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PHYSICAL MAPPING AND INHERITANCE STUDIES OF *CRY1AC* GENE ON TRANSGENIC COTTON CHROMOSOMES

S. Noreen[1], Mahmood-ur-Rahman[2*] and T. Husanin[3]

[1]Department of Chemistry, University of Sargodha, Main Campus, Sargodha, Pakistan
[2]Department of Bioinformatics and Biotechnology, GC University – Faisalabad, Allama Iqbal Road, Faisalabad-38000, Pakistan
[3]National Centre of Excellence in Molecular Biology, 87-West Canal Bank Road, Lahore-53700, Pakistan
[*]Corresponding Author's E-mail: mahmoodansari@gcuf.edu.pk

ABSTRACT

The presence of Bt gene, *cry1Ac* in transgenic lines of *Gossypium hirsutum* L. was determined by molecular and cytogenetic analysis. Fluorescence *in situ* hybridization (FISH) was carried out to study the location of transgene on chromosomes. Metaphase chromosomes prepared from root tips and pollen grains showed more condensation, and hence more karyotypically differentiated. The five transgenic lines of cotton showed stable integration of transgene, *cry1Ac*. Either the lines were heterozygous (CEMB-3010-14, CEMB-3013-10, CEMB-3016-13 and CEMB-3037-2) or homozygous (CEMB-3034-10) showing single and double genes respectively at interphase and metaphase FISH while the control lines showed no fluorescent signal for transgene at all. Metaphase chromosomes prepared from pollens were also analyzed and single copy of *cry1Ac* was mapped on chromosome 10 which showed the stable inheritance of transgene in the progeny. The information obtained during study may be helpful to understand the possible effects of copy number variation and position effect of transgene in Bt cotton. The study may also help to comprehend the process of transgene inheritance in Bt cotton lines.

Keywords: Karyotyping, Fluorescence *in situ* hybridization, transgene, *cry1Ac*, physical mapping
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INTRODUCTION

Cotton (*Gossypium sp.)* is considered as an important raw material for textile industry. It is a natural fiber being used in many products ranging from clothing to home furnishings. Cotton is not only used as a source of raw material to the local textile industry but also is known as the main export item (Sabah *et al.,* 2015). There are 4 domesticated species of cotton. *Gossypium arboreum* and *G. herbaceum* are diploids (2n=26) and inhabitant to the Old World, which are primarily grown-up in India. *G. barbadense* and *G. hirsutum* are New World cotton species, and are allelo-tetraploids (2n = 4x = 52) (Poehlman and Sleper, 1995). Diploid cotton species are considered as the reservoirs of the disease and pest resistant genes and have enhanced fiber and agronomic traits. So, they could be used to study the gene structures and functions by using the advanced techniques called gene knockouts (Sakhanokho *et al.,* 2004).

Genetic modification of crop plants has become very popular. However, the inability of the researchers to determine where a transgene will be incorporated followed by selection of putative transgenic plants with stable integration is a major challenge. To study the integration of alien gene into the host genome, lots of efforts were made (Somers and Makarevistch, 2004). Since predictable transgene integration and expression is the main objective of genetic transformation, it is greatly desired to study behavior of gene during interphase and metaphase stage. It is anticipated that localizing foreign genes on metaphase chromosomes could be useful to study transcriptional efficiency, and the information could be used to predict the behavior of alien genes.

Usually, PCR is used to detect transgenes in genetically modified plants followed by Southern blotting to determine copy number; however, very recently, FISH is being used to identify both presence and copy number of transgenes in the host plant genome (Liu *et al.,* 2020). The procedure has been used in rice, barley and wheat (Anand *et al.,* 2003) to determine copy number and chromosomal location of alien genes (Perdersen *et al.,* 1997). Several transgenes have been reported by using FISH including *Xa21* or *GUS* (Kharb *et al.,* 2001), *GFP* (Chen *et al.,* 2003), chitinase and *β-1,3-glucanase* target genes (Anand *et al.,* 2003) and *GUS* and *bar* genes (Liu *et al.,* 2020). Gene expression in transformed plants is strongly dependent on the integrity and organization of the transgenic locus. Visualization of foreign genes by FISH (fluorescence *in situ* hybridization) on metaphase chromosomes is very helpful in this regard (Abranches *et al.,* 2000; Travella *et al.,* 2005; Mahmood-ur-Rahman *et al.,* 2010 and 2012). Transgenes within a genome of plants and mammalian species were physically located by using the direct method called FISH (Jiang and Bill, 1994; Leggett *et. al.,* 2000; Mahmood-ur-Rahman *et al.,* 2010

and 2014). It is known as the method of detection and localization of the specific DNA sequences in cell and morphologically preserved tissues by hybridization of the complimentary nucleotide probe to the gene of interest (Mahmood-ur-Rahman *et al.,* 2014).

FISH is the most direct method to physically locate transgenes within the genome (Jiang and Bill, 1994; Leggett *et. al.,* 2000; Mahmood-ur-Rahman *et al.,* 2010 and 2014) of plants as well as in mammalian species. Visualizing or mapping of transgenes is important to determine their actual location on the chromosomes and their genetic analysis (Latif *et al.,* 2015; Ali *et al.,* 2016). FISH is an efficient method to construct physical maps of the genes regardless of the plant species. In this study, the *cry1Ac* gene was localized on the metaphase chromosomes by FISH in transgenic cotton lines.

MATERIALS AND METHODS

Plant Material: Seeds of *G. hirsutum*, control and transgenic lines CEMB-3034-10, CEMB-3037-2, CEMB-3016-13, CEMB-3013-10 and CEMB-3010-14 containing *cry1A*c gene were acquired from National Centre of Excellence in Molecular Biology, Lahore, Pakistan. Firstly, seeds were germinated in Petri-dish on moist filter paper and then seedlings were transferred to pots in greenhouse.

Isolation of Genomic DNA and confirmation of presence of *cry1Ac* gene: DNA from non-transgenic (control) and transgenic plants was isolated according to Dellaporta *et al.,* (1983) and PCR analysis was performed by using the sequence specific primers for *cry1Ac* gene. The specific primers were; 5'-ACAGAAGACCCTTCAATATC- 3' (forward) and 3'-GTTACCGAGTGAAGATGTAA-5' (reverse) for amplification of 565 bp fragment of the gene. The DNA was used as a template for PCR analysis following the conditions described by Mahmood-ur-Rahman *et al.,* (2012).

Fluorescence in situ hybridization of cotton chromosomes: Transgene was physically mapped on the chromosomes of Bt cotton by using Fluorescence *in situ* Hybridization (FISH) following the method described by Mahmood-ur-Rahman *et al.,* (2010).

Fluorescent signal detection: Fluorescent microscope (Olympus, BX61) was used to detect the fluorescent signals. CCD camera attached to the microscope was used to take the pictures of fluorescence signal and they were enlarged and analyzed by using the software Genus 3.7 provided by the Cytovision Applied Imaging Systems, USA.

Determination of transgene copy number and location: Copy number and location of gene was determined by direct visualization of fluorescent signals on the transgenic cotton metaphase chromosomal spread. Computer software Genus 3.7 (Cytovision Applied Imaging Systems) was used as the image analysis tool for the captured images and karyotype analysis.

RESULTS AND DISCUSSION

Confirmation of *cry1Ac* gene in cotton: PCR analysis of transgenic lines was performed to confirm the stable integration of transgene. Amplification of *cry1Ac* gene showed the integration of Bt gene in host genome. PCR was carried out with *cry1Ac* specific primers. The primers amplified 565bp internal fragment of the *cry1Ac* gene. DNA of the control plants was used as a negative control which showed no amplification while plasmid DNA containing transgene was used as a positive control (Figure-1). Genomic DNA obtained from all lines of transgenic plants CEMB3034-10, CEMB3037-2, CEMB3016-13, CEMB3013-10 and CEMB3010-14 gave clear amplified bands of 565 bp for *cry1Ac* gene (Figure-1). Alien genes are believed to transform randomly into the host genome. It is considered as a major source of genetic variation due to location as well as position of the transgene. At the same time, it is a major limitation in technology for plant transformation. So, it is important to choose the putative transgenic lines having desired traits at an early growth stage by prediction of transgene location and copy number. It is technically more difficult due to presence of repetitive DNA which is some time more than 90% (Heslop-Harrison, 2000).

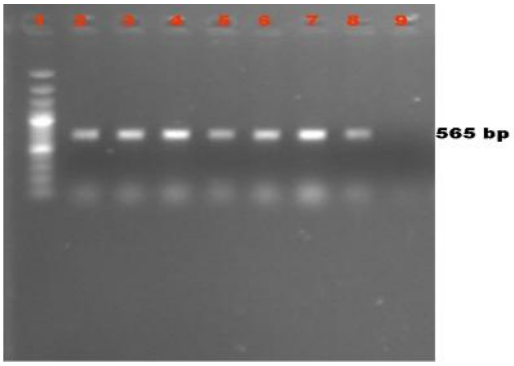

Figure-1: PCR analysis of transgenic and control plants revealing the presence of *cry1Ac* gene in transgenic plants. Lane 1: 100 bp DNA Ladder Marker, Lane 2-6: Samples from transgenic line, Lane 7: Positive Control (confirmed transgenic plant), Lane 8: Plasmid DNA and Lane 9: Negative Control

Mitotic chromosome preparation: Mitotic chromosomes were prepared by using the method described by Abbasi *et al.,* (1999) with little modifications and observed under flourescent microscope (Carl Zeiss,

AXIO 100). Metaphase spread of chromosomes from different transgenic lines and non-transgenic control were prepared. Digital camera attached with the microscope was used to take the pictures which were further analyzed. The metaphase chromosomes from root tips and pollen grains showed more condensation and were prominent. So, they were used in FISH analysis (**Figure-2**).

Figure-2: Metaphase chromosome preparation of cotton from meristematic root tissue.

Fluorescence *in situ* hybridization (FISH): FISH is very useful technique to determine the copy number of foreign gene (Santos *et al.,* 2002) by physically mapping them (Abranches *et al.,* 2000; Mahmood-ur-Rahman *et al.,* 2012). Chromatin organization, transgene location and copy number may regulate the gene expression (Sproul *et al.,* 2015) which is very much important when we study the behavior of transgenes. In this case, ribosomal genes are the prominent example in plant genomes (Shaw and Doonan, 2005).

FISH was performed on metaphase chromosomes prepared from root tips and pollen grains. Images were analyzed to determine the mode of distribution of fluorescent signals which were found on the chromosomes of transgenic cotton after *in situ* hybridization. Clearly visible metaphase spreads of hybridized chromosomes were selected and analyzed by karyotyping using Genus 3.7 (Cytovision Applied Imaging Systems). Different transgenic lines showed transgene at different chromosomes and at different chromosomal positions (Table-1). One copy of the *cry1Ac* gene was present on the chromosome number 5 in CEMB3010-14. The line showed heterozygosity for the transgene (Figure-3A & Figure-4A). CEMB3013-10 contained three copies of the cry*1Ac* gene on chromosome number 1, 2 and 22. The line showed heterozygosity for the transgene (Figure-3B & Figure-4B). CEMB3016-13 contained two copies of the *cry1Ac* gene on chromosome number 2 and 3. The line showed heterozygosity for the transgene (Figure-3C & Figure-4C). In line CEMB3034-10, two copies of transgene were present at chromosome number 18. The line showed homozygosity for the transgene on chromosome number 18 (Figure-3D & Figure-4D). CEMB3037-2 had three copies of the *cry1Ac* gene on chromosome number 2, 10 and 11 and was heterozygous for the transgene (Figure-3E & Figure-4E). While control plants did not show any signal of transgenes. However, only one copy of the *cry1Ac* gene was present on the chromosome number 10 in pollen grains of the transgenic plant. The line showed heterozygosity for the transgene on chromosome number 10 (Figure-5). FISH has become the method of choice to determine position and copy number of transgene integrated in the host plant genome. Very recently, several studies have been conducted to detect foreign genes by using FISH e.g. *CRSP-1* and *CRSP-2* (Ali *et al.,* 2016), phytochrome B gene (Rao *et al.,* 2013) and *CpEXPA1* gene (Yaqoob *et al.,* 2020) in cotton.

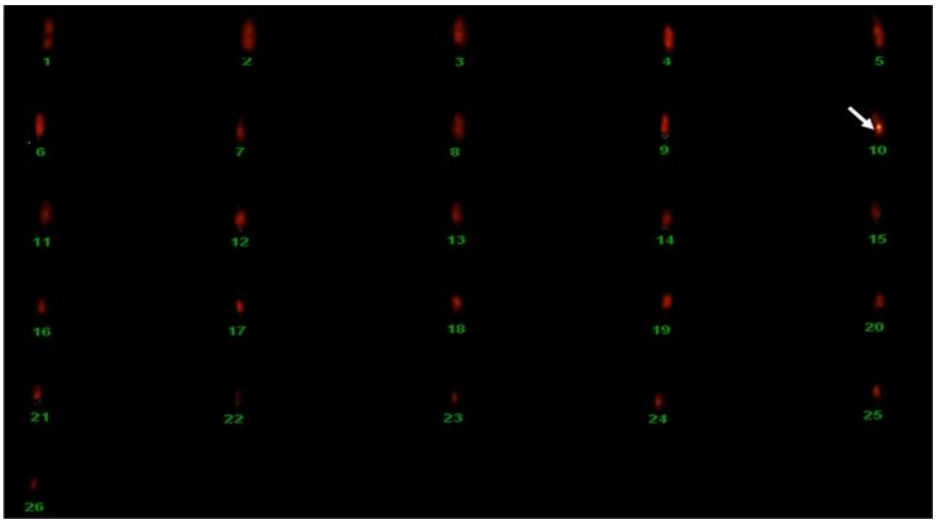

Figure-5: FISH analysis of transgenic pollen to study copy number and position of *cry1Ac* gene.

The insert in this study was equal to 10 kb in size and contained the genes *cry1Ac* and *cry2A* with *CaMV35S* and *ubiquitin* promoters and *nos* terminator. It was investigated in this study that in all five lines (CEMB-

3010-14, CEMB-3013-10, CEMB-3016-13, CEMB-3034-10 and CEMB-3037-2) tested by single color FISH, the signals of *cry1Ac* gene were orange with red background of Propidium Iodide stain. Transgenic cotton lines showed tremendous variations in terms of integration of *cry1Ac* gene on different chromosomes and their copy number (**Table-1; Figure-3 & 4**).

Table-1: Copy number and position of *cry1Ac* gene in genetically modified cotton lines after FISH analysis.

S. No.	Line	Copy Number	Chromosome Number
1.	CEMB-3010-14	1	5
2.	CEMB-3013-10	3	1, 2 and 22
3.	CEMB-3016-13	2	2 and 3
4.	CEMB-3034-10	2	18
5.	CEMB-3037-2	3	2, 10 and 11

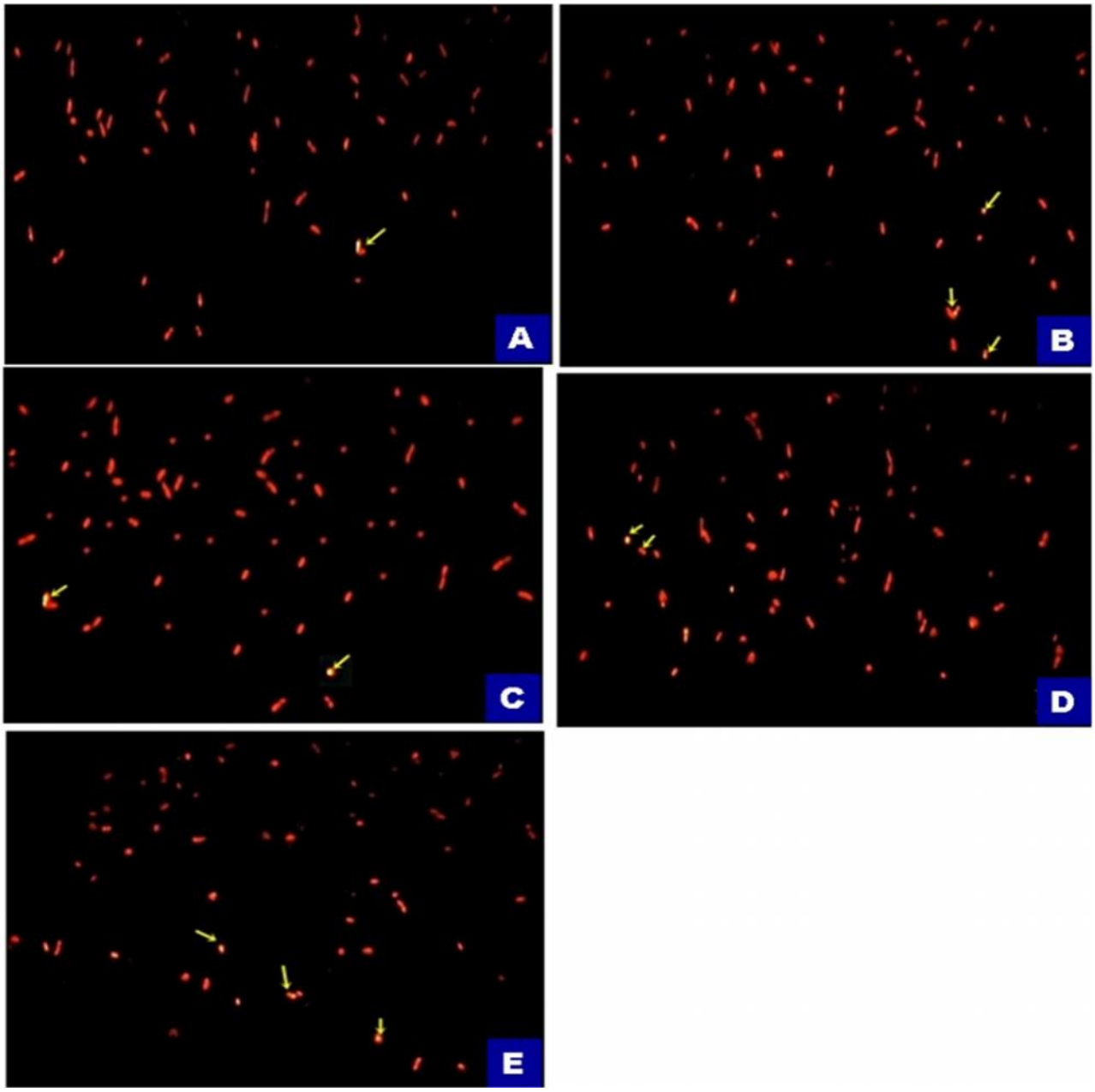

Figure-3: FISH analysis of transgenic cotton lines to determine copy number of *cry1Ac* gene. FISH analysis of A: CEMB-3010-14-line, B: CEMB-3013-10-line, C: CEMB-3016-13-line, D: CEMB-3034-10 line and E1: CEMB-3037-2 line.

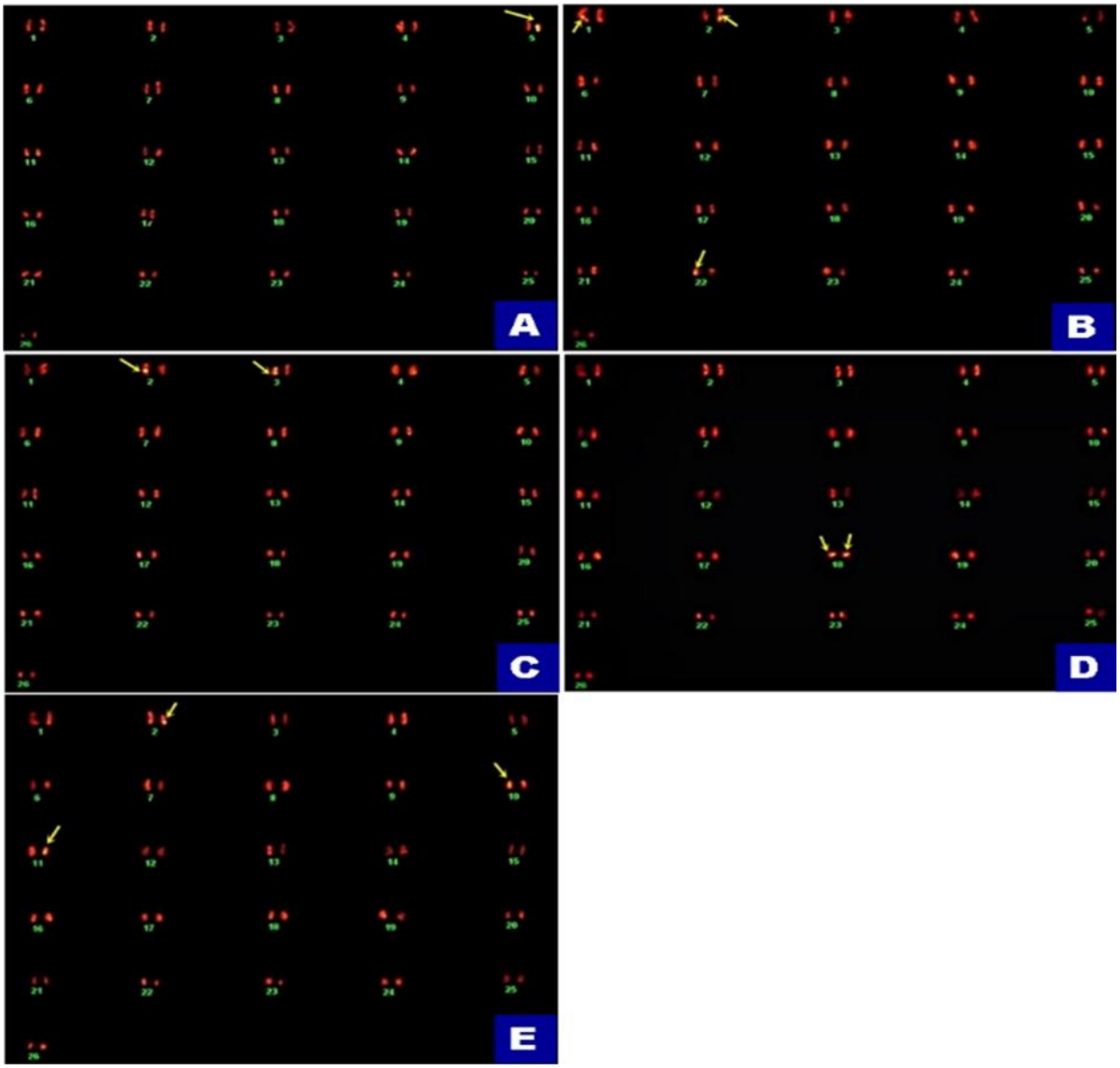

Figure-4: Karyotype analysis of transgenic cotton lines to determine copy number and position of *cry1Ac* gene. Karyotype analysis of A: CEMB-3010-14-line, B: CEMB-3013-10-line, C: CEMB-3016-13-line, D: CEMB-3034-10 line and E: CEMB-3037-2 line.

The variation in transgene position and copy number may be the source of gene expression variability in the transgenic lines (Mahmood-ur-Rahman *et al.,* 2012). FISH is an important tool which is used for the physically mapping of genes on chromosomes in mammalian species and humans. However, the applications of the FISH technique have been restricted in plant species (Jiang *et al.,* 1995). We compared the information obtained from metaphase FISH (Mahmood-ur-Rahman *et al.,* 2012; Latif *et al.,* 2015; Ali *et al.,* 2016) to better explain the phenomenon of position effect and effect of transgene copy number. Our results are very similar to already published data (Rao *et al.,* 2013; Ali *et al.,* 2016; Yaqoob *et al.,* 2020) and confirm the idea of copy number variation. There are lots of techniques which are developed to visualize the single copy of foreign DNA in plant species. The practical applications of our studies have applied perspective as well as increase in basic knowledge.

The information can determine the stability of transgene expression. It may also add to basic information of the relationship between chromosome behavior and gene expression.

Conclusion: Transgenic plants are gaining popularity due to their various benefits. However, it is very important to understand the behavior of transgene after transformation experiments. The localization and copy number of gene is very crucial for its expression. We discussed the position and copy number of insect resistant gene in locally developed transgenic cotton. Five advanced transgenic lines were analyzed and position of transgene was studied on different chromosomes. Different transgenic cotton lines were also found to have the single or multiple copies of transgene. Transgene was transferred from one generation to the next by following Mendelian inheritance pattern.

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