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Variation in Expression of *Phytochrome B* Gene in Cotton (*Gossypium hirsutum* L.)

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ABSTRACT

Transgene integration and expression in host plant is quite unpredictable and is considered as the major problem in plant transformation. The variation in transgene copy number in transgenic plants influences the expression level and is one of such complication. In many plant species, the analysis of transgenic plants has shown that independent transgenic plants have one to many copies of transgenes. This study focused on molecular characterization of difference in copy number of transgenes and its impact on expression level on mRNA basis. Four advanced transgenic lines of *phytochrome B* were analyzed for the integration of the gene. These transgenic lines were taken out on the basis of difference in copy number as determined by Southern blot analysis and Fluorescence *in situ* hybridization (FISH) for transgene expression. Results taken by both real time PCR and Northern blot analysis determined high expression in Line QCC11 having two copies of transgenes in homozygous condition while the least expression was seen in lines QCC10 showing three copy number in heterozygous condition as multiple copies can be incorporated from one to few insertion sites.

Keywords: Chromosome, Copy number, Transgenic, Plant, Transformation.

INTRODUCTION

The primary photoreceptors involved in the regulation of red/far-red light induction are called phytochromes. Among phytochromes, *phytochrome B* has different domains which are involved in controlling different physiological responses depending upon light conditions (Usami *et al*., 2007). *Phytochrome B* controls the response regulator 4 in *Arabidopsis thaliana,* which specifically interacts with the extreme amino-terminus of the photoreceptor (Rao *et al*., 2011b).

Expression of the transgene in plants is variable, even among the plants that are independent events to each other. Also, it cannot be assured that primary transformants, which are performing best of their expression, will give rise to progeny with similar characteristics. Stable and high expression of gene in transgenic plants is the main goal of crop improvement programs based on genetic engineering. Transgenic expression can vary due to many factors including the proportion of exogenous DNA which shows reshuffling prior to integration, position effects, transgenic copy number, and DNA methylation (Meyer, 1998). It is, therefore, necessary to know much about integration, stability, structure, and organizational effect of the expression of transgene in crop plants.

The gene transformation methods commonly in use are *Agrobacterium*- and repeated particle bombardment–mediated transformation that result in variable copies of the transgene at the same or different integration sites (Srivastava *et al*., 1999; Kohli *et al*., 1998). Manifold copies of the transgene

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are the main cause of silencing in transgenic plants (Vaucheret *et al*., 1998; Iyer *et al*., 2000; Flavell, 1994). Southern blot analysis is usually used to find out the transgene copy number. While regular and consistent, the method is time consuming, lengthy, and requires greater quantities of DNA. Since, Fluorescence in situ hybridization (FISH) has some advantages over Southern hybridization analysis in measuring copy number, a firm conclusion as to the number of repeats could be derived from future FISH analysis on the whole rDNA region. (Tsuchiya et al., 2001).

The present study was carried out to investigate the variation in PhyB transgene expression in cotton. The primary objective was to (1) determine the copy number of the PhyB transgene, (2) study the variation in expression of PhyB transgene, and (3) study the correlation between the copy number of transgene and its expression.

MATERIAL AND METHODS

Plant Materials

Cotton variety CIM - 482 was transformed through *the Agrobacterium shoot apex method* as described by Rao *et al*. (2011b) with some modifications. *Phy B* gene was transformed into mature embryos of CIM - 482 cotton variety by using LBA 4404 strain as Shown in Figure 1. Four advanced transgenic lines having *Phy B* gene were selected for this study.

Southern Blot Analysis

Southern blot analysis was performed as described by Southern (1975) by extracting genomic DNA from apical leaves of four advanced lines of transgenic cotton along with untransformed control plants. DNA was extracted by CTAB method. Genomic DNA (20µg) was digested with *Kpn1* for *Phy B* according to the supplier's instructions (Enzyme production lab CEMB) to separate the gene construct from the cotton genome (Figure 1). *Phy B* was cut from the unique site by *Kpn1* enzyme. The color was detected by BCIP/NBT Tablets (Sigma B5655) dissolved in water according to the instructions provided by manufacturer.

Fluorescence *In Situ* Hybridization (FISH)

Fluorescence in situ hybridization (FISH) of transgenic plants was done according to the procedure described by Mahmood-ur-

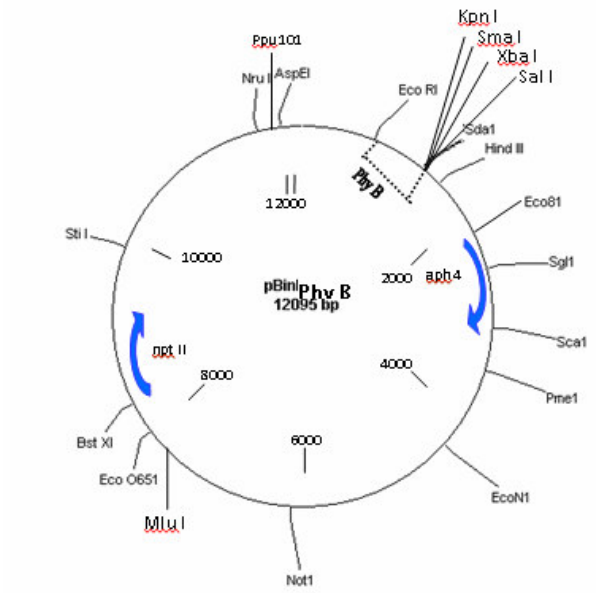

Figure 1. A full map of construct *pBinPhyB* with restriction sites.

Rahman *et al*. (2010).

Total RNA Extraction

Total RNA extraction from cotton leaves was performed as described by Jaakola *et al*. (2001) for four advanced transgenic lines along with un-transformed control plant. After extraction, the pellets were dried and re-suspended in water and the volume was adjusted according to the size of the pellet and stored at -20°C and DNAse treatment was done to remove the contamination.

Northern Blotting

Northern analysis was performed in preparation of poly (A+) RNA from cotton leaves as described by (Heyer and Gatz, 1992a). 1.2% formaldehyde gel was prepared and 15 µg RNA samples of both control and Phy B plants were loaded on the gel in each well. The gel was run overnight at 15V. The gel was transferred to nitrocellulose membrane (Hybond N). BCIP/NBT was used as substrate for color detection.

mRNA Quantification by Real Time PCR

Complementary DNA Preparation

Complementary DNA (cDNA) was synthesized by using Fermentas cDNA synthesis kit. cDNA was synthesized by using 1 µg of DNase treated RNA, 1 µl of 20 µM anchored oligo dt primers and 12 µl DEPC-treated water was used. Later, the reaction mixture was incubated at 70ºC for five minutes, then, quickly chilled by placing on ice. Four µl of 5X reaction buffer, 1 µl of ribonuclease inhibitor (20 u ul^{-1}) and 2 µl of 10mM dNTP mix was added and incubated at 37 ºC for 5 minutes. Finally, 1 µl of H minus M-MuLV reverse transcriptase (200 u μl^{-1}) was added and the mixture was incubated at 42ºC for 60 minutes. Heating was done at 70ºC for 10 minutes to stop the reaction.

Quantitative RT–PCR

Primers used for RT-PCR was as follows Forward Primer: 5'*CTCCTGGCTGAGTTTCTGCT*3' Reverse Primer: 5'*GCTTGTCCACCTGCTGCTAT*3' Real-time PCR reactions were carried out in an iQ5 cycler (Bio-Rad) with a 96-well plate (Bio-Rad) using the IQTM SYBR Green Super mix (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) having oligos sequence Forward Primer 5'*AGGAAGAGCTGCTTCGTTCA*3' Reverse Primer 5'*CCGCCTTAATAGCAGCTTTG*3'was used as an internal control to normalize the data. 50 ng of cDNA was used in each reaction. The reaction conditions were as follows: initial denaturation was done as 95°C for 3 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds and final elongation step at 72°C for 10 minutes. A melting curve analysis was approved out by constantly monitoring fluorescence between 60 and 95°C with 0.5°C increments every 30 seconds. Statistical analysis of the real-time results was performed using iQ5 software (Bio-Rad) version 1.0 on the basis of CT values of the gene in different samples of cotton converted to their linear form normalized with GAPDH gene. Analysis of variance (ANOVA) was performed to analyze significant differences in transcript expression in leaves of the control and transgenic plants (Figure 2).

Null hypothesis and alternative hypothesis were adopted as follows to check the significance of the results:

Null Hypothesis (H0): There is no significant difference in *PHY–B* gene expression in all experimental plants.

Alternative Hypothesis (H1): There is significant difference in *PHY–B* gene expression in all experimental plants.

RESULTS

Detection and Determination of Copy Number of *Phytochrome B* Gene in Cotton Plants

The insertion of Arabidopsis *Phy B* gene in cotton plants was detected by PCR. The 380bp fragment was amplified with internal gene specific primers. A total of four advanced lines, namely, QCC2, QCC7, QCC10, and QCC11 were detected positive in Putative transgenic cotton plants. No amplification was detected in the negative control (Figure 3). The stable integration of Arabidopsis *Phy B* gene in cotton plant genome and transgene copy number was confirmed by Southern blot analysis. Copy number of *Phy B* gene was obtained by a specific probe which highlighted a different copy number based on restriction digestion of genomic DNA with unique sites using *Kpn1*. It is clear from the Figure 4 that all the plants which were PCR positive i.e. QCC2, QCC7, QCC10, and QCC11 showed a different copy number. Moreover, in transgenic lines QCC7 and QCC11, two copies of the *Phy B* transgene were detected, while QCC2 showed less than two and QCC10 showed more than two copies of the transgene.

Location of *Phytochrome B* Gene in Cotton

All *phytochrome B* transformed plants which had already shown signal in Southern blot analysis were subjected to FISH to determine the transgene copy number. FISH was carried out at different stages of cell divisions (prophase, interphase, and telophase). All *phytochrome B* plants showed signal in the nucleus, but no signal was observed in the control plants. The copy number of the *Phy B* transgene was assorted in different transgenic plant lines. In transgenic line QCC2, the cells were analyzed at interphase showing one copy of the transgene (Figure 5-A). In transgenic lines QCC7 and QCC11, two copies of the *Phy B* transgene were detected at late telophase stage (Figures 5-B and -C). These lines

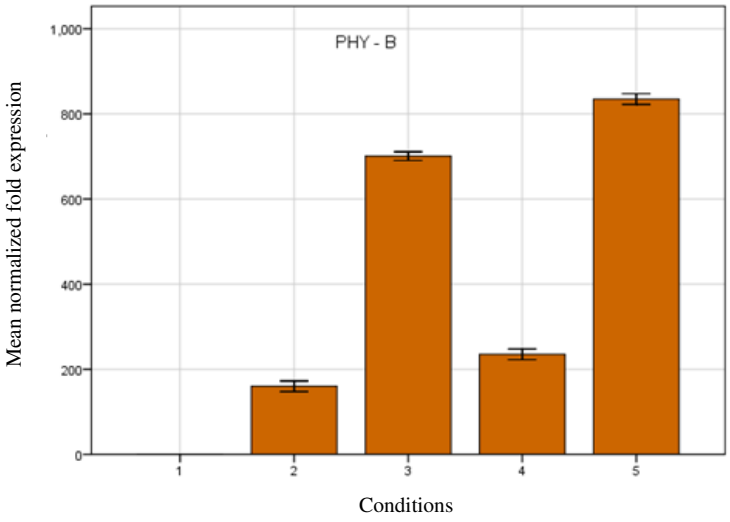

Figure 2. Expression of *Phy B* gene in different transgenic lines: Lane 1 shows the negative control. Lane 2 shows Line QCC 2, Lane 3 shows Line QCC 7, Lane 4 shows line QCC 10 and Lane 5 shows QCC 11 transgenic lines.

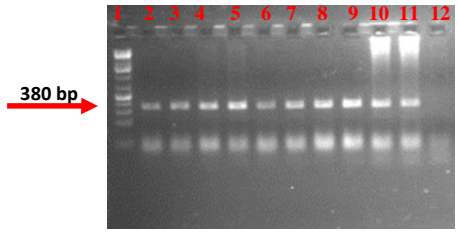

Figure 3. PCR detection of *Phy B* transgenic lines. Here, Lane 1 shows 1 Kb Plus marker. Lanes 2-3 show Line QCC2; Lanes 4-5 represent Line QCC7; Lanes 6-7 show Line QCC10; Lanes 8-9 depict Line QCC11. Whereas Lanes 10-11 is the positive control, Lane 12 is un-transformed plant DNA as the negative control.

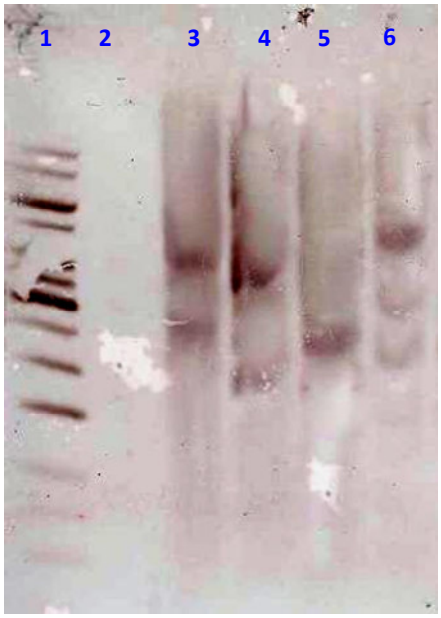

Figure 4. Copy number of *Phy B* gene in different lines by Southern blot. Lane 1 shows 1 Kb DNA marker; Lane 2 depicts Non Transgenic Plant DNA as the Negative Control; Lane 3 shows QCC7; Lane 4 contains QCC11; Lane 5 represents QCC2 , and Lane 6 contains QCC10.

showed the homozygosity for the transgene. QCC10 showed more than two copies of the transgene at late telophase stage when subjected to FISH analysis. One daughter cell had one copy while the second cell contained three copies of the transgene. It showed that line QCC10 was heterozygous for the transgene (Figure 5-D). However, the control plants (CIM-482 variety) did not show any signals when subjected to FISH analysis (Figure 5-E).

Detection of *Phytochrome B* Gene Transcripts

Northern Blotting

Northern blot analyses of Phy B transformed plants and non-transformed cotton plants were conducted to check *Phy B* mRNA expression. The PCR amplified DIG labeled fragment of the *Phy B* gene was used as Probe. The *Kpn1* digested gel eluted 3.8 kb fragment of *Phy B* was used as the positive control and non transformed cotton plant was used as the negative control. In Figure 6, lane one is the negative control and lane 6 determines the positive control, while all the other lanes are Phy B plants mRNA. It is clear from Figure 6 that the expression of *Phy B* mRNA in QCC11 is much higher as compared to the other Phy B plants.

Quantification of *Phytochrome B* Gene RNA in Transgenic Cotton Plants

Quantitative real time PCR was used to check the expression levels of *Phy B* in cotton leaf samples of four transgenic lines. GAPDH gene was used as the indication gene to normalize the expression levels. Figure 2 shows that all the lines expressed different levels of *Phy B* mRNA expression but the plant lines QCC7 and QCC11 showed much higher levels of expression of *Phy B* as compared to other lines of Phy B plants. The figure also determines the lowest expression of *Phy B* in lines QCC10, which has three copies of transgene.

Statistical Results

On way ANOVA was performed with the help of SPSS version 16.0. According to the results, the *p*-value was < 0.00 1, indicating statistically significant differences (Table 1). As *p*-value was < 0.001, Null hypothesis (H0) was rejected in favor of Alternative hypothesis (H1).

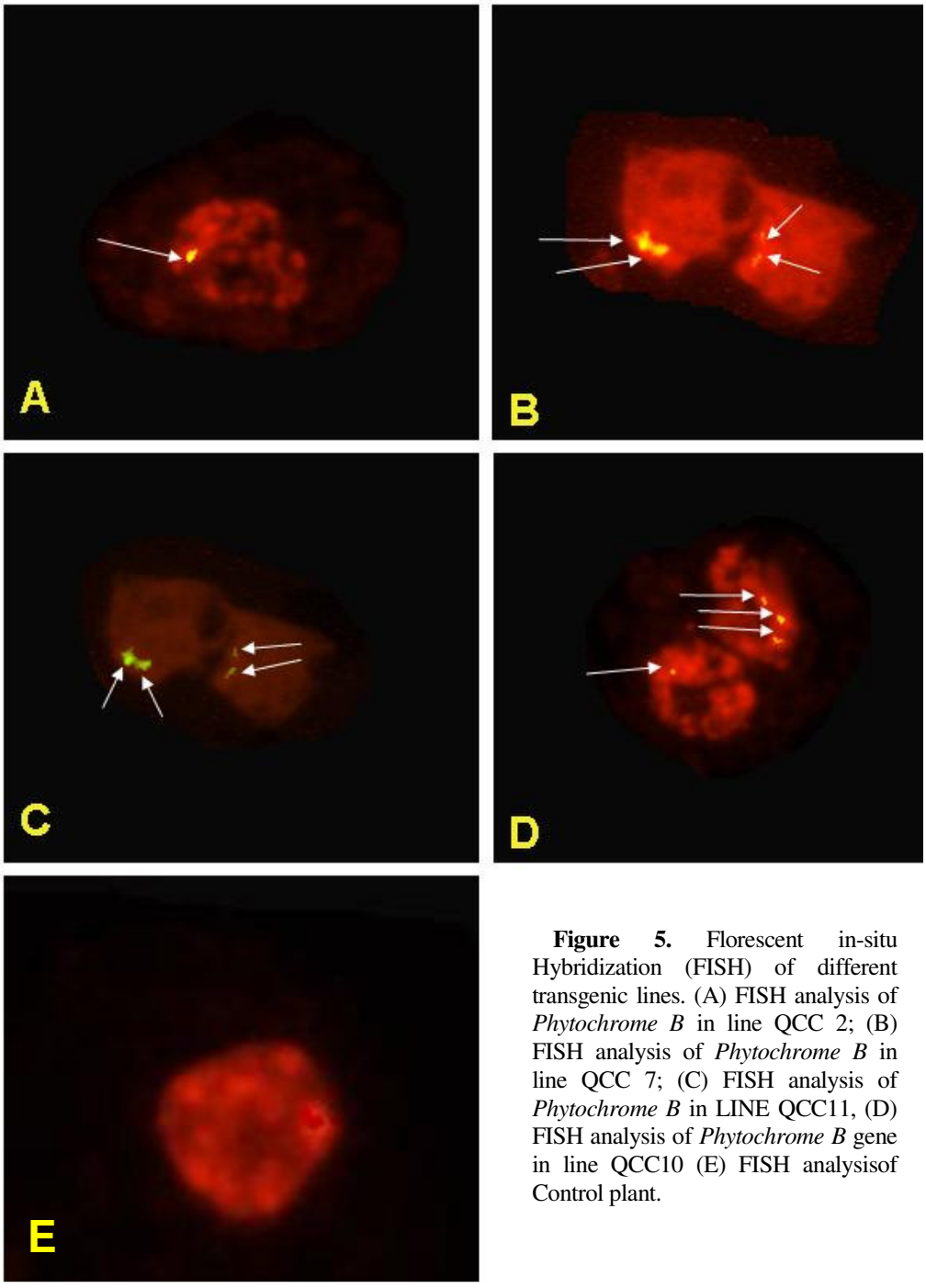

Figure 5. Florescent in-situ Hybridization (FISH) of different transgenic lines. (A) FISH analysis of *Phytochrome B* in line QCC 2; (B) FISH analysis of *Phytochrome B* in line QCC 7; (C) FISH analysis of *Phytochrome B* in LINE QCC11, (D) FISH analysis of *Phytochrome B* gene in line QCC10 (E) FISH analysisof Control plant.

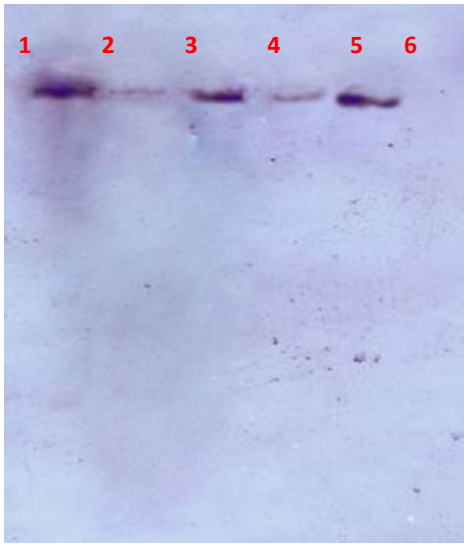

Figure 6. Northern Blot Analysis of Phy B transgenic plants. Lane 1 shows the positive control. Lane 2, 3, 4 and 5 show, QCC 2, QCC 7, QCC 10 & QCC 11, respectively. Lane 6 shows the negative control.

DISCUSSION

Over the last few years, however, there have been a number of detailed studies involving combined molecular and cytogenetic analysis. These studies have provided more insight into the role of transgene copy number in gene expression, in particular, the use of Southern blot hybridization in combination with fluorescence in situ hybridization (FISH), methods that discriminate higher order transgene organization in the context of nuclear architecture.

Southern blot hybridization is a common technique used in many aspects of genome analysis (Southern, 1975; Sambrook *et al*., 1989). In this study, Phy B transgenic plants were analyzed by Southern blot hybridization and the results indicated the integration of the vector construct *pBinPhyB* in plant genome and variation in copy number of the gene (Rao *et al*., 2011b).

Northern analysis provides the way for the evaluation of message abundance between plant samples. The Northern blotting procedure is clear-cut and provides opportunities to assess progress at various points. Plants QCC2, QCC7, QCC10, and QCC11 were analyzed for their mRNA expression along with the negative control by Northern blot analysis (Heyer and Gatz, 1992a). It is also clear from results of Northern blot analysis (Figure 6) that expression level of mRNA in QCC11, which is lane 4, is much higher as compared with other Phy B plants (Rao *et al*., 2011a). This may be due to the reason that lane QCC11 has two copies of transgenes in the homozygous condition. These results are comparable with the results of Warren (2007) in which he determined the variation in expression level of cells.

The results of qRT real time PCR have revealed that the QCC11 exhibit high level of mRNA expression as compared to other plants, whose mRNA expression is quite low. The results clearly showed that expression level was variable among different lines of *Phy B*, with the expression of QCC7 and QCC11 being much higher than the other lines (Figure 2).

Dong and Li (2007) obtained the same results in which they determined that the expression level can vary based on different

Table 1. Analysis of variance (ANOVA) showing Significant difference in PHY – B gene expression.

Mean normalized fold expression					
	Sum of Squares	*df*	Mean square	*F*	*Sig.*
Between Groups	1553749.7	4	388437.4	1223.04	P< 0.001*
Within Groups	3176.0	10	317.6		
Total	1556925.7	14			

* *P*-value is < 0.001 is considered statistically significant.

factors such as altering nucleotide sequence of the gene, promoter and insertion point genes in host DNA, copy number of the transgene, and several external as well as internal factors (Rao *et al*., 2011a; Warren, 2007; Rao, 2005).

The transformation of the transgene into a recipient genome comprises a series of complicated events which depends on the transgene itself and the host genome. The transgene expression level may vary extremely, depending on a number of factors (Kohli *et al*., 1999; Yin *et al*, 2005) in which the copy number of transgene plays a major role.

Transformation through particle bombardment results in multiple copies of the gene being incorporated into the plant genome. If the gene copy number is low, the transgene integration in plant will be stable (Jones, 2005). Shakiness of transgene expression in plants is connected with multiple copies of transgenes being integrated at the same locus.

Yet, the unpredictability in gene expression of transgene plants carrying the same copy number has appeared too large to show any insulating effect of the matrix attachment regions (MAR) sequence (Allen *et al*., 1993). Therefore, countless copies of the same DNA sequence would be troublesome to determine the relative involvement of each gene to the total level of gene expression. The change in copy number may result from change in plant material, cultivation environment, the type of *Agrobacterium,* and selection protocol (Heberle-Bors *et al*., 1988). The differences in copy number distribution would thus seem to be important factors in plant transformation strategies in case of integration and subsequent expression of the transgene.

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تغییرات بیان (تظاهر) ژن فیتوکروم B در پنبه(*Gossypium hirsutum* L.)

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چکیده

ادغام ژن تراریخته و بیان آن در گیاه میزبان بسیار غیر قابل پیش بینی است و این امر از مسایل عمده تراریختی (تغییر شکل) گیاه است. تغییرات در تعداد نسخه ژن تراریخته در گیاه تراریخته بر سطح بیان یا تظاهر ژن اثر میگذارد و یکی از مشکلات و مسایل مزبور می باشد. در بسیاری از گونه های گیاهی، تحلیل گیاهان تراریخته نشان داده است که گیاهان تراریخته مستقل یک تا چندین نسخه از ژن تراریخته دارند. هدف مطالعه حاضر تشخیص و شناسایی ملکولی تفاوت در تعداد نسخه ژن های تراریخته و تاثیر آن بر سطح بیان بر مبنای آر.ن. ای پیک mRNA بود.تعداد چهار لاین (رگه)تراریخته پیشرفته فیتوکروم B از نظر ادغام ژنی مورد تحلیل قرار داده شد. این لاین های(رگه های) تراریخته بر مبنای تفاوت در تعداد نسخه (copy number) به روش پایه لکه گذاری ساترنی و دورگه سازی درجای فلور سنتی(FISH) برای بیان تراریختگی برداشت شده بودند. نتایج به دست آمده از اندازه گیری زمان واقعی پی سی آر و روش لکه گذاری نورترن نشانگر بیان زیاد (high expression) در لاین QCC 11بود که دارای دو نسخه ژن تراریخته در شرایط خالص بود در حالی که کمترین بیان در لاین QCC10 مشاهده شد که سه تعداد نسخه در شرایط خالص را نشان می داد، همانا که چندین نسخه را می توان از یک یا چند جایگاه رخنه(جایگاه درج) معدود وارد کرد.