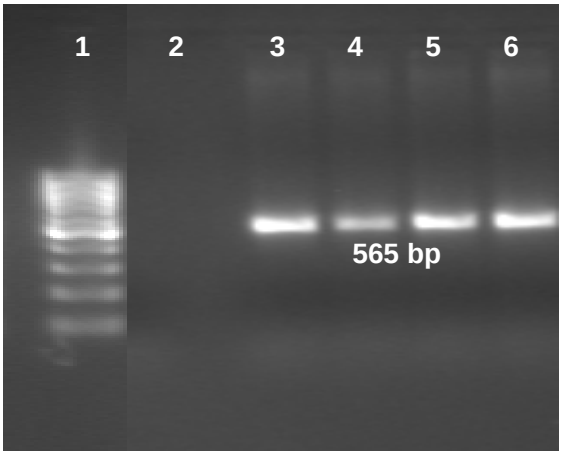


**Figure 1. Polymerase Chain Reaction (PCR) of Transgenic and Control Plants of rice revealing the integration of Bt gene. Lane 1: 100 bp DNA Ladder Marker, Lane 2: Negative Control (Basmati-370), Lane 3-5: Transgenic rice samples & Lane 6: Positive Control (Plasmid DNA).**

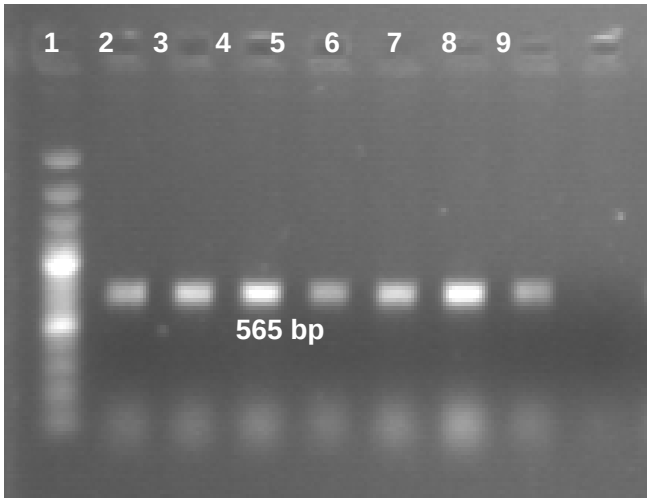


**Figure 2. Polymerase Chain Reaction (PCR) of Transgenic and Control Plants of cotton revealing the integration of Bt gene. Lane 1: 100 bp DNA Ladder Marker, Lane 2-7: Transgenic cotton samples, Lane 8: Positive Control (Plasmid DNA) & Lane 9: Negative Control (CIM-482).**

Metaphase chromosomes of PCR positive plants were prepared. The rice diploid genome has 24 chromosomes (2n=24) (Figure 3a) while cotton diploid genome has 52 chromosomes (2n=52) (Figure 3b). The selected slides of mitotic chromosomes were subjected to FISH analysis.

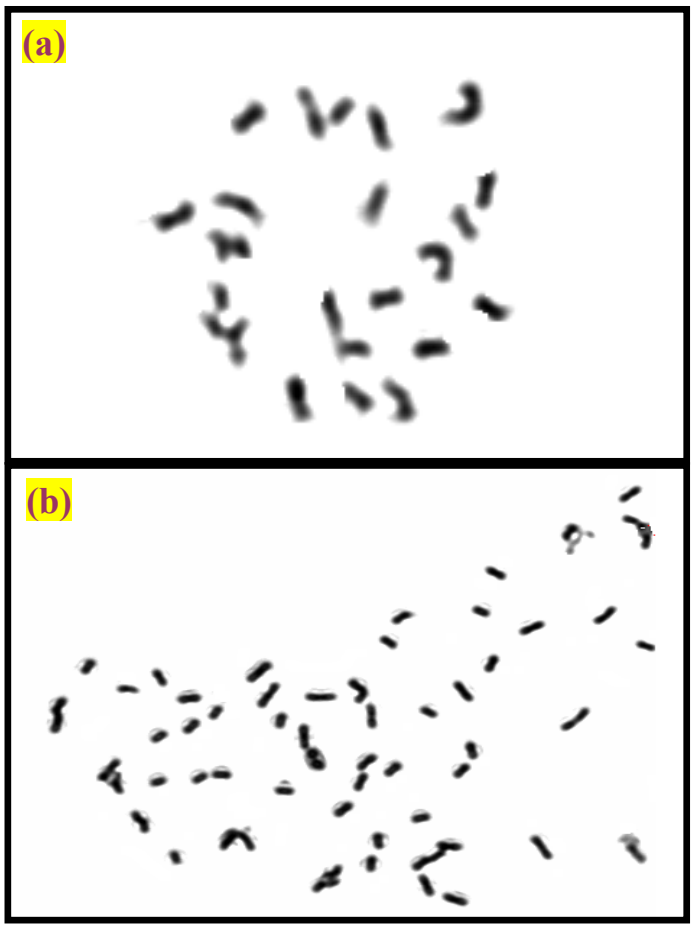


**Figure 3. Metaphase chromosome spread of transgenic (a) rice and (b) cotton.**

Fluorescence *in situ* hybridization (FISH) was carried out for selected lines of transgenic plants. The single color FISH procedure was adopted, the signals of *Bt* gene were in yellow the chromosomes were counterstained with red (Propidium iodide). The transgenic rice was homozygous for Bt gene having two copies. The transgenic rice showed stable integration of transgene on homologous chromosome number 4 (Figure 4a,b). In cotton, three copies of the Bt gene were found. On chromosome 4, one copy was present while on chromosome 18 two copies were detected (Figure 5a,b). The karyotypeing of transgenic plants made this protocol easy to determine the location of *Bt* gene on chromosome.

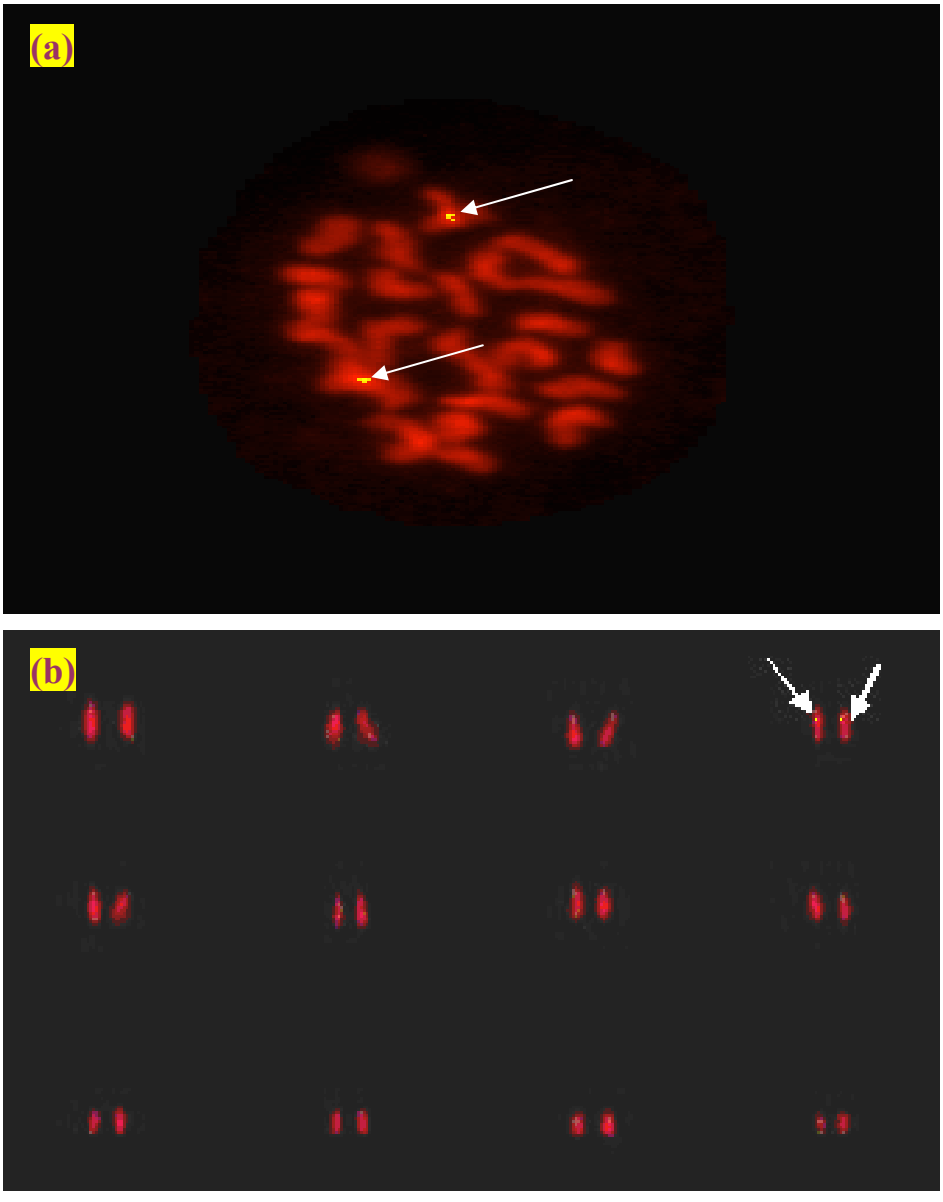


**Figure 4. Fluorescence *in situ* hybridization (FISH) analysis of Bt gene in rice. (A): FISH showing *Bt* gene signals on transgenic rice mitotic chromosomes. (B): Karyotype analysis of transgenic rice showing position of the Bt gene on mitotic chromosome.**

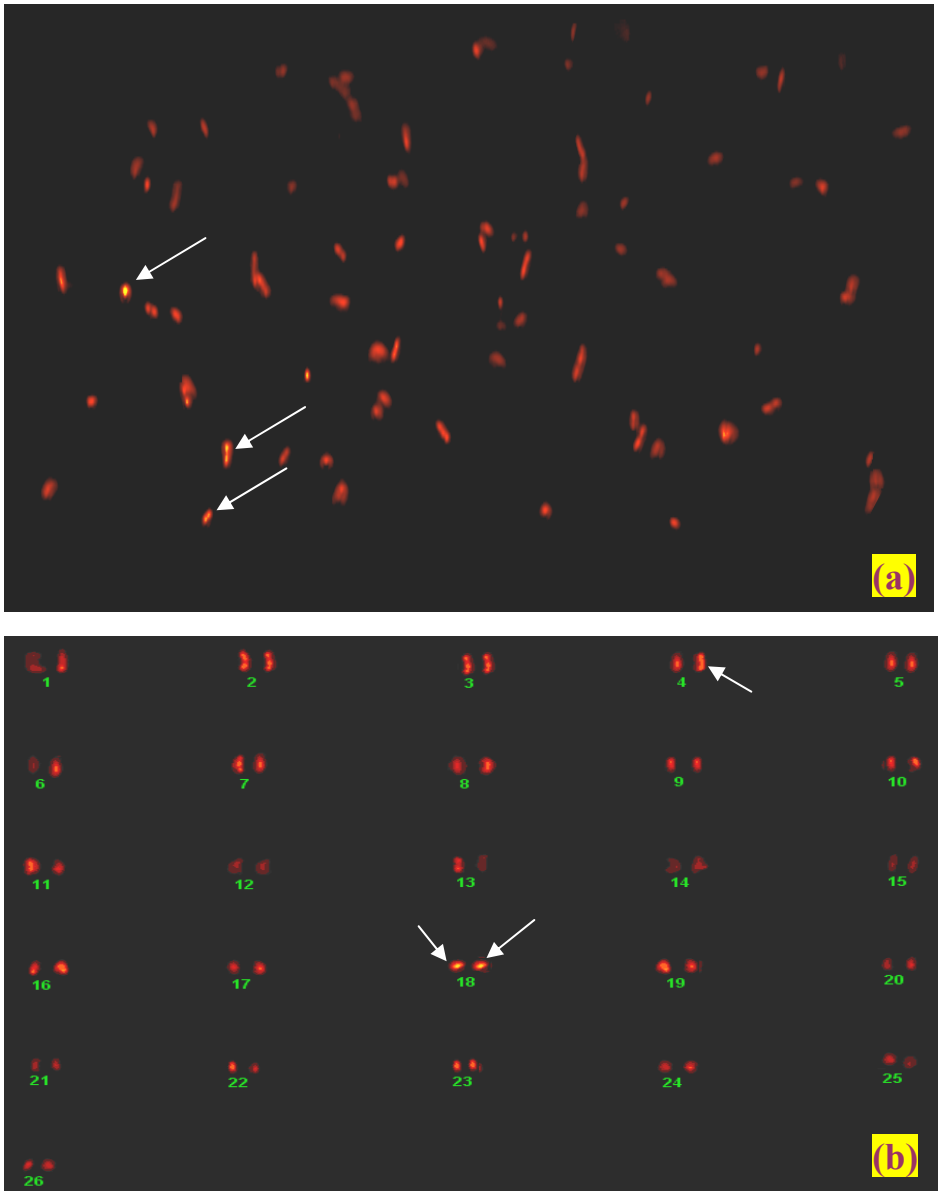


**Figure 5. Fluorescence *in situ* hybridization (FISH) analysis of Bt gene in cotton. (A): FISH showing *Bt* gene signals on transgenic cotton mitotic chromosomes. (B): Karyotype analysis of transgenic cotton showing position of the Bt gene on mitotic chromosome.**

## Discussion

Alien genes in transgenic plants when transferred by any suitable procedure always result in partial integration and rearrangements (Jakowitsch et al., 1999). Their site of insertion is more important for a researcher to study the "position effect" of the alien gene in transgenic plant. Many scientists observed phenotypic variations in transgenic plants (Sachuh et al., 1993; Bao et al., 1996; Jiang et al., 2000; Shu et al., 2002; Bashir et al., 2005; Mahmood-ur-Rahman et al., 2007). Possible reasons for these phenotypic variations may be somaclonal variation (Larkin and Scowcroft, 1981), breakdown of plant genes caused by transgene insertion or insertion mutagenesis (Van et al., 1991), pleiotropy or transgene induced endogenous silencing (Matzke et al., 2000), and position effect (Bhattacharyya et al., 1994) of transgene. In this study the

integration sites of transgene were directly identified in rice and cotton in rapid and efficient way as compared to available protocols (Fukui et al., 1987; Jiang et al., 1995; Jin et al., 2002). We used fluorescein labeled probes instead of DIG-labeled or that of other methods available. It is direct, fast and efficient procedure of detection and lengthy methods for detection may be avoided. So, we report it as a fast method as compared to already published ones.

Copies of *Bt* genes were found interspersed in different regions of chromosomes or on different chromosomes. The results were consistent with those of other researchers, whose genetic mapping has indicated that the insertion sites are widely dispersed in plant chromosomes (Deroles et al., 1988; Heberle-Baors et al., 1988). As reported previously, the frequency of homologous recombination also showed similar variation to that of reported for integration of alien genes from centromeres to chromosome ends. This indicates that the process of alien gene integration resembles that of homologous recombination, even though transgene may be non-homologous to genomic DNA. Therefore, it could be concluded that transgene recognition sites occur all along chromosomes causing variations in morphology of plants.

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