VALIDATION AND EVENT CHARACTERIZATION OF *Cry* 1Ac UASD Bt COTTON EVENT NO. 78

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CERTIFICATE

This is to certify that the thesis entitled "**VALIDATION AND EVENT CHARACTERIZATION OF *Cry* 1Ac UASD Bt COTTON EVENT NO. 78**" submitted by **Mr. BASAVANAGOUDA PATIL** for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **GENETICS AND PLANT BREEDING** to the University of Agricultural Sciences, Dharwad is a record of research work done by him during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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DHARWAD
NOVEMBER, 2019 ***(BASAVANAGOUDA PATIL)***

CONTENTS

LIST OF ABBREVIATIONS

Bt	- *Bacillus thuringiensis*
CTAB	- Cetyl trimethyl ammonium bromide
ELISA	- Enzyme-linked immunosorbent assay
GMO	- Genetically Modified Organism
PCR	- Polymerase chain reaction
TAIL	- Thermal Acyclic Interlaced
RBFl	- Right Border Flanking
LBFl	- Left Border Flanking
NRB	- Near to Right Border
ARB	- Away from Right Border
NLB	- Near to Left Border
AD	- Arbitrary Degenerate
BLASTn	- Basic Local Alignment
ABI	- Applied Bio-System
BG-I	- Bollgard I
BG-II	- Bollgard II
bp	- Base pairs
µg/ml	- Microgram per ml
Cry	- *Cry*stal protein
rpm	- Revolutions per minute

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1. INTRODUCTION

Environmental stresses, population detonation and food scarcity have caused serious problems to mankind on the globe. Producing crops with improved quality and quantity is imperative for the growing demands. Genetic engineering along with conventional selection and breeding techniques can help for sustainable agriculture. The future is very promising for GM technologies to meet the increasing global needs for food, feed and fibre sustainably and responsibly.

Presently GM crops are cultivated in Australia, Mexico, China, USA, Argentina, Canada, Colombia, Costa Rica, Mexico, Paraguay and South Africa covering 181.5 million hectares area (Anon 2019). Transgenic cotton resistant to lepidopteran pests are commercially cultivated in Australia, Mexico, China, USA, Argentina and including India. The maximum area under Bt cotton is in USA (72%) followed by South Africa (40%), Australia (30%) and Mexico (25%) (Anon., 2019). The area under herbicide-resistant transgenic cotton is restricted to the USA. In India cotton is the only GM crop cultivated and occupies 99 per cent of cultivated cotton area (Anon., 2019).

Genetic engineering facilitates the transfer of desired characteristics across species not possible through conventional plant breeding. The first transgenic plant was developed in 1983 in tobacco (Fraley *et al.*, 1983) and the first transgenic food available in the market was tomatoes in the US in 1994 (Teisl *et al*., 2003). Later on, in 1996, seven major crops such as soybean, cotton, canola, tomato, potato, maize and squash were used for generating transgenic crops (Xia *et al*., 2010). Thereafter, the world area of transgenic crops grew enormously. Development of transgenic biotechnology has promoted the commercialization of genetically modified crops to a great extent (Ahmad *et al*., 2012).

Cotton is the crop which consumes excessive pesticides and insecticides to manage insect pests. Lepidopteran group of insect pests are the major group of insects which attacks cotton crop. Among the lepidopteran pests, major pests are cotton bollworm (*Helicoverpa* armigera), pink bollworm (*Pectinophera gossypiella*), spotted bollworm (*Earias insulana*) and tobacco caterpillar (*Spodoptera litura*). Transgenic Bt cotton, which induces inbuilt resistance in the host has gained wider adaptability as a means of avoidance of losses due to bollworm because the technology operates at seed level (Anon., 2019). ‘Bt’ refers to ubiquitous soil bacterium, *Bacillus thuringiensis*, a gram-positive and spore-forming parasporal Crystals during the stationary phase of its growth cycle. The synthesized Crystalline proteins called ‘endotoxins' are highly toxic to certain insects while the other beneficial insects are unaffected by these proteins. These proteins are characterized by their insecticidal activity into four classes *i.e.* Lepidoptera-specific (*Cry* I), Lepidoptera and Dipteran-specific (*Cry* II), Coleoptera-specific (*Cry* III) and Dipteran-specific (*Cry* IV). The gene bank database of Bacillus Genetic Stock Centre (BGSC) has a list of *Cry* (*Cry*stal), Cyt (Cytolytic) and Vip genes either synthetic or modified versions from *B. thuringiensis*. About 22 classes of *Cry* including 126 *Cry* genes have been registered along with a *Cry* gene and 3 Vip (Vegetative insecticidal protein) genes (Anon., 2019).

In cotton, the first transgenic plant was developed in 1987 in the U.S.A. by Monsanto, Delta and Pine companies (Benedict *et al.*, 2001). Later on, the research work on the development of transgenics was intensified all over the globe and several transgenic plants were developed. However, bollworm resistant Bt transgenic cotton has spread to several countries. The era of transgenic cotton began in 1990 with the introduction of *Cry* 1A (b) and *Cry* 1A (c) genes into cotton plants and transformed plants showed a high level of resistance to *Helicoverpa*. In the USA, the first product ‘bollgard' was launched in 1996 by the Monsanto company after extensive testing and approval of the US Environmental Protection Agency (EPA), the USDA and US Food and Drug Administration (Anon., 2019)

The work on Bt cotton in India was first initiated in 1994 at CICR, Nagpur with World Bank aided Biotechnology Project. Department of Biotechnology (DBT) approved MAHYCO (Maharashtra Hybrid seed Company), a premier Indian Seed Company in March 1995 to import 100 g. of the Bt cotton variety Coker 312, transformed by Monsanto, U.S.A. which contained Bt gene *Cry* 1Ac for introgression into elite parents of MECH and hybrid development (Benedict *et al.*, 2001,Mayee, C.D *et al.*, 2002). After extensive testing of these Bt hybrids in AICCIP and farmers field, the Government of India approved commercial cultivation of Bt cotton during 2002.

Till now five Bt gene through six events such as *Cry*1Ac through MON531 (Monsanto), truncated *Cry*1Ac through Event-1 (JK Agri Genetics Ltd.); *Cry*1Ac + *Cry*2Ab through MON15985 (Monsanto); *Cry*1Ab + *Cry*1Ac through GFM*Cry*1A (Nath Seeds Ltd.); and *Cry*1C through event 9124 (Metahelix), have been approved. However, based on only Monsanto's *Cry*Ac + *Cry* 2Ab, thousands of hybrids are commercially grown in India.

The widespread cultivation of Bt transgenic cotton based on single event increases the risk development of insect resistance against Bt toxin. The development of events with higher toxin expression, diversification and multiple gene sources, gene pyramiding can help to avoid resistance development and sustainable use of transgenic technology in cotton.

The best-known and closest to commercialisation is the vegetative insecticidal protein (VIP) 3A, which has been engineered into cotton to protect against lepidopteran pests (Estruch *et al.*, 1996; ICAC, 2003). They bind to receptors in the insect gut, but not the same receptors as *Cry* proteins (Lee *et al.*, 2003, 2006), and *Cry*1Ac-resistant strains of *Heliothis virescens* are susceptible to VIP3A as *Cry*1Ac-susceptible strains of this pest (Malone *et al.*, 2008)

Efforts were made at UASD to develop transgenic Bt cotton with *Cry* 1Ac (with double promoter) gene obtained from ICGEB. Among the many positive events developed, "UASD Bt Cotton Event No. 78", has been identified as potential one owing to significantly higher *Cry* protein expression with corresponding bioefficacy throughout crop duration and all the plant parts studied in comparison to Monsanto's BGII (Manjunath Swamy, 2014). This Event No. 78 can successfully be promoted from the public sector to farmers for which all the regulatory procedures including event characterization need to be conducted.

Commercialization of transgenic crops can be achieved only after regulatory approval which requires the rigorous assessment of their safety. Due to the massive plantation and wide application of genetically modified organisms (GMOs), appropriate laws and regulations for strengthening the management of importation and exportation of transgenic plants and corresponding products are being developed all over the world

(Xu *et al.*, 2013). Molecular characterization of transgenic events is an important analysis towards this goal and is conducted at two stages: first, for the selection of desirable events and later for the characterization of the selected lead event(s) to support regulatory submissions. A thorough molecular characterization of the transgene locus, determining its sequence, integrity and its location in the genome, is a critical step in the safety assessment process. This characterization also addresses mandatory analysis that determines whether the transgene expression cassette is inserted into the host genome as a single copy, is intact across generations, has made any unintended alterations to the host genome due to insertion, and whether it lacks the backbone sequences derived from the plasmid vector used for the transgenesis (Guttikonda *et al.*, 2016).

Identifying the event-specific insertion sites upstream or downstream of an exogenous insertion in a GM crop provides direct evidence of the unknown flanking regions and conclusive evidence of the identity of the given GM crop. For this purpose, many PCR-based genome walking methods, such as, Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995), Ligation mediated PCR (LM-PCR) (Mueller and Wold, 1991), Inverse PCR (IPCR) (Ochman *et al.*, 1988), and Site finding PCR (Tan *et al.*, 2005) have been originally adopted. Later on, more and more modified or improved PCR-based methods, high throughput or high-efficiency Tail PCR (Liu and Yuanling 2007; Singer and Burke, 2003), and adaptor mediated PCR (Huang *et al.*, 2007) were developed for routine molecular characterization with high throughput and/or high efficiency. These PCR-based methods rely on known sequence information of the exogenous insertion, and no single method can be applied universally (Yang *et al.*, 2011). Each method possesses its characteristics, and combinations of different methods adopted to increase efficiency (Li *et al.*, 2017).

Among all the methods TAIL-PCR is the best and quite efficient method there is no need for DNA digestion, DNA ligation, PCR product cloning and vector screening which was followed in rest of the methods.

The present investigation involves the confirmation of the presence of the gene in UASD Event No.78, event characterization using TAIL-PCR and zygosity studies with the following objectives.

1. Confirmation of UASD transgenic Event No.78, with *Cry* 1Ac gene through PCR with gene-specific primers
2. Event characterization of the *Cry* 1Ac UASD transgenic Event no.78 through TAIL-PCR.
3. Confirmation of zygosity status of plants with *Cry* 1Ac gene of event No.78

2. REVIEW OF LITERATURE

The present study aimed at PCR confirmation and Event characterization of Cotton transgenic Event, “UASD Bt Cotton Event No.78” developed at the university of agricultural science Dharwad. The literature reviewed in line with the below objective has been summarised in this chapter.

2.1 Confirmation of UASD transgenic Event No.78, with *Cry* 1Ac gene through PCR with gene-specific primers

2.2 Event characterization of the *Cry* 1Ac UASD transgenic Event no.78 through TAIL-PCR.

2.3 Confirmation of zygosity status of plants with *Cry* 1Ac gene of event No.78

2.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was developed by Kary Mullis in 1985 (Mullis, 1990). The technology is used to know the presence of a transgene which is one of the major applications of PCR. The PCR results in discriminating the amplification of a transgene if a suitable primer is designed from any part of the transgene can be chosen. Two short oligonucleotides must hybridize to the DNA molecule, one to each strand of the double helix. These oligonucleotides, which act as primers for the DNA synthesis, restrict the region that will be amplified. Amplification is generally carried out by the DNA polymerase-I enzyme obtained from *Thermus aquaticus*, which lives in hot springs and many of its enzymes, including Taq polymerase, are thermostable, meaning to say they are resistant to denaturation by heat treatment. PCR is the most practical, low-cost and broadly used technique among other methods for Genetically Modified Organism (GMO) detection. This can be applied to a diverse kind of samples such as seeds, leaf, processed food/feed *etc*, and provides very specific and accurate results in a short time. Multiplex PCR, qRT-PCR and QC-PCR are the major types used for Genetically Modified Organism (GMO) screening (Filiz *et al.*, 2011).

At the end of a PCR, a sample of the reaction mixture is usually analysed by agarose gel electrophoresis. DNA of amplified fragment in sufficient concentration viewed as a discrete band after staining with ethidium bromide. This provides useful information about the presence of transgene that has been amplified (Sangannavar, 2008).

PCR technology is a major tool for screening of genetically modified organisms and being accepted as the most sensitive and reliable method for detection of GM (Genetically Modified)-derived material in routine applications. PCR-based GMO (Genetically Modified Organism) tests can be grouped into at least four categories corresponding to their level of specificity (Shreevidya *et al.*, 2012).

Each category corresponds to the composition of the DNA fragment that is amplified in the PCR.

The four categories are as follows.

1. Screening methods-This method screens GMO’s by PCR amplification of Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S) and/or the CaMV 35S (T35S) or the Agrobacterium tumefaciens nopaline synthase terminator (T-Nos) *etc*., as almost every transgene will have any of these as a part.

2. Gene-specific methods-Most commonly used the method as it concentrates on amplification of the gene of interest directly.

3. The construct-specific methods-This method will target junctions between adjacent elements of the gene construct, for example between the promoter and the gene of interest. With these methods, a positive signal will only appear in the presence of GM-derived material.

4. Event-specific methods-The unique signature of a transformation event is the junction at the integration locus between the recipient genome and the inserted DNA. This junction is the target of event-specific methods (Arne *et al.*, 2003).

Pidugu *et al.* (2012) developed a reliable detection method for genetically modified cotton events MON 531 and MON 15985 by using multiplex PCR reaction technique. A simple and efficient multiplex PCR method for specific detection of two GM cotton events was developed. Three primer pairs (CaMv35S, fsACP and *Cry*1AC) were designed by primer-3 (version 0.4.0) software and four primer pairs (Nos, Npt II, ESP 531 and CSP15985) were taken from the literature. All oligonucleotide primers were checked with public database (NCBI BLAST search) and confirmed that no match with an unintended DNA sequence was found. The results showed that each primer set for corresponding GM cotton was able to amplify a specific band. The two cotton events were differentiated by event specific and construct specific primers. This multiplex PCR has been developed for the detection of two GM cotton events. The sensitivity of their assay is 0.03%. Duplex PCR and multiplex PCR methods were standardized. The multiplex PCR method reported in the present study is simple, cost-effective and time-saving. This method could be an effective tool for the detection and evaluation of two specific GM cotton events.

Yang *et al.* (2014) identified Gm soybean event no A2704-12 from other GM soybean events (A5547-127, MON 89788, & GT 540-3-2). They used PCR method to identify event-specific GM soybean. Primer express 3.0 software was used to design two primers, one primer on the 5' flanking sequence of event A27042-12. Determined by using TAIL-PCR. Another primer was specific for event A2074-12. PCR amplification of the target 239 bp fragment with the clear and sharp band. The fragment was obtained from GM soybean A2074-12 & no amplification product was observed from other GM soybean. In conclusion, they developed a high throughput low cost and event-specific qualitative and quantitative PCR detection method for GM soybean A2704-12.

Pan *et al.* (2005) developed an event-specific detection method based on the flanking sequence of an exogenous integrant in the transgenic maize MON863 which contains *Cry*3Bb1 gene expressing a Bacillus thuringiensis *Cry*3Bb1 protein that is selectively toxic to a maize rootworm pathogen. The 3' integration junction between host plant DNA and integrated DNA of transgenic MON863 maize was isolated using thermal asymmetric interlaced (TAIL)-PCR. The event-specific primers and TaqMan probe were designed based upon the isolated 3'-integration junction sequence and qualitative and quantitative PCR systems were established employing these designed primers and probe.

2.1.1 *Cry* Genes

PCR is the preferred method for the identification and quantification of Bt gene because of its versatility, sensitivity, specificity and high throughput applications (Shreevidya *et al.*, 2012). To detect any Bt gene, it is necessary to know the sequence of the genes used in the GM construct. These may include plasmid vector sequences, selectable markers, promoters and terminators (Suchitra and Sher, 2013). Studies those confirm the presence of different *Cry* genes using PCR is presented in Table 1.

2.2 Event characterization of transgenic with special emphasis on TAIL-PCR

Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a powerful tool for the recovery of DNA fragments adjacent to a known sequence. It was developed by Liu and Whittier in 1995 (Liu and Whittier, 1995). Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) is a fast and efficient method to amplify unknown sequences adjacent to known T-DNA sequence. Nested, insertion-specific primers are used together with arbitrary degenerate primers (AD primers), which are designed to differ in their annealing temperatures. Alternating cycles of high and low annealing temperature yield specific products bordered by an insertion-specific primer on one side and an AD primer on the other. Further specificity is obtained through subsequent rounds of TAIL-PCR, using nested insertion-specific primers. The increasing availability of whole-genome sequences renders TAIL-PCR an attractive tool to easily identify insertion sites in large genome tagging populations through the direct sequencing of TAIL-PCR products. For large-scale functional genomics approaches, it is desirable to obtain flanking sequences for each individual in the population in a fast and cost-effective manner (Singer and Burke, 2003) Studies which have used TAIL-PCR to find the flanking sequence of inserted T-DNA are listed in Table 2

Three types of products are expected to occur in TAIL-PCR the preferred specific target products, referred to as type I products, are primed by the insertion-specific primer on one side and a nonspecific AD primer on the other side. Type II products are nonspecific products, primed on both sides by the insertion-specific primer. Another class of nonspecific products, termed type III, are primed on both sides by

Table 1: Plants transformed with various *Cry* genes to confer resistance against insects

Sl. No	Transgene/ Transgenic Event	Transformed Plant	TargetPest/ trait	Technique used to confirm transgene	References
1.	*Cry* 1Ac	Cotton	Helicoverpa armigera	PCR, Southern blotting	Zhang *et al.* (1998)
2.	*Cry* 1F	Cotton	Helicoverpa armigera	PCR	Sangannavar (2008)
3.	*Cry* 2 Aa	Cotton	Helicoverpa armigera	PCR	Sumithra (2008
4.	*Cry* 1Ec	Cotton	Helicoverpa armigera	PCR	Manoj *et al.* (2009)
5.	*Cry* 1Ac+*Cry* 2Aa	Cotton	Helicoverpa armigera	PCR, Southern blotting	Allahbakhsh (2010)
6.	*Cry* 1Ac	Cotton	Helicoverpa armigera	PCR	Shreevidhya *et al.* (2012)
7.	*Cry* 1A	Cotton	Helicoverpa armigera	PCR	Bao *et al.* (2000)
8.	*Cry* 1A	Rice	Yellow stem borer	PCR	Ramesha *et al.* (2004)
9.	*Cry* 1C	Rice	Yellow stem borer	PCR, Southern blotting	Wei (2006)
10.	*Cry* 1C	Rice	Yellow stem borer	PCR	Rongjian *et al.* (2009)
11.	*Cry* 1Ab	Rice	Yellow stem borer	PCR	Qi *et al.* (2009)
12.	*Cry* 1Ac	Soyabean	Pod borer	PCR	Neal *et al.* (1996)
13.	*Cry* 1Ab	Potato	Potato tuber moth	PCR	Meetul *et al.* (2009)
14.	Gluten subunit gene	Wheat	wheat quality	PCR	Xu *et al.* (2013)

Contd…

Sl. No	Transgene/ Transgenic Event	Transformed Plant	TargetPest/ trait	Technique used to confirm transgene	References
15.	Herbicide-resistant bar gene.	Wheat	Herbicide resistant	PCR	Xu *et al.* (2013)
16.	*Cry*3Bb1 gene	Maize	Toxic to maize root worm pathogen	PCR	Pan, A., *et al.* (2005)
17.	herbicide-resistant bar	wheat	Resistant herbicide spray	PCR	Xu *et al.* (2013)
18.	Pcambia2300 construct of UTR NPTII 35S.pro	Cotton	Kanamycin resistance	PCR	Yang, X., *et al.* (2013)
19.	rvl1	Tomato	Resistant to root knot nematode	PCR	Kolkar, R. M. (2012)
20.	*Barnase gene*	Tomato	Resistant basta spray	PCR	Birdar (2008)
21.	Ihp-TRP construct	Tomato	Resistant to tomato leaf curl virs	PCR, dot blot analysis, southern hybridisation& GUS analysis.	Vitthal (2013)

Table 2. TAIL-PCR used in different crops to identify the flanking sequence of inserted T-DNA

Sl. No	Transgeic crop	Blast N analysis of sequence	Transgenic Event/ Lines	Reference
1	Maize	The recovered DNA fragment of the 3’ junction region contained 203 bp originating from the sequence of vector PV-ZMIR13 and 361 bp originating from endogenous maize genomic DNA. Results of the analysis of the 361 was identical to the Zea mays strain NB mitochondrial DNA sequence from 292436 to 292688 (Genbank Accession number AY506529).	MON863	Pan,*et al.*,(2005)
2	Maize	The exact site of the integration T-DNA was located at chromosome 3:196142361- -196144004 in maize genome (B73RefGen_v2).	BVLA430101	Rao *et al.* (2016)
3	Rice	Sequence analysis of right border flanking identified that the sequences between 1 and 166 matched with the position 3,926–4,092 of the T-DNA vector (GenBank Acc. No. AB303069.1), the sequences between 189 and 505 matched with the position 95,684–95,962 of the rice genome chromosome 6 DNA (GenBank Acc. No. AP004749) Sequence of left border flanking analysis identified that the nucleotides between 1 and 283 matched with the position 8,984–8,701 in the rice genome chromosome 9 (GenBank Acc. No.AP009051.1). The nucleotides between 289 and 607 are matched with the position 41–358 of the T-DNA vector (GenBank No. AB303069.1).	KeFeng-6	Wang *et al.* (2011)

4	Wheat	a 14,691-bp sequence of GM wheat B73-6-1 was obtained.The obtained gene sequence with 14,691 bp was subjected to the comparative analysis by BLAST program in GenBank database. The flanking sequence between insert DNA showed high similarity with Triticum turgidum A gene (GenBank: AY494981.1)	B73–6-1	Xu *et al.* (2013)
5	Cotton	The flanking sequences of 5’ were aligned with the known vector sequence, showing overlapping sequences of 431 bp with a similarity of 100%. The remaining sequence is an unknown sequence of approximately 734 bp. According to a blastn search, 626 bp of the sequence exhibits 94.2% similarity to the sequence of Gossypium raimondii chromosome 11, with 97.0% similarity to Gossypium hirsutum L. chromosome D10	BG2-7	Zhang *et al.* (2016)
6	Cotton	Left border of T-DNA integration, located from 6173 to 6198 bp of cottongenom(GenBank accession AF234315) Right borders located from 8637 to 8661 bp of cotton genome (GenBank accession AE007871)	Cotton transgenic lines	Yang, X., *et al.* (2013)
7	Carnation	The 5 junction sequence of an exogenous integrant of Moonlite. sequence consisted of a 93-bp fragment from pCGP1470 and an 887-bp unknown DNA sequence. Because of less sequence information of carnation. The specific primers are designed for this unknown 887-bp unknown DNA sequence and these primers amplified only in carnation not in any of GM crops and non-GM crops	Variety Moonlite	Li, P *et al.* (2012)

8	Maize	Out of 64 specific amplified fragments, 32 and 9 are homologous to the sequences of the maize genome and the expression plasmid, respectively.	70 maize transgenic lines	Yang, L., *et al.* (2011)
9	Rice	BlastN analysis of the right border flanking sequences of the T-DNA indicatesthat the insertion site is at the position 1,838,745.5 of on the short arm of the rice chromosome 1 (GenBank AP003214) Flanking sequence showed homology to the rice sequence from 6,736,067 bp to 6,736,246 bp (GeneBank Acc. No. AP001800)	A1473	Zhu, C., *et al.* (2010)
10	Tobacco	829 bp of left border flanking region which showed homology to putative Leucine-rich repeats.	Tobacco line	Vodnala, S. M. (2008)
11	Tomato	A left border flanking sequence of 22 bp showed homology with tomato genome sequence clone C05_HBa0078I05(Genebank Acc. No. AC238741.21)	RVL1-T0	Kolkar, R. M. (2012)
12	Tomato	Sequence of RB-TAIL-PCR prdoucts show homology to right border sequence of Pnu435r (GeneBank Acc. No. DQ225750).	PT4-T1	Biradar (2008)
13	Tomato	T-DNA right border flanking sequence of A1-42-20 showed homology to tomato genomic region corresponding to clone, *solanum lycopersicum* strain Heinz 1706 chromosome 1 (GeneBank Acc. No. AC246092.2).	A1-42-20	Vitthal (2013)

		T-DNA right border flanking sequence of C47-25-11 showed homology to tomato genomic region corresponding to clone, *solanum lycopersicum* chromosome 12 (GeneBank Acc. No.SL2.40CH12). T-DNA right border flanking sequence of E35-10 showed homology to tomato genomic region corresponding to clone, *solanum lycopersicum* chromosome 2 CLONE C02HBa0007F24 (GeneBank Acc. No. AC21531.2).	C47-25-11 E35-10	
14	Tomato	out of 1639 bp sequence 977 bp sequence showed homology to right border of T-DNA and 662 bp showed homology to genomic region of tomato.	GM Huafan No.1 tomato	Yang, C. (2012)

nonspecific AD primers. In the subsequent TAIL-cycling rounds, the preferred target molecule (type I) is amplified together with nonspecific type II and type III products. TAIL cycling consists of 15 supercycles, in which each super-cycle consists of two high stringency cycles and one low stringency cycle (Liu and Yuanling, 2007).

To increase the yield of the specific type I product and to decrease the amount of contaminating nonspecific products, the second round of TAIL-PCR is performed using a nested insertion-specific primer. The nested primer is designed to hybridize to the known insertion sequence internally of the first primer. The type II nonspecific products were generated through mispriming of insertion-specific primers product of the first round, fail to reamplify with the nested primers in the second round (Kong and Liu, 2009). In the standard TAIL-PCR protocol, the secondary round of TAIL-amplification is followed by a third-round using another set of nested insertion-specific primers. Dilution of the secondary PCR products before the reaction and employing only a limited number of high stringency cycles (20) ensures that all undesired products fail to amplify. Through visual inspection on agarose gels of secondary and tertiary PCR products loaded next to each other, specific products are corroborated through a decrease in product size according to the position of the nested primers (Jia *et al.*, 2017).

Multiple bands are often observed after secondary or tertiary TAIL-PCRs. Those bands are indicative of either multiple insertion sites of the mutagenic DNA into the genome or represent one insertion site primed with nested AD primers. As the latter products represent the same flanking sequence of an insertion site but only differ in length, sequencing of those products with the insertion-specific primer as a sequencing primer usually results in clean sequencing profiles (Wu *et al.*, 2015).

2.2.1 Sequencing of TAIL-PCR products

There is no doubt that since its initiation the polymerase chain reaction (PCR) has revolutionized our ability to detect, analyze, and manipulate DNA and RNA sequences (Saiki *et al.*, 1985). The ability to analyze PCR amplification products is central to PCR's overall utility for increasing the rapidity of data acquisition, especially regarding genomic composition, organization, and regulation. There are many methods by which PCR products can be analyzed directly or indirectly (the following cloning) by using restriction endonuclease mapping, hybridization, and so forth or nucleotide sequencing of PCR products directly (Erlich *et al.*, 1991).

Nucleotide sequencing of PCR products helps to confirm the specificity of amplification, identify genetic variants (polymorphisms, rearrangements, translocations, *etc*.), identify hitherto uncharacterized genes, and map these genes within the organization of the genome (Bevan *et al.*, 2019).

Numerous methods have been developed for direct sequencing of single or double-stranded DNA amplified by PCR, and most require template purification steps before sequencing to remove excess unincorporated primers and dNTPs. Purification methods can be cumbersome, laborious and time-consuming, or yield low DNA recoveries. Strategies for direct sequencing of double-stranded DNA products without purification steps have been described (Meltzer *et al.*, 1990 and Rao *et al.*, 1992), but these require use of end-labelled primers for sequencing, since primers carried over from the amplification reaction will generate superimposed sequence ladders if an unlabeled sequencing primer is used. Primer labelling has the disadvantage that the radioactive pattern of sequencing ladders decreases in strength with increasing distance from the primer. Furthermore, the primer end labelling requires additional manipulations and must be carried out periodically even when using the same primer (Liu *et al.*, 1993).

New sequencing strategy that omits PCR product purification altogether, yet does not require end-labelled primers for generating clear sequencing ladders. A thermal asymmetric PCR method has been described for generating single-stranded DNA (Mazars *et al.*, 1991). The sequencing strategy relies on thermal asymmetric PCR and utilizes one or two relatively short primers in the initial PCR amplification of target DNA. Subsequent sequencing is carried out by temperature cycling (Murray, 1989, Carothers, *et al.*, 1989 and Srivastava *et al.*, 1992) using a longer sequencing primer. Sequencing cycles employ a high annealing temperature to avoid annealing and elongation of carried over short primers.

(Liu *et al.*, 1993) sequenced TAIL-PCR products with the PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit (ABI), 5 µl of unpurified secondary (or tertiary) reaction products were added directly to a 17 µl volume of sequencing mixtures containing 7 µl (1 pmol/pl) of the same specific primer used for the PCR amplification and 10 µl of sequencing mix from the kit. Cycle sequencing was carried out on a GeneAmp System 9600 thermocycler using 25 cycles of 96°C for 15 s, 60°C for 5 s, and 65°C for 4 min. Sequencing using an automated DNA sequencer 373A (ABI) was carried out according to the manufacturer's protocol.

Liu *et al.*, (1993) identified flanking region T-DNA was identified by TAIL PCR. Sequencing was done using the Dye Deoxy Terminator Cycle sequencing kit (ABI) on automated DNA sequencer. With these kits, an unlabelled sequencing primer is used, and the fluorescent label is attached to the chain-terminating dideoxynucleotides. A small amount (5 µl) of the unpurified secondary (or tertiary) PCR product was applied directly to sequencing reactions. Even when using the same specific primer for PCR as for sequencing, unpurified template yielded clear sequencing profiles, reflecting the high specificity of the products.

Alka *et al.* (2014) cloned the PCR amplified product using Ecoli DH5 α and sequenced *Cry*1Ac and *Cry*2Ab specific PCR products having 230bp and 453bp from Bt-cotton (MON 531 and MON 15985). Cloned products were sequenced by using M13F primer in ABI PRISM 3500/3500 genetic analyser by following user guide of big dye Terminator v3.1.

Paschalis *et al.* (2008) performed PCR using either 35S pro5 or 35Spro3 and adopter AP1 in the event MON 1445. The PCR amplified products were analysed in agarose gel and subsequently transferred onto nylon membrane to carry out hybridisation with 35 S specific probe. The probe was obtained by PCR using 35 S pro5 and 35 S pro3 primers with template pBI 121 plasmid vector harbouring a fragment of 35 S promoter. Positive bands were cut out of the gel using a scalpel, purified with the Nucleospin Extract II kit (Macherey Nagel, Duren, Germany) and subsequently cloned into a plasmid vector using the TOPO TA cloning kit (Invitrogen, Paisley, UK). Clones were screened by hybridization with the 35S probe and positive clones were sent for sequencing.

Yang *et al.* (2011) identified the presence of T-DNA and its flanking sequence at both 5' end and 3' end was known by TAIL PCR method. The specificity confirmation by the repeat PCR amplification. The recovered products of specific fragments amplified in the tertiary round TAIL-PCR were cloned into PMD18-T vector (TaKaRa) and sequenced with three replications at SinoGenoMax. After removing the sequences of PMD18-T vector and expression construct, local alignment was conducted between the sequences of the specific fragments and the maize genome (line B73), downloaded from maize Sequence (http://www.maizesequence.org).

Pan *et al.* (2006) determined the 3' flanking region of the MON863 insert sequence using the TAIL-PCR method, as described by Liu *et al.* (1995). The product of third round of TAIL-PCR amplification was analysed on 2% agarose gel electrophoresis and stained with ethidium bromide. Parallel amplification on wild-type MON863 was carried out to identify specific PCR products. These DNA fragments were purified and subsequently cloned into the pMD18-T vector (Takara Biotechnology Co., Ltd, Dalian, China). DNA inserts in this vector were sequenced by using the ABI PRISMe 377 DNA Sequencer.

Wang *et al.* (2011) direct sequencing of PCR product, 12-15 μL of PCR products were purified through a Sephadex G-50 column and subjected to sequencing procedures using SP3 primers designed to known sections of the target sequence, or FSP2 primers designed according to the original FP primers. For indirect sequencing, bands corresponding to the largest products were excised from the agarose gels, purified and cloned into a pMD18-T TA cloning vector (TaKaRa), and then sequenced with M13 forward or reverse primers.

Li *et al.* (2012) the 5’-flanking sequence of the Moonlite exogenous insertion was determined using the TAIL-PCR method, essentially as described by Liu *et al.* (1995). The product of the third TAIL-PCR amplification was analysed on 2% agarose gel electrophoresis and stained with ethidium bromide. The DNA fragments were purified and subsequently ligated into the pMD18-T vector (TaKaRa Biotechnology Co. Ltd.). DNA inserted in this vector was sequenced by using ABI PRISMTM 377 DNA Sequencer (Applied Biosystems).

2.3 Confirmation of zygosity status of transgenic Event No.78

Homozygosity is highly desirable in transgenic plants research to ensure the stable integration and inheritance of transgene(s). Simple, reliable and high-throughput techniques to detect the zygosity of transgenic events in plants are invaluable tools for biotechnology and plant breeding. Currently, several basic techniques are being used to determine the zygosity of transgenic plants in the T1 generation. For successful application of any technique, precision and simplicity of approach combined with the power of resolution are important parameters. Based on simplicity, resolution and cost involved, the available techniques have been classified into three major classes which are conventional methods, current methods and next-generation methods. Conventional methods include antibiotic marker-based selection and the highly labour-intensive Southern blot analysis. In contrast, current methods such as real-time PCR, TAIL PCR and competitive PCR are not only cost-effective but rapid as well. Moreover, methods such as NGS, digital PCR and loop-mediated isothermal amplification are cost-effective, fast and less labour intensive.

This is the list of methods used to identify the zygosity status of transgene

1. Antibiotic selection
2. Southern hybridisation
3. Competitive PCR
4. Real-time PCR
5. TAIL PCR
6. Digital PCR
7. NGS (next-generation sequencing)
8. LAMP (Loop-mediated isothermal amplification assay)

Among these TAIL-PCR is easy when compared to other methods because it requires less amount of DNA, highly specific, can work even transgene integration occurs more than two sites and can easily scale up. Digital PCR, NGS and LAMP are highly specific but it requires sophisticated instruments, highly expensive (Pillai *et al.*, 2008).

Passricha *et al.* (2016) identified the flanking sequence of inserted T-DNA identified by TAIL-PCR method. This information was used to design the primers for zygosity study. Three sets of primers were used in zygosity study first primer being common and present on right border flanking sequence of the inserted T-DNA as 5’ primer. The other two primers were designed as 3’ primers, one specific primer to the inserted T-DNA and the other to left border flanking sequence as 3’ primer, vice versa can also be designed (*i.e.* one common primer as 3’ of left border flanking and other two as 5’ primer one belong to T-DNA and other one to the right border flanking of T-DNA.) These 3 sets of primers are used in single PCR (primer multiplexing) reaction which differentiated the homozygous transgenic plant, hemizygous transgenic plant and wild plant (non-transgenic plant).

Paschalis *et al.* (2008) identified the GM cotton plants having the event mon 1445 that is resistant to the herbicide glyphosate by designing the primers. To prepare an event-specific test for cotton Mon1445 they had designed a specific primer at the 3’flanking region (primer 3cot), amplifying a sequence of 750bp in combination with primer 35Spro3. For a negative control identifying non-GM cotton genomic DNA, a primer designed on genomic DNA flanking the 5’end of the transgene from sequence information in the patent no 6,740,488 (US Patent Office). They designed the 5cot primer amplifying a sequence of ~300bp when applied with 3cot on the non-GM cotton template. The test was performed in three non-GM samples and the isolated transgenic sample. Existence of Mon1445 cotton was confirmed by the presence of a 750bp PCR product, whereas the existence of non-GM cotton was confirmed by the presence of a ~300bp band. Results showed that the event 1445-specific 750 bp band could be amplified from the 35Spositive samples examined. A cotton-specific 300 bp band could be amplified from non-GM plants. Indicating that the presence of the T-DNA is specific to event Mon1445 plants.

Pillai *et al.* (2008) determined the zygosity of transgenic mice. Primers were designed to the 5’ flanking region of T-DNA, along with this primer other two 3’ primers were designed one specific to transgene and the other to the wild type. Genomic DNA was amplified using all three primers in each PCR reaction. Differentially sized bands were produced by the amplification of the uninterrupted wild-type chromosome and the chromosome with the transgene. By this method, a 776-bp fragment alone was detected in the wild-type animal, and a shorter 196-bp fragment alone in the homozygote, while both the bands were amplified in the heterozygote.

3. MATERIAL AND METHODS

The present study was undertaken to identify the flanking genomic regions of inserted T-DNA in transgenic UASD Bt Cotton Event No.78 through TAIL-PCR method and flanking sequence obtained was used to design the primers to confirm the zygosity status of gene *Cry* 1Ac. The material and methods employed are given below;

3.1 Transgenic UASD Bt cotton event No.78

RAH-100 is a *Gossypium hirsutum* variety, released by UAS, Dharwad for irrigated southern zone and the agronomic features are as detailed in table No.3. The transgenic cotton event, UASD Bt Cotton Event No.78 has been developed in the background of RAH-100 and carrying *Cry* 1 Ac gene and is described in Table 4.

3.1.1 *Cry* 1Ac gene sequence (1915bp)

GGCGAATTGGGTACCGGATCCATGGATAACAACCCAAACATTAACGAGTGCA
TTCCATACAACTGCTTGAGCAACCCAGAGGTTGAGGTTCTTGGAGGAGAGCG
CATTGAGACCGGATACACTCCCATCGACATCTCCTTGTCCTTGACTCAGTTCC
TCCTCAGCGAGTTCGTGCCAGGAGCTGGGTTCGTTCTCGGACTTGTTGACATC
ATCTGGGGAATCTTCGGACCATCTCAATGGGACGCCTTCCTCGTGCAAATTGA
GCAGTTGATCAACCGAGGATCGAGGAGTTCGCCAGGAACCAGGCCATCTCTA
GGTTGGAGGGATTGAGCAACCTCTACCAAATCTACGCTGAGAGCTTCAGAGA
GTGGGAGGCCGATCCAACTAACCCAGCTCTCCGCGAGGAAATGCGTATTCAA
TTCAACGACATGAACAGCGCCTTGACCACTGCTATCCCATTGTTCGCCGTGCA
GAACTACCAAGTTCCACTCTTGTCCGTTACGTTCAAGCTGCTAACCTTCACCT
CAGCGTGCTTCGTGACGTTAGCGTGTTCGGCCAAAGGTGGGGATTCGATGCT
GCAACCATCAACAGCCGTTACAACGACCTTACTAGGCTCATTGGAAACTACA
CCGACCACGCTGTTCGTTGGTACAACACTGGCTTGGAGCGTGTCTGGGGACC
CGATTCTAGAGATTGGATCAGGTACAACCAGTTCAGGAGAGAGTTGACCCTC
ACTGTTTTGGACATTGTGTCTCTCTTCCCCAACTACGACTCCAGAACCTACCC
TATCCGTACTGTGTCCCAACTTACCAGAGAGATCTACACTAACCCAGTTCTTG
AGAACTTCGACGGTAGCTTCCGCGGATCTGCTCAGGGCATCGAGGGCTCCAT
CAGGAGCCCACACTTGATGGACATCTTGAACAGCATAACTATCTACACCGAT

Table 3. Agronomic features (source-Release proposal and Registration) of RAH-100 genotype used to generate primary transformation event No. 78 to be evaluated in BRL-1 trial

Sl. No.	Character of Variety	RAH-100
1	Plant height	150-155 cm
2	No. of monopodia	1-2
3	No. of sympodia	18-20
4	Leaves	Medium sized, dark green
5	Stem	Hairy
6	Flower Petal Color	Yellow
7	Pollen color	Cream
8	Calyx	Green
9	Boll Shape	Large, round with pointed tip
10	Boll rind	Thick
11	Days to 50% flowering	70-75 days
12	Maturity	160-165 days
13	Maturity group	Medium
14	Reaction to major diseases	Moderately tolerant
15	Reaction to major insect pests	Moderately tolerant
	Agronomic Features	
16	Sowing date	May-June
17	Spacing	90 cm X 30 cm.
18	Fertilizer	80:40:40 kg. NPK/ha.
19	Seed rate	2.5 kg./ha
20	Quality of produce	Bright white cotton with good boll opening, superior medium staple cotton suitable for 40s-50s counts
21	Yield (Seed cotton)	15q/ha (average of 7 years in AICRP)

Table 4: Details of transgenic Event-78

Event Identity	**Insecticidal Gene**	**Gene Source**	**Genetic Background**	**Binary vector used for *Cry* 1Ac gene transformation**
UASD Bt Cotton Event No.78	*Cry* 1Ac with double promoter	ICGEB	RAH 100	pBI 121

Cry- *Cry*stal protein.

ICGEB- International Centre for Genetic Engineering and Biotechnology.

pBI 121-a binary vector

GCTCACAGAGGAGAGTACTACTGGTCTGGACACCAGATCATGGCCTCTCCAG
TTGGATTCAGCGGGCCCGAGTTCACCTTCCCACTCTACGGAACTATGGGAAA
CGCCGCTCCACAACAACGTATCGTTGCTCAACTTGGACAGGGAGTCTACAGG
ACCTTGTCTTCCACCTTGTACAGAAGGCCCTTCAACATCGGAATCAACAACCA
GCAACTTTCCGTTCTTGACGGAACTGAGTTCGCCTACGGAACCTCTTCCAACT
TGCCATCCGCTGTTTACAGAAAGAGCGGAACCGTTGATTCCTTGGACGAGAT
CCCACCACAGAACAACAATGTGCCACCCAGGCAAGGATTCTCCCACAGGTTG
AGCCACGTGTCCATGTTCCGTTCCGGATTCAGCAACAGTTCCGTGAGCATCAT
CAGAGCCCCTATGTTCTCTTGGATTCACCGTTCTGCCGAGTTCAACAACATCA
TCGCTTCTGATAGCATTACTCAGATCCCAGCCGTGAAGGGAAACTTCCTTTTC
AACGGAAGCGTTATCAGCGGACCAGGATTCACTGGCGGAGACCTTGTGAGAC
TTAATAGCTCTGGCAACAACATTCAGAATAGAGGCTACATCGAGGTTCCTAT
CCACTTCCCATCCACATCTACTAGATATAGAGTTAGGGTTAGATACGCCTCTG
TGACCCCAATCCACCTTAACGTGAACTGGGGCAACTCATCTATCTTCTCCAAC
ACCGTTCCAGCTACTGCTACCTCTCTTGATAACCTTCAATCCAGCGATTTCGG
ATACTTCGAGAGCGCCAACGCTTTCACTTCTTCCTTGGGCAACATCGTGGGAG
TTAGGAACTTCAGCGGTACTGCAGGAGTGATCATTGACAGATTCGAGTTCATT
CCAGTTACTGCCACTCTTGAGGCTGAGTACAACCTTTAAGAGCTCCAGCTTTT
GTTCCCTTTAGTGAGGGTTAATT

3.1.2 pBI 121 vector description

Map of pBI 121 vector was used to clone the m*Cry*1A/c gene to create binary vector pBI121-m*Cry*1A/c in Fig. 1 (Marja *et al.,* 1989).

3.2 Confirmation of *Cry* 1AC in UASD Bt cotton Event No.78

To confirm the presence of transgene, PCR analysis of Event No 78 was done by extraction of genomic DNA from leaf tissue according CTAB method (Sambrook and Russell, 2001) using *Cry* 1Ac specific primers.

3.2.1 DNA extraction

DNA extraction was done with CTAB method with few modifications (Sambrook and Russell, 2001).

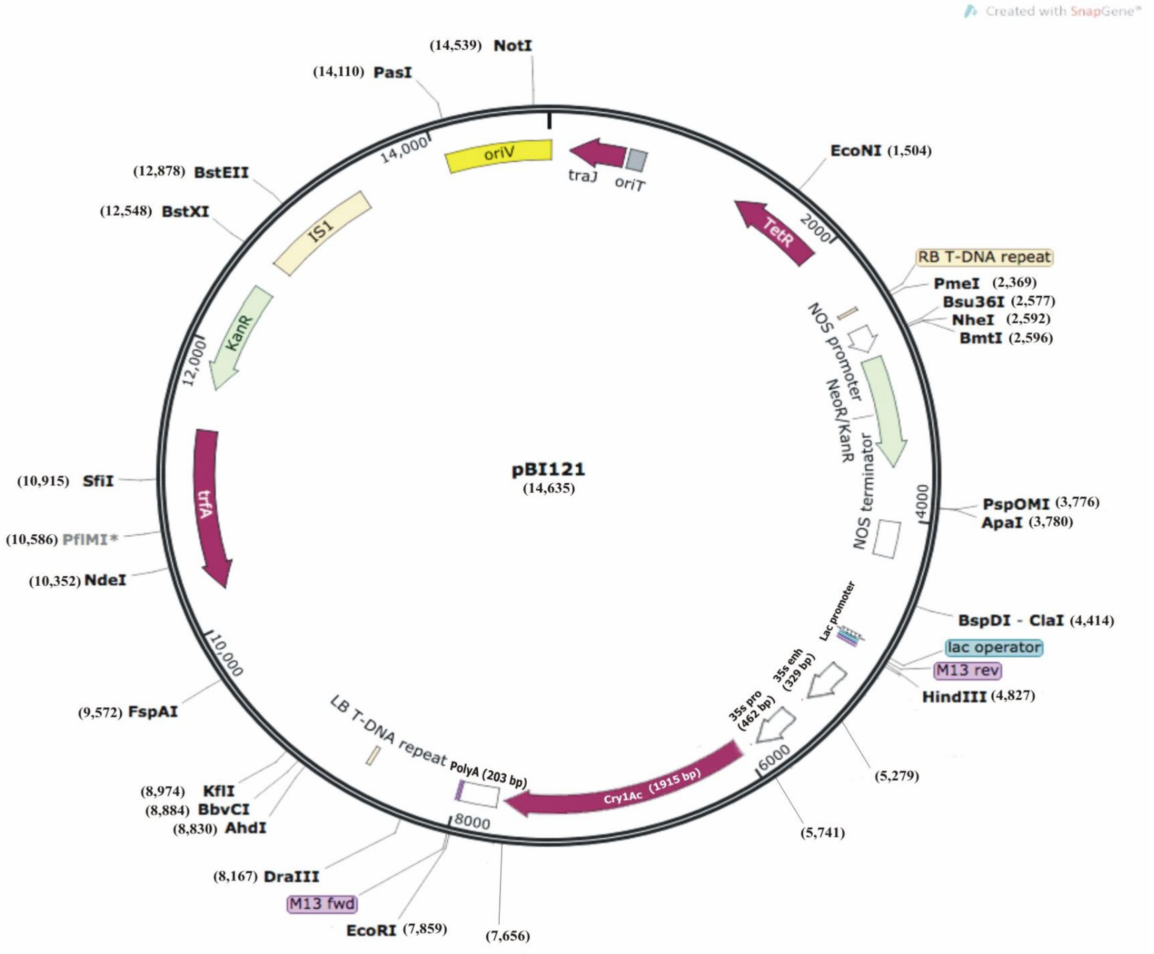


Fig. 1: Map of pBI121with *Cry* 1Ac gene which was used for transformation

1. Leaf samples for DNA extraction were collected from the seedling at two to three leaf stage in eppendorf tube with proper label.
2. 400 ml of CTAB buffer (Appendices II) was added to Eppendorf tubes containing the sample.
3. The tissue was crushed until it becomes homogenized.
4. The samples were kept in water bath at 65˚ C for 30 min.
5. Centrifuged for 15 min at 13, 000 rpm.
6. Supernatant was collected in another Eppendorf tube.
7. Equal volume of chloroform: IAA (Iso amyl alcohol) in the ratio of 24:1 was added to the tube and centrifuged at 13, 000 rpm for 15 min.
8. Supernatant was transferred to new tube.
9. Equal volume (100 µL) of pre-chilled Iso propanol was added, rested for 30 min for precipitation of nucleic acids and centrifuged for 10 min at 13, 000 rpm.
10. Supernatant was decanted.
11. The DNA pellet obtained was washed with 70 per cent ethanol and the tubes were inverted on blotting paper to air dry the pellet.
12. Later, DNA was suspended in 50 µl $T_{10}E_1$ buffer (Appendices-IV) and stored at 20^0C (pH 8.0).
13. Two microlitre of RNase was added and incubated for 65 ^{0}C for 15 min and 35^0C for 20 min and DNA samples were stored in deep freezer -20 ^{0}C.

3.2.2 DNA quantity and quality estimation

The concentration of DNA was assessed spectrophotometrically in Thermo Scientific™ NanoDrop 2000 and also by gel electrophoresis using 0.8 per cent agarose gel with known quantity of DNA.

To test the quality of DNA, samples were run on 0.8 per cent agarose gel prepared in 1X TAE buffer and stained with ethidium bromide (Appendices-V). Even though the DNA was treated with RNAase, it was checked for contamination by RNA (which usually runs ahead of DNA) visually. The DNA was evaluated by comparing it with a standard undigested DNA sample. Serial dilutions were carried out to get desired quantity of DNA for PCR.

3.2.3 PCR analysis of transgenic event No.78 using *Cry* 1Ac gene specific primers

The DNA isolated from cotton plants was tested for the presence of transgene using *Cry*1Ac specific primers as listed below (Table 5) and the details of components of reaction mixture followed for PCR is detailed in the Table 6 (Manjunathswamy, 2014).

3.2.4 PCR programme

Specific primers were designed for the sequence *Cry* 1AC gene which was used to develop UASD Bt Cotton Event No.78. The details of the PCR programme are presented in Table 7.

3.2.5 Agarose Gel Electrophoresis

1. Sufficient 1x electrophoresis TAE buffer (Appendices –V) was prepared from 50x stock solution.

2. Agarose powder was added (2%) to TAE buffer (1x) and was dissolved by melting at 100˚C. The solution was cooled to 50˚ C and ethidium bromide was added (5 µl/200 ml of agarose) and the comb was positioned at 0.5-1.0 mm above the Plate. Then agarose solution was poured into the gel frame and was allowed to polymerize. The gel tank was filled with TAE buffer (1x) just enough to cover the surface of the gel to a height of 1 mm.

3. PCR product was mixed with gel loading dye (Appendices –III) and were loaded into the wells of the submerged gel using disposable microtips. Quick-Load® 100 bp DNA Ladder was used as molecular weight marker.

4. The system was connected to the power supply and electrophoresis was carried out at 65 volts for 70-75 min.

5. It was examined in gel documentation system.

Table 5: Sequence of gene specific primers used for confirmation of *Cry* 1Ac gene with enhancer

Gene	Primer	Primer Sequence
Cry 1Ac with enhancer	Forward Reverse	5' CAGGATCGAGTTGATGCTCAC 3' 5' GATGCATAGGGATTCTG 3'

Table 6: Components of PCR reaction mixture

Sl No.	Components	Concentration
1	DNA (30 ng/l)	2 µl
2	Primers	
	Forward Primer (5pM/µl)	0.5 µl
	Reverse Primer (5pM/µl) 0.5 l	0.5 µl
3	Taq polymerase (3U/l) 0.5 l	0.5 µl
4	10x Standard Taq buffer 2 l	2 µl
5	dNTP's (2.5 mM each) 2 l	2 µl
6	Double distilled water 12.5 l	12.5 µl
7	Total volume	20 µl

Table 7: PCR programme for *Cry* 1Ac with enhancer

Z	**Step**	**Temperature(^{0} C)**	**Duration (min)**	**No. of. cycles**
I	Initial Denaturation	95.0	5	1
II	1. Denaturation	94.0	0.40	33
	2. Annealing.	61.7	2.00	
	3. Extension.	72.0	1.30	
III	1. Final extension	72.0	10.0	1
	2. Hold	4.0	-	-

3.3 TAIL-PCR and sequencing

TAIL-PCR was used to isolate the T-DNA flanking sequence with some modification of both the original (Liu and Whittier, 1995) and modified TAIL-PCR protocols (Singer and Burke, 2003). Two different types of primers were used, one is the nested primer which is insertion specific, long and with a higher annealing temperature whereas second set of primers are called arbitrary degenerate (AD) primers, which are shorter, having low melting temperatures and possibly can randomly bind throughout the genome. T-DNA border-specific primers and arbitrary degenerate (AD) primers used in the present study are described in Table 8a and Table 8b. The final concentration of specific primers was adjusted to 0.2μM in primary and secondary (or tertiary) reactions, and that of AD was 3–4 μM depending on its degeneracy. TAIL-PCR was performed in the 96-well Plate, in each PCR tube separate AD primers (AD1, AD2, AD3 and AD6) were used versus nested specific primer. T-DNA flanks were amplified with 1 unit of *Taq* DNA polymerase using 10–30 ng of genomic DNA as a template. This technique uses the advantage of high and low annealing temperatures to amplify the target sequences and consists of three rounds reaction. First round starts with 5 cycles with high annealing temperatures where insertion specific primer binds and linear amplification occurs producing specific single stranded products. Second round consists of 1-2 cycles of low annealing temperature and producing primer specific target sites. Further tail cycling preferred target site is amplified which consists of 15 super cycles in which each single super cycle has two cycles with high annealing temperature and one cycle with low annealing temperature. Three types of products *viz.*, type-1, type-2 and type-3 products are amplified. Type-1 products called specific products primed by insertion specific primer on one side and a non-specific AD primer on other side. Type two products are called non-specific primers primed on both sides by insertion specific primers. Type-3 products are also called nonspecific products primed on both sides by non-specific AD primers on both sides (Fig. 2). The TAIL-PCR was performed using a BIO-RAD T100™ Thermal Cycler with thermal conditions as detailed in Table 9b and reaction mixture as detailed in Table 9a. In order to increase the amount of specific target products, second round of TAIL-PCR was performed with insertion specific nested primers which as explained by Singer and Burke (2003). Third round TAIL-PCR products were run on agarose gel, single band amplification PCR products were directly taken for sequencing by purifying Third round PCR products using MinElute PCR Purification Kit (Cat No./ID: 28004) according to the manufacturer's recommendation and upon getting multiple bands, only the desired amplified product is eluted using MinElute Gel Extraction Kit (Cat No./ID: 28604), and the eluted products were used for sequencing with care taken to get the eluted product in sufficient quantity of DNA concentration as mentioned by sequencing protocols. The PCR products were sequenced using Big Dye terminator v 3.1 cycle sequencing (sequencing chemistry) kit user guide in ABI (Applied Biosystems) 3500\3500 Genetic analyser capillary electrophoresis.

3.3.1 Primary TAIL-PCR

The flanking genomic regions to right border in transgenic plant was amplified through TAIL-PCR assay. In primary TAILPCR, right border specific reverse primer RB1 (5'TTCACTCGAACGACGTCACC-3') and AD1 (5'-NGTCGASWGANAWGAA-3), AD2 (5'-TGWGNAGSANCASAGA-'), AD3(5'-AGWGNAGWANCAWAGG-3) and AD6 (5-GTGNAGWANCANAGA-3) arbitrary primers were used. PCR reaction components for primary TAIL-PCR were mixed as shown in Table 9a, the PCR assay protocol followed for primary TAIL-PCR is as detailed in Table 9b. After the reaction, the primary TAIL-PCR product was checked using electrophoresis on 1% agarose gel. PCR product of primary TAIL-PCR was diluted in 1:10 ratio with sterile water and used as template for secondary TAIL-PCR.

3.3.2 Secondary TAIL-PCR

Secondary TAIL-PCR was performed to amplify flanking genomic region with higher specificity using primary TAIL-PCR product as a template. For secondary TAIL-PCR, right border sequence specific reverse primer RB2 with AD1, AD2, AD3 and AD4 arbitrary primers were used. PCR reaction components for secondary TAIL-PCR were mixed as shown in Table 9a, the PCR assay protocol followed for secondary TAIL-PCR is as detailed in Table 9b. After the reaction, the secondary TAIL-PCR product was checked using electrophoresis on 1% agarose gel. PCR product of secondary TAIL-PCR was diluted in 1:10 ratio with sterile water and used as template for tertiary TAIL-PCR.

Table 8a: Sequences and characteristics of the AD primers used for TAIL-PCR

Sl. No.	**Primer name**	**Sequence (5’-3’)**	**Average T_m (^{0}C)**	**Length**	**Degeneracy**
1	AD1	5'-NGTCGASWGANAWGAA-3	46.6^0 C	16 bp	128-fold
2	AD2	5'-TGWGNAGSANCASAGA-3’	49.2^0 C	16 bp	128-fold
3	AD3	5'-AGWGNAGWANCAWAGG-3	46.6^0 C	16 bp	128-fold
4	AD6	5.-WGTGNAGWANCANAGA-3	45.3^0 C	16 bp	256-fold

W=A or T, S=G or C, N= A or T OR G or C.

AD: arbitrary degenerate

Table 8b: Sequences and characteristics of the, specific primers near to right border of T-DNA (NRB)

Sl. No.	**Primer name**	**Sequence (5’-3’)**	**Average T_m (^{0}C)**	**Length**	**GC%**
1	NRB1	5’ TTCACTCGAACGACGTCACC 3’	60.32^0 C	20 bp	55
2	NRB2	5’ GCTGTATGCGTTGGTGCAAT 3’	59.8^0 C	20 bp	50
3	NRB3	5, AATCTTGCTCGTCTCGCTGG 3’	58.6	20 bp	55
4	NRB4	5’ CAGATCTGGGGAACCCTGTG 3’	59.7^0 C	20 bp	60

Right border specific for T-DNA binary vector pBI 121

NRB: near to right border

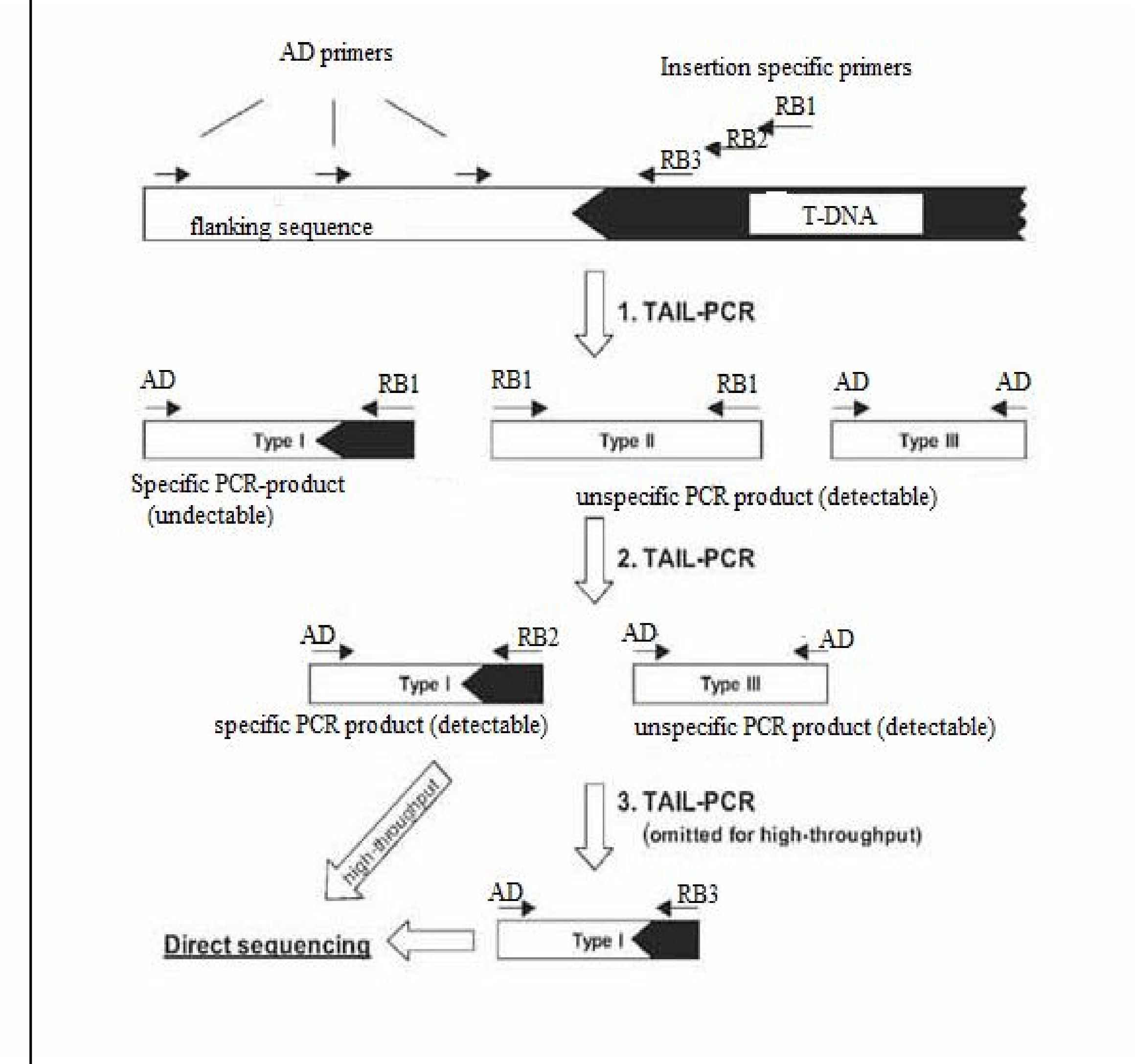


Fig 2: Schematic representation of primer binding sites in TAIL-PCR and TAIL-PCR products. One side of the T-DNA insertion and the T-DNA specific insertion primers (RB 1, RB2, RB3) are shown exemplary for any T-DNA insertion. For clarity only, one side of the T-DNA insertion and the RB primers are depicted

3.3.3 Tertiary TAIL-PCR

Tertiary TAIL-PCR was performed to amplify flanking genomic region in a more specific manner using secondary TAIL-PCR product as a template. For Tertiary TAIL-PCR right border sequence specific reverse primer RB4 with AD1, AD2, AD3 and AD4 arbitrary primers were used. PCR reaction components for tertiary TAIL-PCR were mixed as shown in Table 9a and the PCR assay protocol followed for tertiary TAIL-PCR is as detailed in Table 9b. After the reaction, the PCR product was checked using electrophoresis on 1% agarose gel. The selected amplicon from tertiary TAIL- PCR product,~1kb amplicon for sample Event No.78 was excised from low melting agarose gel (0.8%) with a sharp sterile scalpel blade by keeping gel on low intensity UV-transilluminator. The excised gel containing fragment was collected in a sterile pre-weighted 2 ml microcentrifuge tube. The DNA fragment was extracted from gel using MinElute Gel Extraction Kit (Cat No./ID: 28604).

3.4 Gel purification of TAIL-PCR Products

Protocol for precipitating tertiary Tail-PCR products before loading on agarose gel to be used for sequencing:

Sufficient amount of DNA is needed from tertiary Tail-PCR products, so several rounds of Tertiary TAIL-PCR products were precipated to obtain pure DNA by removing excess primers, nucleotides *etc*. The protocol followed is as given below,

1. The volume of all Tertiary Tail-PCR products was measured and put in to an Eppendorf tube
2. Double the volume of ethanol was added to the above tube
3. 10% percent of 5M sodium acetate was added (according to the total volume of PCR products)
4. The tube was incubated for twenty minutes at -80 ^{0}C
5. It was centrifuged for twenty minutes at 4 ^{0}C @ 13000 rpm

Table 9a: Reaction mixture for successive TAIL-PCR steps (primary, secondary and tertiary)

Reagents/stock solutions	**Primary TAIL-PCR**		**Secondary TAIL-PCR**		**Tertiary TAIL-PCR**	
	1_X Reaction vol	**Final concentration**	**1_X Reaction vol**	**Final concentration**	**1_X Reaction vol**	**Final concentration**
dH20	4.7 µL		5.7 µL		10.4 µL	
10_x PCR buffer	1.0 µL	1_X	1.0 µL	1_X	2.0 µL	1_X
10mM dNTP working solution	0.2 µL	0.2 µM	0.2 µL	0.2 µM	04 µL	0.2 µM
50mM $MgCl_2$	0.3 µL	1.5 µM	0.3 µL	1.5 µM	0.6 µL	1.5 µM
10 µM RB primer	0.2 µL	0.2 µM	0.2 µL	0.2 µM	0.4 µL	0.2 µM
AD primer	**2.5 µL**	**3-4** µM	**1.5** µL	**1.8-2.4** µM	**5 µL**	**3-4** µM
5I/µL Taq polymerase	0.1 µL	0.5 U	0.1 µL	0.5 U	0.2 µL	1 U
Template	1 µL		1 µL		1 µL	
Total vol	10 µL		10 µL		10 µL	

6.

Table 9b: Thermal conditions for TAIL-PCR cycle (primary, secondary tertiary)

Primary TAIL-PCR	**Secondary TAIL-PCR**	**Tertiary TAIL-PCR**
1. 94C for 3 min	1. 94^0C for 3 min	1. 94^0 C for 3 min
2. 94^0C for 30 s.	2. 94^0C for 10s.	2. 94^0 C for 10s
3. 55^0 C for 1 min.	3. 56^0C for 1 min	3. 44^0 C for 1 min
4. 72^0 C for 2.30 min.	4. 72^0C for 2.30 min	4. 72^0 C for 2 min
5. Five cycles ofsteps 3-5	5. Five cycles of **steps 2-4**	**5.** 20 cycles of **steps 2-4**
6. 94^0 C for 30s.	6. 94^0C for 10s	6. 72^0C for 5 min
7. 68^0 C for 1 min.	7. 64^0C for 1 min	7. 4^0 C hold
8. 72^0 C for 2.30 min.	8. 72^0C for 2.30 min	
9. Two cycles of **steps 7-9**	9. 94^0C for 10s	
10. 94^0 C for 10s.	10. 64^0C for 1 min	
11. 68^0 C for 1 min.	11. 72^0C for2. 30 min	
12. 72^0 C for 2.30 min.	12. 94^0C for 10s	
13. 94^0 C for 10s.	13. 44^0C for 1 min	
14. 68^0 C for 1 min.	14. 72^0C for 1 min	
15. 72^0 C for 2.30 min.	15. 15 cycles of **steps 6-14**	
16. 94^0 C for 10s.	16. 94^0C for 10s	
17. 44^0 C for 1 min.	17. 44^0C for 1 min	
18. 72^0 C for 2.30 min.	18. 72^0C for 3 min	
19. 15 cycles of **steps 11-19**	19. Five cycles of **steps 16-18**	
20. 72^0 C for 5 min.	20. 72^0C for 5 min	
21. 42^0 C hold.	21. 4^0C hold	

7. The pellet was washed with 70 % ethanol and centrifuged at room temperature for five minutes at 14000 rpm.

8. The pellet was dried and dissolved in required volume of nuclease free water.

3.4.1 Purification of amplicon by gel elution for sequencing:

1. The tertiary PCR products run after precipitation on agarose gel, produce amplicons which were purified with MinElute Gel Extraction Kit (Cat No./ID: 28604).

2. The band of desired length was cut out from the gel using a sharp sterile scalpel blade by keeping gel on low intensity UV-transilluminator. The excised gel was weighed out and three volumes (three times to amount of excised weight of gel containing band) of QG buffer was added to Eppendorf tube containing the gel with band.

3. The tube was incubated at 60 ^{0}C for 10 min, with intermittent vertexing

4. One volume of isopropanol was added, mix thoroughly and the sample was loaded in to the column provided with kit.

5. The tube was centrifuged for 30 seconds at 13000 rpm at room temperature.

6. The column was washed with required amount of PE buffer and centrifuged for 30 seconds @13000 rpm

7. A second spin of one minute at 13000 rpm was given and the column was transferred to new Eppendorf tube.

8. 20µL of nuclease free water was added and incubated for one minute at room temperature, centrifuged for two minutes and finally DNA was collected in Eppendorf tube

3.4.1.2 TemPlate quantity of gel eluted product of tertiary TAIL-PCR product

After the gel elution of required band of desirable bp, the concentration of eluted DNA was assessed spectrophotometrically in Thermo Scientific™ NanoDrop 2000 and also by gel electrophoresis using 0.8 per cent agarose gel. The quantity of eluted DNA template used in a single cycle sequencing reaction are shown in Table 10. The quantity of PCR product was optimized to maximize the number of primer binding sites for the BigDye™ reaction and is dependent on the length and purity of the PCR product.

Note: In general, higher DNA quantities give higher signal intensities

Table 10: DNA quantities of gel purified product of tertiary TAIL-PCR product

DNA template	Quantity (nano gram)
PCR product	
1000 bp	10-40 ng

3.4.2 Sequencing of TAIL-PCR products

Tertiary TAIL- PCR products were sequenced using standard sequencing procedures. RB3 primer insertion-specific primer was used as the sequencing primer. Sequencing was performed in Genomics lab of Institute of Agricultural Bio-technology (IABT) at UASD according to the preferred protocol where the sequencing reactions were performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit and on the capillary Electrophoresis an Applied Biosystems (ABI)™ 3500/3500xL Genetic Analyzer with 3500 Series Sequencer.

3.4.2.1 Preparation of chemical reaction mixture for Cycle sequencing

The sequencing reactions were set up with the following notes and procedures:

The dye terminators were protected from light. Reaction mix and sequencing Plates were covered with aluminium foil before use.

1. Completely thawed contents of the BigDye™ Terminator v3.1 Cycle Sequencing Kit and primers were kept on ice.
2. The tubes were vortexed for 2 to 3 seconds, then centrifuged (2 to 3 seconds) on a benchtop microcentrifuge to collect contents at the bottom of the tubes.

3. Components were added as indicated in Table 11.
4. Pipette tips were changed after each transfer.
5. 4 µL of the control primer was added to 20 µL reaction mixture.
   Control primer concentration = 0.8pmol/µL.
6. The Plate was sealed with MicroAmp™ Clear Adhesive Film.
7. The Plate was vortexed for 2 to 3 seconds, then centrifuged in a swinging bucket centrifuge (5 to 10 seconds) at 1,000 x g.

3.4.2.2 Cycle sequencing

1. The tubes or Plate(s) were placed in a thermal cycler
2. Cycle sequencing was performed as mentioned in Table 12.

3.4.2.3 Ethanol-EDTA precipitation of Cycle sequencing PCR product

1. A 125 mM EDTA solution was prepared from 0.5 M EDTA, pH 8.0.
2. 70% ethanol was prepared using absolute ethanol.
3. The sequencing Plate was centrifuged in a swinging bucket centrifuge (5 to 10 seconds) at 1,000xg.
4. The MicroAmp™ Clear Adhesive Film was removed from the Plate.
5. The following chemical components were added in order as shown in Table 13.
6. The Plate was sealed with MicroAmp™ Clear Adhesive Film.
7. The Plate was vortexed for 2 to 3 seconds, then centrifuged (5 to 10 seconds) at 1,000 x g.
8. The Plate was incubated at room temperature for 15 minutes.
9. The Plate was centrifuged in a swinging bucket centrifuge at 1,870 x g (4°C) for 45 minutes.

Table 11: Components used for cycle sequencing step

Component	Standard reaction (20µL)		
	Quantity per reaction	Forward	Reverse
BigDye™ Terminator 3.1 Ready Reaction Mi	8 µL	8µL	8 µL
Forward primer (3.2 µM	3.2 pmol	2 µL	-
Reverse primer (3.2 µM)		-	2 µL
Deionized water (RNase/DNase-free)	Varies based on template and primer volume	8 µL	8 µL
Template	**		
Total volume	20 µL	20 µL	20 µL

Table 12: PCR Programme for cycle sequencing

Parameter	Stage\step				
	Incubate	25 cycles			Hold
		Denature	Anneal	Extend	
Ramp rate	-	1^{0}C/second			
Temperature	96^{0}C	96^{0}C	50^{0}C	60^{0}C	4^{0}C
Time (mm:ss)	1.00	00.10	00.05	04.00	Hold until ready to purify

10. The MicroAmp™ Clear Adhesive Film was slowly removed to prevent disruption of the pellet 4 layers of absorbent paper was placed into the centrifuge and the Plate was inverted carefully onto the paper without dislodging the pellet. Centrifuged at 185 × g for 1 minute.

11. 6µL of 70% ethanol was added to each well.

12. The Plate was sealed with MicroAmp™ Clear Adhesive Film and centrifuged at 1,870xg (4°C) for 15 minutes.The MicroAmp™ Clear Adhesive Film was removed slowly to prevent disruption of the pellet. 4 layers of absorbent paper was placed into the centrifuge and the Plate inverted carefully onto the paper towel without dislodging the pellet. Centrifuged at 185xg for 1 minute.

13. The Plate was allowed to air dry, by facing up and protected from light, for 5 to 10 minutes at room temperature.

14. The Plate was sealed with MicroAmp™ Clear Adhesive Film, stored at 4°C and protected from light,

Table 13: Chemical components for ethanol/EDTA precipitation

Component	**Volume per 20 µL reaction**
sequencing reaction (starting volume	20 µL reaction
125 mM EDTA solution	5 µL
absolute ethanol	60 µL
Total volume	85 µL/well

EDTA was dispensed directly into the sample in centrifuge Plate wells and was centrifuged to ensure that the EDTA mixes well with the sequencing reactions.

3.4.3 Sequencing of TAIL-PCR product

The tertiary TAIL-PCR product was sequenced according to the BigDye™ Terminator v3.1 Cycle Sequencing Kit on the capillary Electrophoresis an Applied Biosystems (ABI)™ 3500/3500xL Genetic Analyzer with 3500 Series Sequencer. NRB4 (Table 8b) insertion specific primer on T-DNA was used as sequencing primer.

3.4.3.1 Sequence analysis

The sequences were then analysed using BLAST tools in NCBI database

3.4.4 Primers designing for left border flanking and PCR confirmation of left border flanking region

The right border flanking sequence was identified from TAIL-PCR and point of integration of T-DNA in Cotton genome was known by BLAST analysis. Primer for left border flanking sequence of inserted T-DNA designed (using NCBI primer design tool) away from the point of insertion of T-DNA, by taking care of probability of loss of bp at junction of T-DNA integration in cotton genome. LBFl primers (left border flanking) are listed in Table 14 and Primers were designed for left border of T-DNA as NLB primers (near to left border) listed in Table 15. Schematic representation of LBFl primers on Cotton genome and NLB primers on inserted T-DNA region presented in Fig. 3. PCR was done with NLB primers and LBFl primers, the details of the components used for PCR and thermal conditions followed for the PCR cycle, are mentioned in (Table 6) and Table 16, respectively.

Table 14: List of Left border flanking primers (LBFL)

Sl. No	Primer name (r)	Sequence (5’-3’)
1	LBFl 5	**
2	LBFl 6	**

r: reverse primer and ** sequence information of flanking primers not published because UASD Bt Cotton Event No.78 needs to be patented.

Table 15: List of Near to left border primers (NLB)

Sl. No.	Primer name (f)	Sequence (5’-3’)
1	NLB 1	5’GGTTCACGTAGTGGGCCATC3’
2	NLB 2	5’TTTTCGCCCTTTGACGTTGG3’
3	NLB 3	5’CACTCAACCCTATCTCGGGC3’

f: forward primer

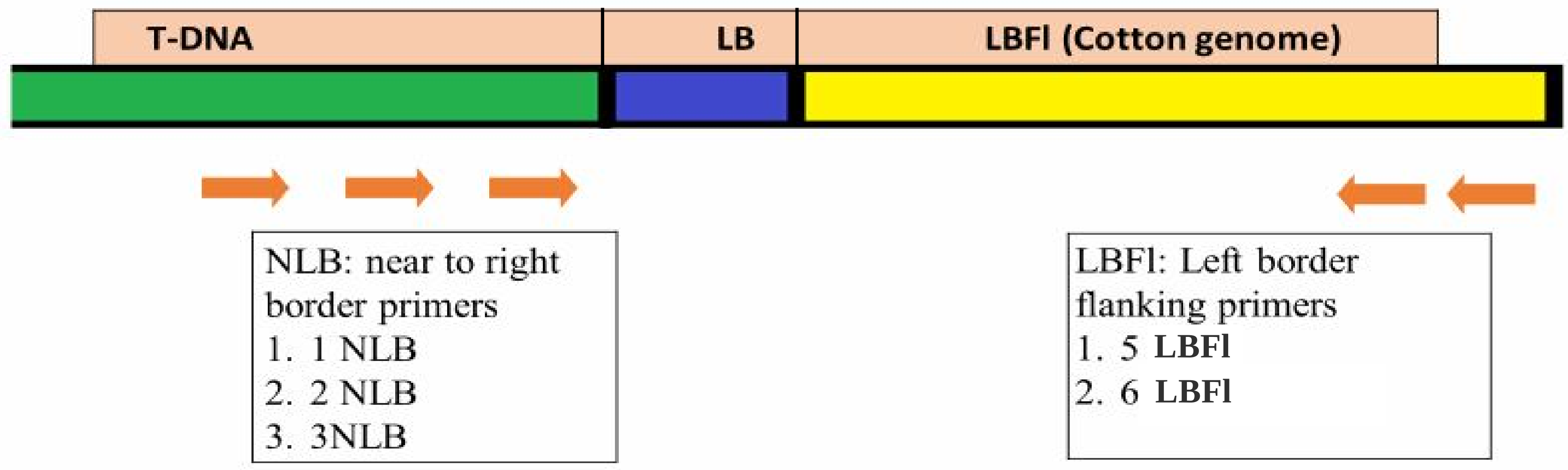


Fig 3: Schematic representation of primers designed on LBFl (left border flanking)

Table 16: PCR programme for confirmation of left border flanking region

Sl. No.	Step	Temperature (^{0}C)	Duration (min)	No. of. cycles
I	Initial Denaturation	95.0	5	1
II	1. Denaturation	94.0	0.40	
	2. Annealing.	53.5	2.00	33
	3. Extension.	72.0	1.30	
III	1. Final extension	72.0	10.0	1
	2. Hold	4.0	-	-

3.4.5 PCR confirmation of right border flanking sequence.

Primers were designed for the sequence of right border flanking region (identified from TAIL-PCR) on Cotton genome using NCBI primer design tool (Table 17). NRB primers (Table 8b) and the primers designed for right border flanking were used in PCR reaction for the DNA of Event No.78. Components used for PCR are mentioned in Table 6. PCR programme for confirmation of right border flanking sequence is shown in Table 18. The amplified PCR product was sent for sequencing through outsourcing.

Table 17: Right border flanking primers (RBFL) list

Sl. No.	Primer name (f)	Sequence (5'-3')
1	RBFl 1	**
2	RBFl 2	**
3	RBFl 3	**
4	RBFl 4	**

f: forward primer and ** sequence information of flanking primers is not published because UASD Bt Cotton Event No.78 needs to be patented

Table 18: PCR programme for confirmation of right border flanking region

Sl. No.	Step	Temperature (^{0}C)	Duration (min)	No. of. cycles
I	Initial Denaturation	95.0	5	1
II	1. Denaturation	94.0	0.40	33
	2. Annealing.	55	2.00	
	3. Extension.	72.0	1.30	
III	1. Final extension	72.0	10.0	1
	2. Hold	4.0	-	-

3.4.6 Sequence analysis of right border flanking sequence.

BLSTn analysis was done using sequence data of RBFl obtained by using specific primer in place of AD primer, against specific primer of T-DNA (NRB).

3.5 Zygosity study

A diploid organism is homozygous at a gene locus when two identical alleles of the gene are present. A plant is called homozygous for a particular gene when both alleles at given locus are similar (dominant or recessive). A homozygous plant maintains a high degree of consistency for particular characters determined by the gene throughout the subsequent generations (true to type progenies-pure lines). In a breeding program, homozygous lines have many advantages such as uniformity in maturity, height and in all yield attributing characters. High yielding pure line (homozygous) method of breeding crop varieties is therefore preferred by the plant breeders wherever feasible. Whenever transgenic plants undergo sexual reproduction “selfing”, they give rise to plants of diverse genotypic constitutions - hemizygous, homozygous and negative for the transgene. As per Mendelian genetics, single gene segregating population (monohybrid) produce 1:2:1 (genotypic ratio) for transgene. This indicates that only 25% plants are homozygous for transgene (dominant or recessive forms each) and are further useful for crop improvement. There are few reports where heterozygous and homozygous plants may have different phenotypes (depending on degree of dominance) and or due to a transgene dosage effect. Therefore, it is necessary to identify homozygous and heterozygous plants among the descendants of each original transgenic plant by designing the primers to the flanking sequence of T-DNA which was identified by the TAIL-PCR method (Nishant, 2016).

3.5.1 Primers used for zygosity study

Primer P {Right border flanking primers (RBFl) (Table 17)} and Q {Left border flanking primers (LBFl) (Table 14)} are flanking sequence primers. Primer S {Near to right border (NRB) {Table 8b} and away from right border (ARB) (Table 19)} are transgene specific primers (Fig. 4).

Table 19: Away from right border primers (ARB) list

Sl. No.	Primer name(r)	Sequence (5’-3’)
1	ARB 1	5’-CAGACAATCGGCTGCTCTGA-3’
2	ARB 2	5’-AAAAGCGGCCATTTTCCACC-3’
3	ARB 3	5-TCGGTCATTTCGAACCCCAG-3’
4	ARB 4	5’-TCGGTCATTTCGAACCCCAG-3’

r-reverse primer

3.5.2 Method

Three sets of primers were used in zygosity study, first one is being common on right border flanking sequence denoted as P primers (Table 17). Among the remaining 2 primers, one is specific primer to the inserted T-DNA denoted as S primers (Table 8b and Table 19), other is left border flanking sequence denoted as Q primer (Table 14). These three sets of primers were tested with DNA of Event No.78, F_1 of Event No.78, commercial Bt hybrid of Event MON 531 (Ajeet 1190 and Ajeet 1192) and RAH-100 (as negative non GM) to identify best 3 primers combination for single PCR (primer multiplexing) reaction which will differentiate the homozygous transgenic plant (Event No.78), hemizygous transgenic plant (F_1 of Event No.78), wild plant (RAH-100) and commercial transgenic hybrid Event MON 531 (Ajeet 1900 and Ajeet 1192). Schematic representation of zygosity evaluation is shown in Fig. 5.

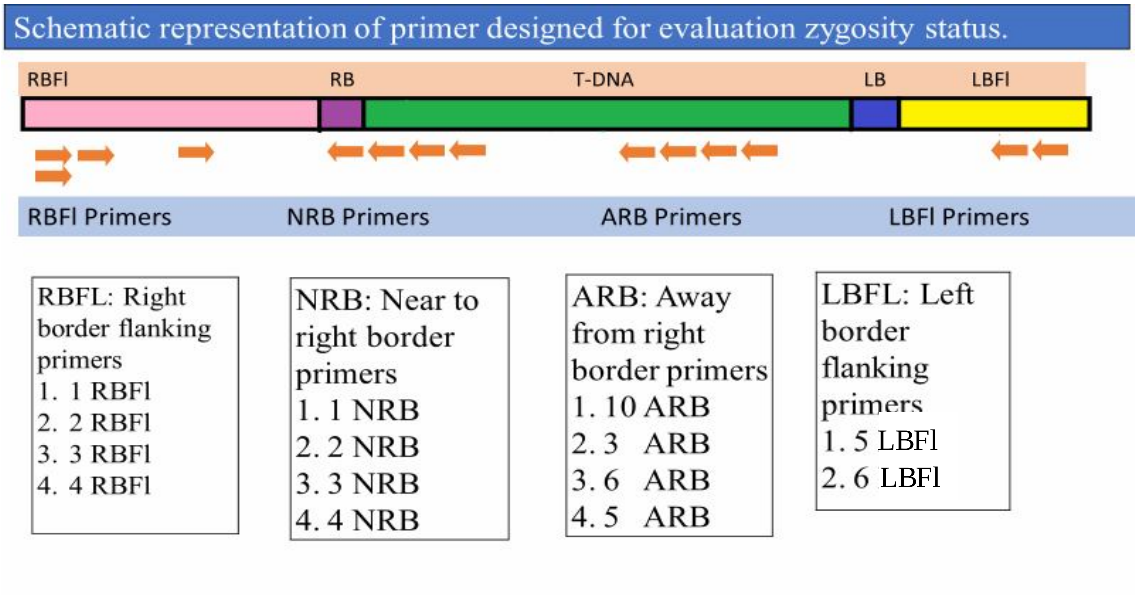


Fig. 4: Schematic representation of primer designed for evaluation of zygosity status

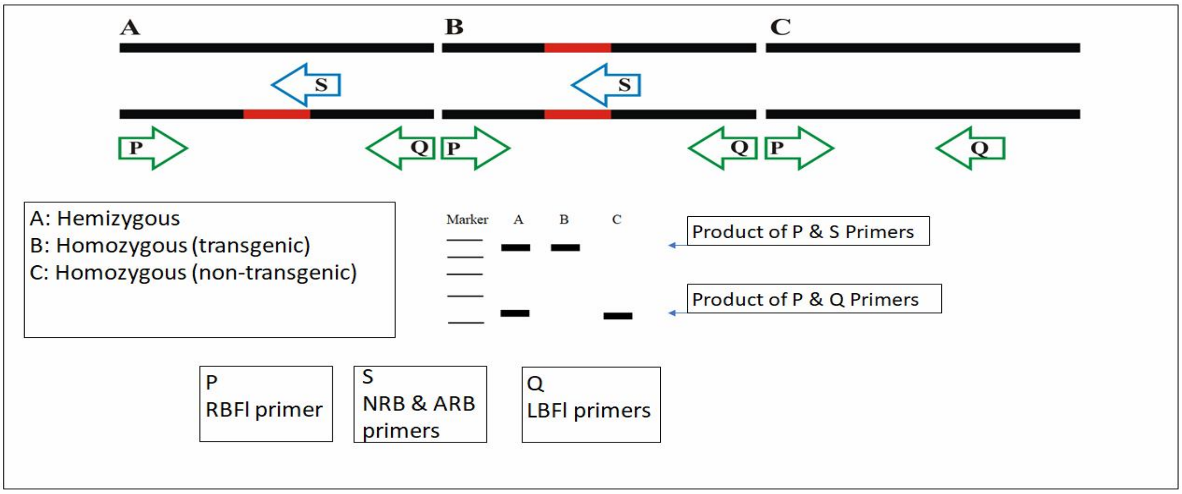


Fig 5: Schematic representation of zygosity evaluation. P set of primer as forward, S set of primers as reverse and Q set of primers as reverse in a single PCR (primer multiplexing) here P set of primer is common which will amplify with S set of primer in homozygous transgenic plant and also with Q set of primer in hemizygous transgenic plants and non-transgenic or other transgenic events. vice versa can also be designed (*i.e.* Q primer as common for P and S set of primers but here care has to take that S set of primer now has designed as forward)

4. EXPERIMENTAL RESULTS

The results of laboratory experiments on Bt cotton Event No.78 with respect to transgene confirmation, TAIL-PCR to find the flanking sequence of the inserted T-DNA and confirmation of zygosity status were carried out at Agriculture research station, Dharwad farm and IABT during 2018-19 are represented under this chapter.

4.1 Polymerase Chain Reaction based confirmation of transgenic events

4.1.1 PCR analysis of transgenic events with *Cry* 1Ac

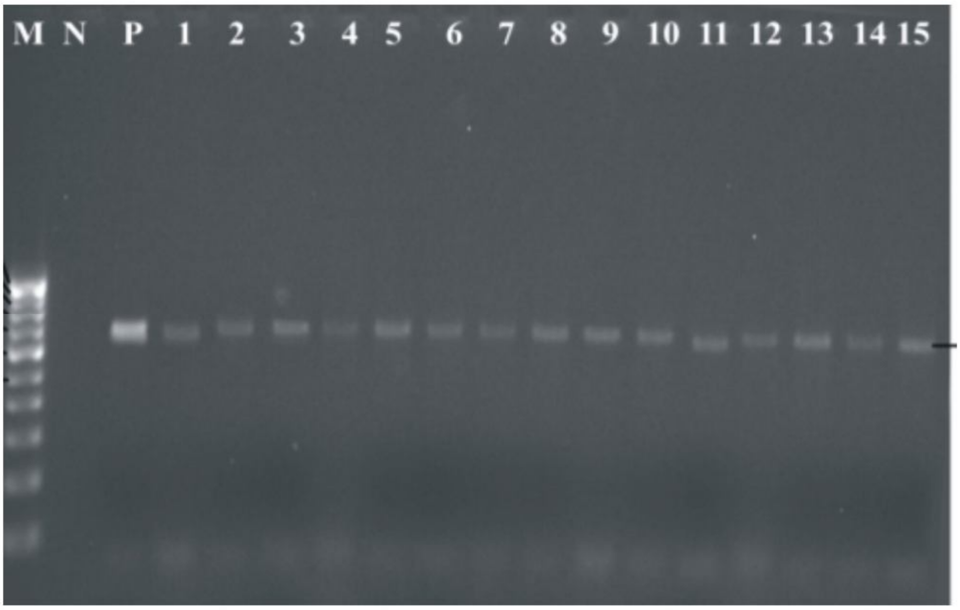
In the present study of UASD Bt cotton Event, No.78 was used for the characterization. Event-78 was transformed with *Cry* 1Ac with double promoter gene obtained from ICGEB (International Centre for Genetic Engineering and Biotechnology). PCR tests involved the amplification of the DNA with transgene specific primers (Table 5). The amplification of the genomic DNA to show the expected amplicon size indicates the presence of the transgene. Event-78 showed positive amplification of the corresponding *Cry* gene and confirmed the presence of *Cry* 1Ac gene in this Event No.78 (Plate 1).

4.2 TAIL-PCR analysis

The site of insertion of T-DNA/*Cry* 1AC in the cotton genome was identified by recovering the genomic sequence flanking the right border (RB) of T-DNA by TAIL-PCR in Event No.78. Primary TAIL-PCR produced a smear with NRB1 _AD1, AD2, AD3 and AD4 (Plate 2a). Secondary TAIL-PCR showed multiple bands with NRB2 _ AD1, AD2, AD3 and AD4 (Plate 2b). However, the tertiary TAIL-PCR amplified an amplicon of 1kb bp and 700 bp with NRB4_AD4 (Plate 2c).

4.2.1 Sequencing of third TAIL-PCR product

The tertiary TAIL-PCR product was run on 0.8% agarose gel for elution of 1kb bp (Plate 3). The eluted product of 1kb bp was sequenced with NRB4_AD4 by using capillary electrophoresis genetic analyser 3500/3500 using BigDye™ Terminator v3.1



M:100 bp DNA ladder
N: Negative control (Non-transgenic plant)
P: Positive control (plasmid DNA "pBI121"
1 to 15: Cotton transgenics harbouring *Cry* 1Ac gene

Plate 1. PCR confirmation of UASD Bt Cotton transgenic Event No.78

L 100 b. p ladder
1.NRB1_AD1
2.NRB1_AD2
3.NRB1_AD3
4.NRB1_AD4

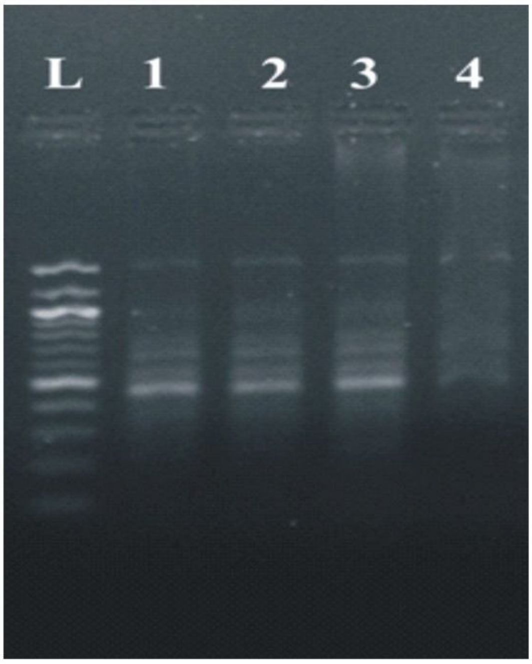
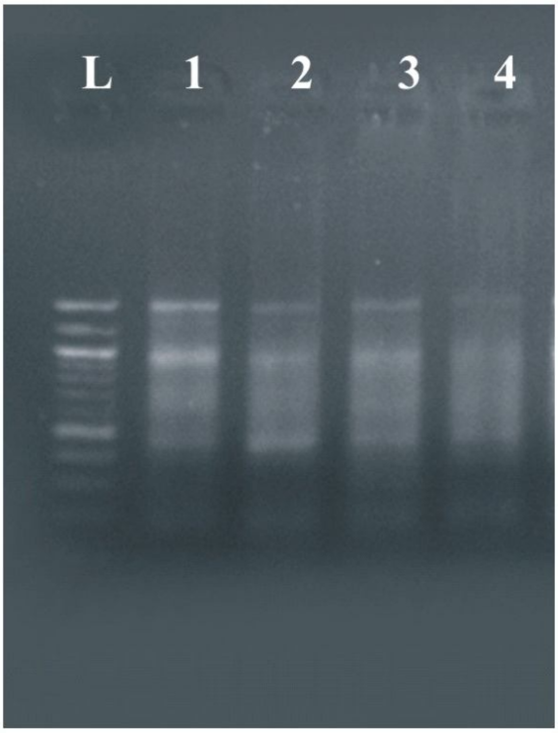
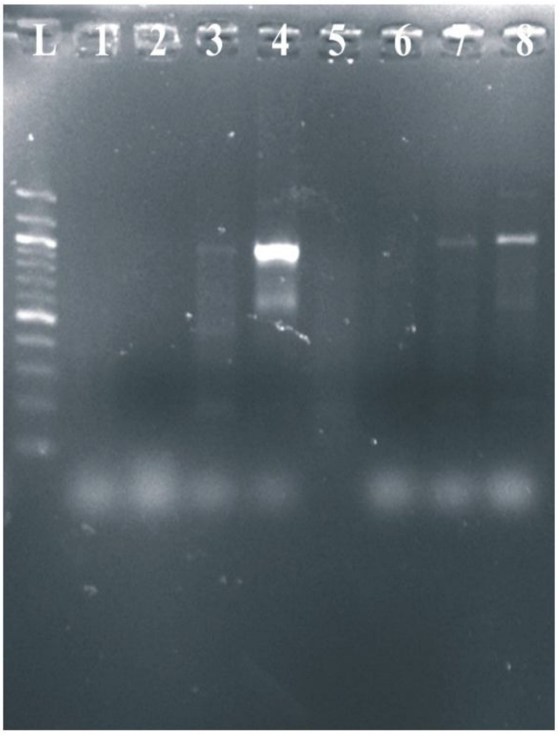


Plate 2a: Products of primary TAIL-PCR of Event No.78 (*Cry* 1Ac) transgenic plant with Near to right border specific primer and arbitrary degenerate primers



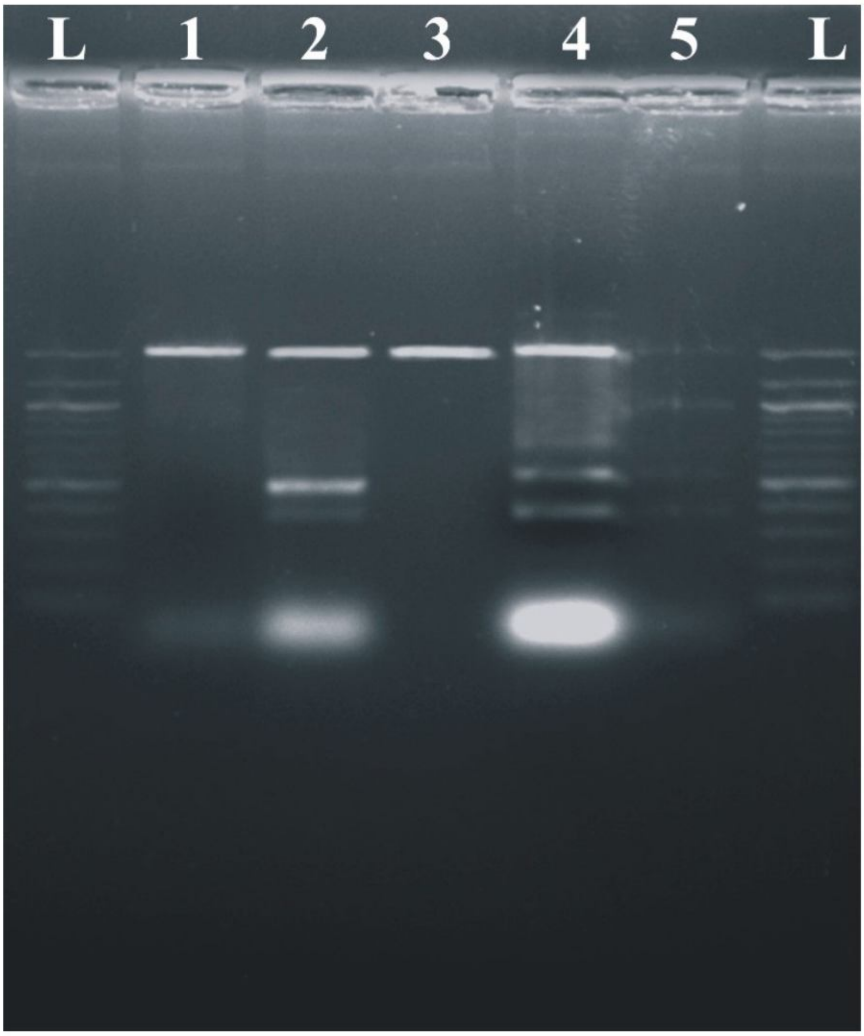
L 100 b.p ladder
1. NRB2_AD1
2. NRB2_AD2
3. NRB2_AD3
NRB2_AD6

Plate 2b: Products of secondary TAIL-PCR of Event No.78 (*Cry* 1Ac) transgenic plant with Near to right border specific primer and arbitrary degenerate primers



L 100 bp ladder
1. RB4_ AD1
2. RB4_AD2
3. RB4_AD3
4. RB4-AD4
5. RB4_AD1
6. RB4_AD2
7. RB4_AD3
8. RB4-AD4

Plate 2c: Products of tertiary TAIL-PCR of Event No.78 (*Cry* 1Ac) transgenic plant with Near to right border specific primer and arbitrary degenerate primers



L 100 bp ladder
1. NRB4-AD4
2. NRB4-AD4
3. NRB4-AD4
4. NRB4-AD4
5. NRB4-AD4

Plate 3: Gel elution of Products of tertiary TAIL-PCR of Event No.78 (*Cry* 1AC) transgenic plant with NRB4 _AD4.

Cycle Sequencing Kit User Guide. The Sequence information obtained by sequencing has not been presented as the event needs to be patented.

4.2.2 Sequence analysis

The BLAST result of Right border flanking sequence of 713 bp showed homology to *Gossypium hirsutum* accessions. From the BLAST analysis data, it was identified that out of 713 bp sequences, from 28 to 691 bp was covered the *Gossypium hirsutum* genome.

The Right border flanking sequence of 713 bp when BLAST analysis was done against pBI121, showed homology from 688 to 708 bp from the point of 2362 to 2343 of pBI121 (Genbank acc. No. AF485783.1) (Fig. 6).

4.2.3 PCR Confirmation of left border flanking sequence amplification

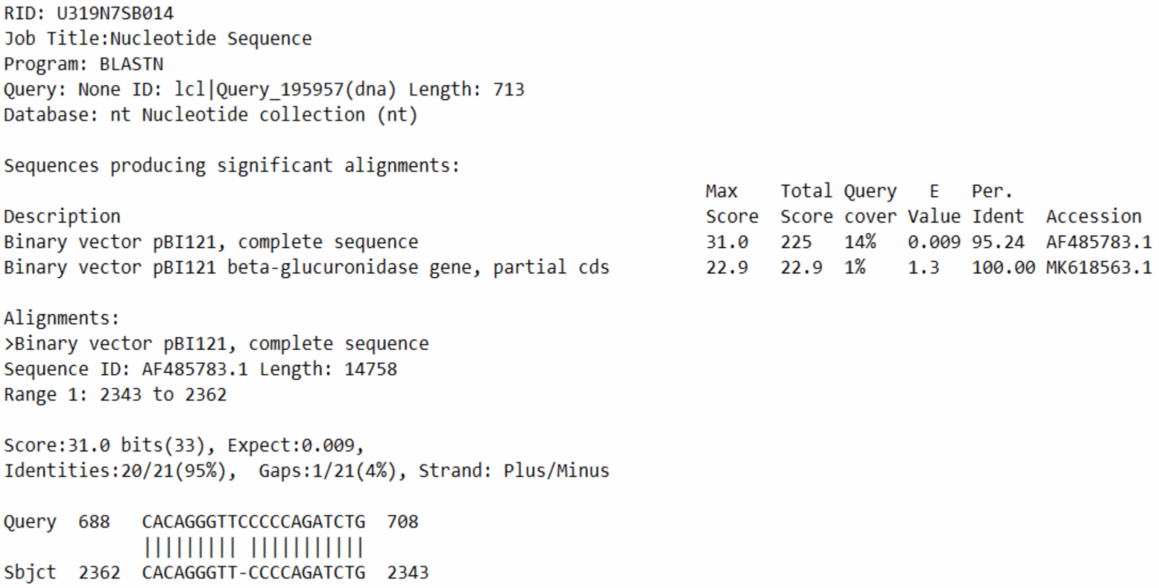
PCR carried out with near to left border (NLB) specific primer (as a forward primer) and left border flanking (LBFl) sequence primer (as a reverse primer) resulted in an expected amplicon of 500bp and 600bp when DNA from Event N.78 was used as a template (Plate 4a and 4b). The PCR, when performed using non-Bt DNA (RAH-100), did not show amplification.

4.2.4 PCR Confirmation of right border flanking sequence amplification

PCR with near to right border (NRB) specific primer (as reverse) of inserted T-DNA and right border flanking primers which were designed using sequence information obtained by sequencing TAIL-PCR product, gave an expected base pair of 550bp when DNA from Event No.78 was used as a template (Plate 5).

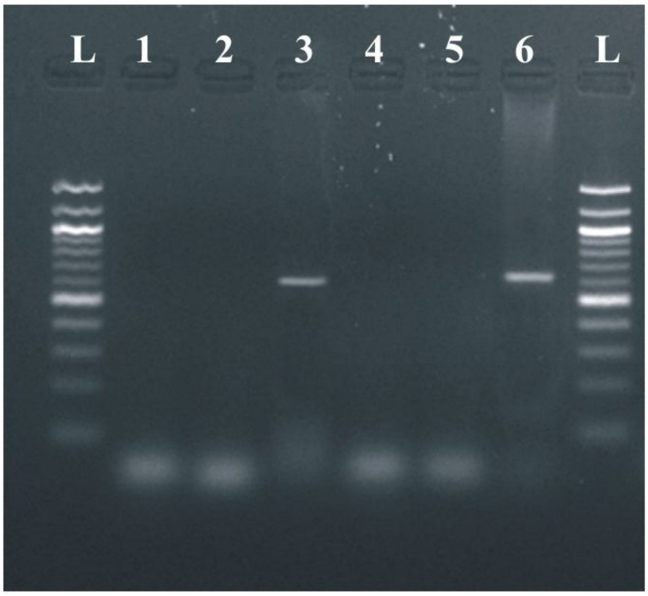
4.2.5 Reconfirmation of Right border flanking sequence through outsourcing.

The PCR amplicon obtained using RBFl 4 (a forward primer on flanking sequence) and NRB 4 (a reverse primer on T-DNA) was outsourced for sequencing. The 550bp sequence was aligned to the TAIL-PCR sequence of 713bp by sequence alignment software of NCBI which revealed 88.45 % homology.



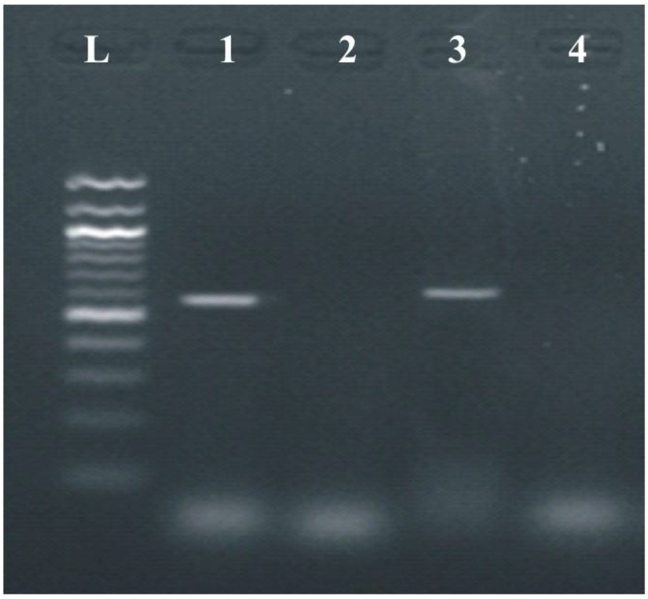
```
RID: U319N7SB014
Job Title:Nucleotide Sequence
Program: BLASTN
Query: None ID: lcl|Query_195957(dna) Length: 713
Database: nt Nucleotide collection (nt)

Sequences producing significant alignments:
                                                                  Max    Total Query   E    Per.
Description                                                       Score  Score cover Value Ident  Accession
Binary vector pBI121, complete sequence                           31.0   225   14%   0.009 95.24  AF485783.1
Binary vector pBI121 beta-glucuronidase gene, partial cds         22.9   22.9  1%    1.3   100.00 MK618563.1

Alignments:
>Binary vector pBI121, complete sequence
Sequence ID: AF485783.1 Length: 14758
Range 1: 2343 to 2362

Score:31.0 bits(33), Expect:0.009,
Identities:20/21(95%),  Gaps:1/21(4%), Strand: Plus/Minus

Query  688   CACAGGGTTCCCCCAGATCTG  708
             ||||||||| |||||||||||
Sbjct  2362  CACAGGGTT-CCCCAGATCTG  2343
```

Fig. 6: Blast result of 713bp sequences against pBI 121



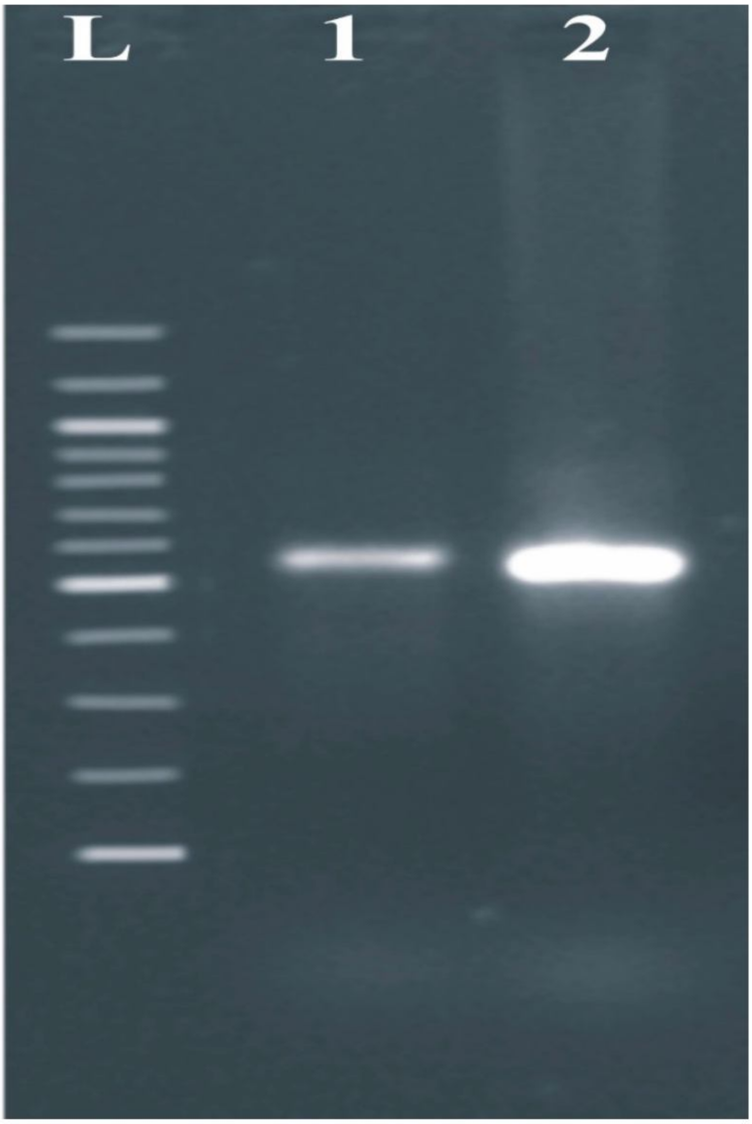
Sl.no	Primer combination	Type of DNA used
1.	NLB 1F_ 5R LBFl	Bt DNA of Event No.78
2.	NLB 2F_ 5R LBFl	Bt DNA of Event No.78
3.	NLB 3F_ 5R LBFl	Bt DNA of Event No.78
4.	NLB 1F_ 6R LBFl	Bt DNA of Event No.78
5.	NLB 2F_ 6R LBFl	Bt DNA of Event No.78
6.	NLB 3F_ 6R LBFl	Bt DNA of Event No.78
L.	100 bp Ladder	

Plate 4a: Confirmation of left border flanking sequence amplification by using NLB primers with LBFl



1	NLB 3F_ 5R LBFl	Bt DNA of Event No.78
2	NLB 3F_ 5R LBFl	non Bt DNA(RAH-100)
3	NLB 3F_ 6R LBFl	Bt DNA of Event No.78
4	NLB 3F_ 6R LBFl	non Bt DNA (RAH-100)

Plate 4b: Confirmation of amplified primers of NLB primers of T-DNA and left border flanking primers



Sl.no	Primer combination		Type of DNA used
L.	100 bp Ladder		
1.	RBFl 1F_ 4R	NRB	Bt DNA of Event No.78
2.	RBFl 1F_ 4R	NRB	Bt DNA of Event No.78

Plate 5: The PCR product was directly sequenced with with RBFl 1F_ 4R NRB

The BLAST analysis of this sequence again hit to same *Gossypium hirsutum* which was done earlier using TAIL-PCR sequence. The BLAST analysis of Event No.78 sequence data against pBI121 again aligned at bp starting from 2362 to 2343 (Fig. 7) which was in the coordination with the sequence data obtained through TAIL-PCR. These results reconfirmed the flanking sequence information obtained in the present study.

4.3 Identification of primer combination for multiplexing and zygosity studies

4.3.1 Evaluation of P, S and Q set of primers

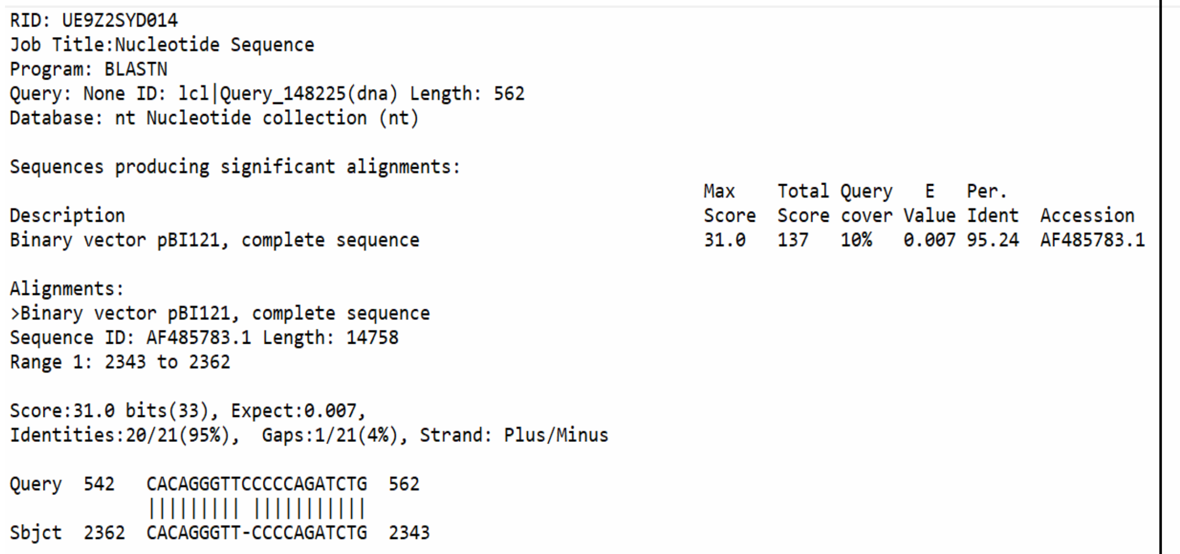
PCR amplification with Event No.78 DNA using P, S and Q (as detailed in the Materials and methods) set of primer combinations produced an expected amplicon size of different bps.

4.3.1.1 Evaluation of P and S set of primers in Event No.78

Results of the PCR of P and S set of primers with the DNA of F_1 derived from Event No.78 amplified amplicons of the size 550bp (RBFl1F with 4RNRB), 1.1kb (RBFl1F with 2RARB), 650bp (RBFl4F with 1R), 600bp(RBFl 4Fwith 2R NRB), 500bp (RBFl 4F with 3R NRB), 200bp (RBFl 4F with 4R NRB) and 1kb (RBFl 4F with 3R ARB) (Plate 6). The P and S set of primers which showed amplification were reconfirmed by repeating the PCR amplification. The results of this PCR along with amplicon size is presented in Plate 7a and 7b. The results of PCR with primer combinations P and S set of primers (RBFl 4F with 4R NRB and RBFl 1F with 3R) amplified an amplicon of 650bp and 1.1 kb, respectively (Plate10a and 10b) only in Bt DNA of Event No.78.

4.3.1.2 Evaluation of P and Q set of primers in F_1 of Event No.78

Results of PCR of P and Q set of primers with the DNA of F_1 derived from Event No.78 produced amplicons of size 550bp (RBFl 1F with 5R LBFl), 500bp (RBFl 1F with 6R), 220bp (RBFl 4F with 5R LBFl) and 210BP (RBFl 4F with 6R LBFl) only in F_1 Bt DNA (Plate 8). The results were once again reconfirmed by repeating the PCR using the P and Q set of primers (Plate 9).



```
RID: UE9Z2SYD014
Job Title:Nucleotide Sequence
Program: BLASTN
Query: None ID: lcl|Query_148225(dna) Length: 562
Database: nt Nucleotide collection (nt)

Sequences producing significant alignments:
                                                                  Max    Total Query   E    Per.
Description                                                       Score  Score cover Value Ident  Accession
Binary vector pBI121, complete sequence                           31.0   137   10%   0.007 95.24  AF485783.1

Alignments:
>Binary vector pBI121, complete sequence
Sequence ID: AF485783.1 Length: 14758
Range 1: 2343 to 2362

Score:31.0 bits(33), Expect:0.007,
Identities:20/21(95%),  Gaps:1/21(4%), Strand: Plus/Minus

Query  542   CACAGGGTTCCCCCAGATCTG  562
             ||||||||| |||||||||||
Sbjct  2362  CACAGGGTT-CCCCAGATCTG  2343
```

Fig. 7: BLAST result of Right border flanking against Pbi121 Binary vector

4.3.1.3 Evaluation of P and Q set of primers in Negative control {RAH-100 and MON 531 (Ajeet 1192 and Ajeet 1190)}

Results of PCR of P (PRBFl 4F) and Q (6R NRB) set of primers produced an amplicon of size of 210bp in F_1 Bt DNA of Event No.78, non-Bt DNA (RAH-100), and MON 531 (Ajeet 1192 and 1190) showed in Plate 11a.

PCR with primer combination P (RBFl 1F) and Q (6R ARB) produced an amplicon of size of 550bp in F_1 Bt DNA of Event No.78, non-Bt DNA (RAH-100), and MON 531 (Ajeet 1192 and 1190) showed in Plate 11b.

The PCR results for P, S and Q set of primers produced different amplicon size is as detailed in Table 20.

Table 20. Amplicon size of PCR of P, S and Q set of primers with respective amplicon size

Sl. No	Primer combination	Amplicon size
1.	RBFl 1F-4R NRB (P_S)	550 bp
2.	RBFl 1F_3R ARB (P_S)	1.1kb
3.	RBFl 4F_3R ARB (P_S)	1 kb
4.	RBFl 4F_1R NRB (P_S)	650bp
5.	RBFl 4F_2R NRB (P_S)	600bp
6.	RBFl 4F_3R NRB (P_S)	500bp
7.	RBFl 4F_3R NRB (P_S)	200bp
8.	RBFl 1F_5R LBFl (P_Q)	550bp
9.	RBFl 1F_6R LBFl (P_Q)	500bp
10.	RBFl 4F_5R LBFl (P_Q)	220bp
11.	RBFl 4F_5R LBFl (P_Q)	2210bp

4.3.2 Identification of P, S and Q sets of primers for primer multiplexing.

Out of the above listed primers, two sets of 3 primers (one each from P, S and Q) are identified which clearly differentiates the homozygous (UASD Bt Cotton Event NO.78), hemizygous (F_1 derived of UASD Bt Cotton Event No.78), wild (RAH-100 non transgenic) and commercial Bt Cotton hybrid Event MON 531 (Ajeet 1190 and Ajeet 1192). The results of PCR amplification of these two sets of primer combinations is confirmed and described as under.

4.3.2.1 Set I primer multiplexing for zygosity studies.

4.3.2.1.1 PCR confirmation of set I primer multiplexing in event No.78

Only one amplicon of 600bp size amplification was observed in Event No.78 plants. This amplicon was the result of RBFl 4F (P) and NRB 4R (S) primers (lane 1 of Plate 12).

4.3.2.1.2 PCR confirmation of set I primer multiplexing in F_1 of Event No.78.

In F_1 of Event No.78, two amplicons of 600bp and 210bp were observed. The 600bp is an amplicon derived by RBFl 4F (P) and NRB 4R (S) primers, which is from the chromosome carrying *Cry* 1 Ac gene.

The 210 bp amplicon is the result of RBFl 4F (P) and LBFl 6R (Q) primers located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome with out *Cry* 1Ac gene in the hemizygous F_1 DNA (lane 2 of Plate 12).

4.3.2.1.3 PCR confirmation of set I primer multiplexing in RAH-100.

In the RAH-100, one amplicon of 210bp was observed. This amplicon is the result of RBFl 4F (P) and LBFl 6R (Q) located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome without *Cry* 1Ac gene in the negative RAH-100 DNA showed in lane 3 of Plate 12.

4.3.2.1.4 PCR confirmation of set I primer multiplexing in MON 531 (Ajeet 1192 and 1190)

In the MON 531 (Ajeet 1192 and 1190), one amplicon of 210bp was observed. This amplicon is the result of RBFl 4F (P) and LBFl 6R (Q) located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome without *Cry* 1Ac gene in MON 531 DNA showed in lane 4 and 5 of Plate 12.

4.3.2.2 Set II primer multiplexing for zygosity studies

4.3.2.2.1 PCR confirmation of set I primer multiplexing in event No.78

Only one amplicon of 1.1kb size amplification was observed in Event No.78 plants. This amplicon is the result of RBFl 1F (P) and NRB 3R (S) primers showed in lane 1 of Plate 13.

4.3.2.2.2 PCR confirmation of set I primer multiplexing in F_1 of Event No.78

In F_1 derived of Event No.78, two amplicons of 1.1kb and 550 bp were observed. The 1.1kb is an amplicon derived by RBFl 1F (P) and NRB 3R (S) primers, which is from the chromosome carrying *Cry* 1 Ac gene.

The 550 bp amplicon is the result of RBFl 1F (P) and LBFl 6R (Q) primers located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome without *Cry* 1Ac gene in the hemizygous F_1 DNA showed in lane 2 of Plate 13.

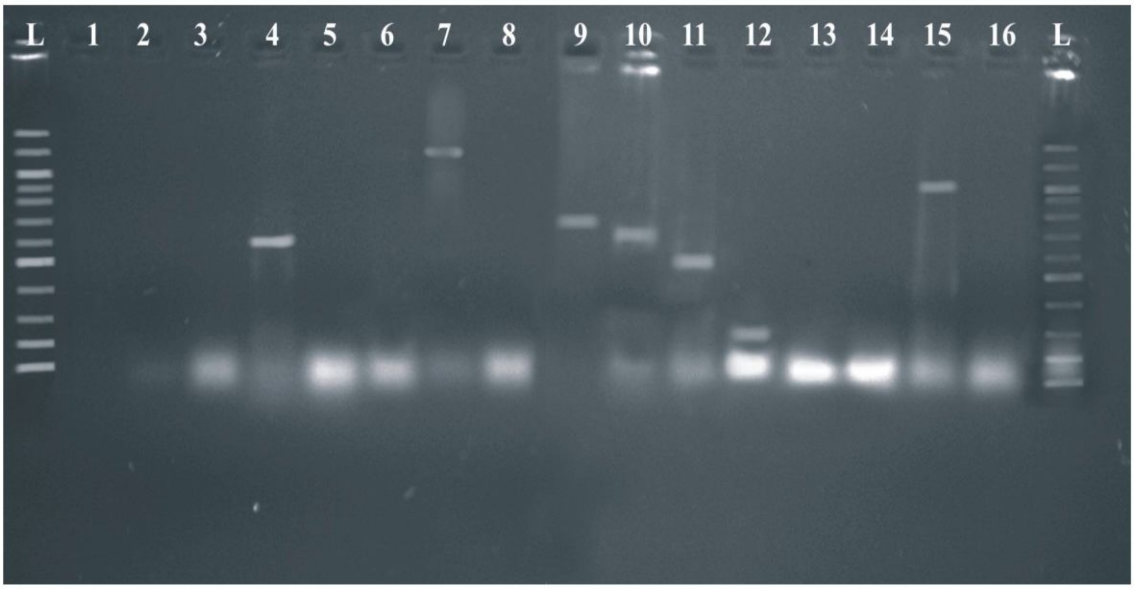
4.3.2.2.3 PCR confirmation of set I primer multiplexing in RAH-100

In the RAH-100, one amplicon of 550bp was observed. This amplicon is the result of RBFl 1F (P) and LBFl 6R (Q) located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome without *Cry* 1Ac gene in the negative RAH-100 DNA showed in lane 3 of Plate 13.

4.3.2.2.4 PCR confirmation of set I primer multiplexing in MON 531 (Ajeet 1192 and 1190)

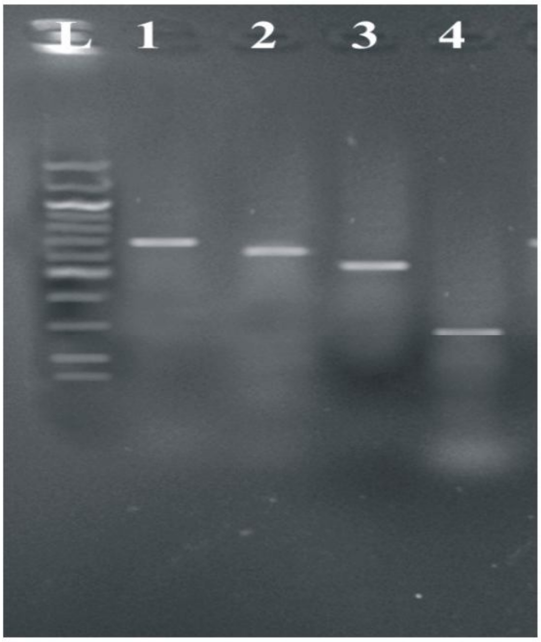
In the MON 531 (Ajeet 1192 and 1190) one amplicon of 550bp was observed. This amplicon is the result of RB1l 4F (P) and LBFl 6R (Q) located on Right and left flanking sequence. This short amplicon is the amplification of the chromosome without *Cry* 1Ac gene in the negative RAH-100 DNA showed in lane 4 and 5 of Plate 13.

These identified combinations of primers can be utilized for detecting the zygosity status of the Event in breeding and maintenance programmes.



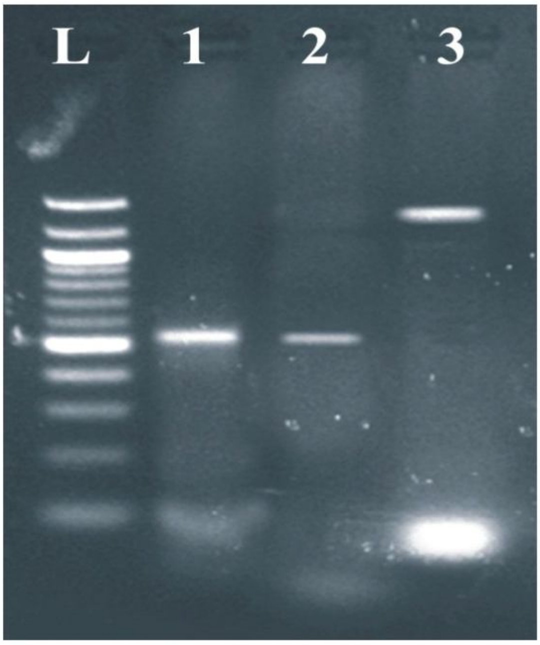
Sl.No	Primer combination	Type of DNA used
	L 100 B.P LADDER	
1.	RBFl 1F_ 1R NRB	Bt DNA of Event No.78
2.	RBFl 1F_ 2R NRB	Bt DNA of Event No.78
3.	RBFl 1F_ 3R NRB	Bt DNA of Event No.78
4.	RBFl 1F_ 4R NRB	Bt DNA of Event No.78
5.	RBFl 1F_ 1R ARB	Bt DNA of Event No.78
6.	RBFl 1F_ 2R ARB	Bt DNA of Event No.78
7.	RBFl 1F_ 3R ARB	Bt DNA of Event No.78
8.	RBFl 1F_ 4R ARB	Bt DNA of Event No.78
9.	RBFl 4F_ 1R NRB	Bt DNA of Event No.78
10.	RBFl 4F_ 2R NRB	Bt DNA of Event No.78
11.	RBFl 4F_ 3R NRB	Bt DNA of Event No.78
12.	RBFl 4F_ 4R NRB	Bt DNA of Event No.78
13.	RBFl 4F_ 1R ARB	Bt DNA of Event No.78
14.	RBFl 4F_ 2R ARB	Bt DNA of Event No.78
15.	RBFl 4F_ 3R ARB	Bt DNA of Event No.78
16.	RBFl 4F_ 4R ARB	Bt DNA of Event No.78

Plate 6: Evaluation of primer for RBFl, ARB and ARB primers for zygosity test



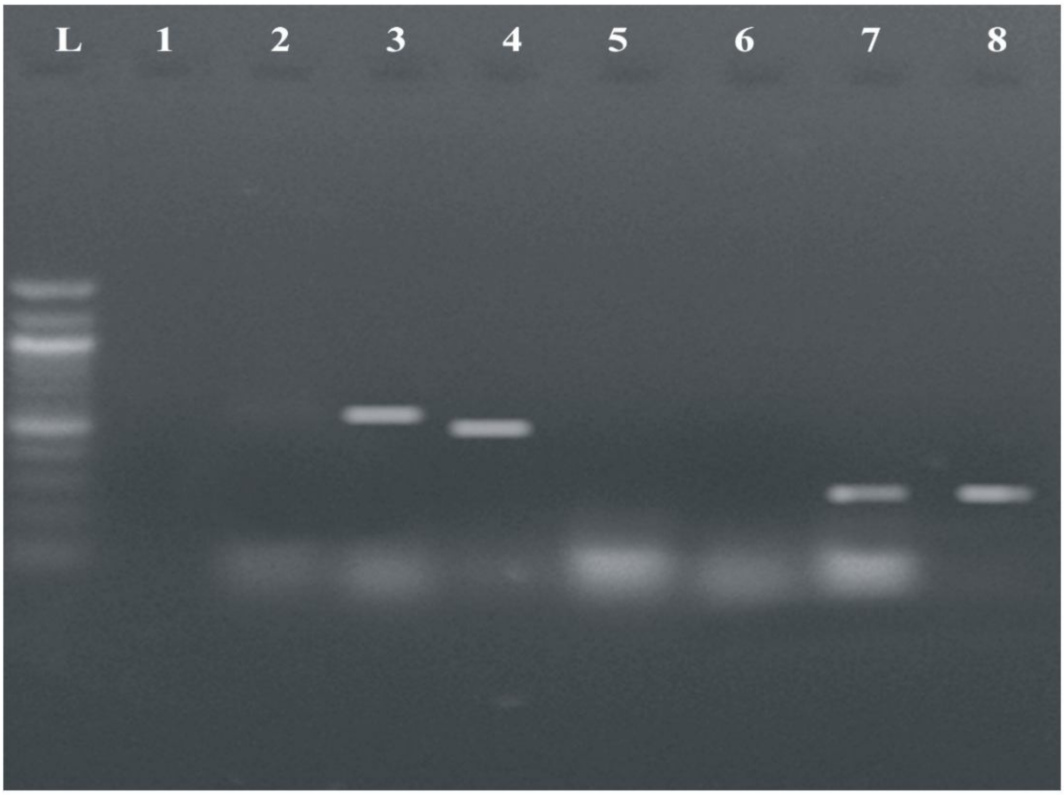
Sl. No.	Primer combination	Type of DNA used
	L.100 bp Ladder	
1.	RBFl 4F_ 1R NRB	Bt DNA of Event No.78
2.	RBFl 4F_ 2R NRB	Bt DNA of Event No.78
3.	RBFl 4F_ 3R NRB	Bt DNA of Event No.78
4.	RBFl 4F_ 4R NRB	Bt DNA of Event No.78

Plate 7a: Confirmation of RBFl (P) primers with NRB (S) primers



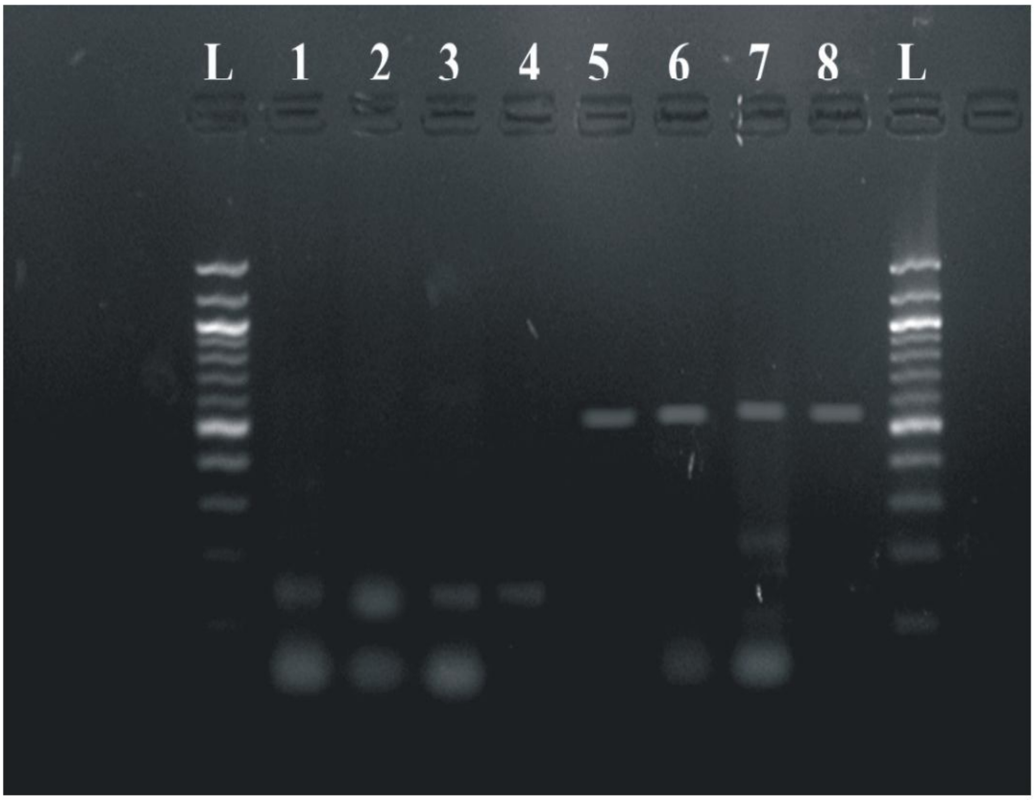
Sl. No.	Primer combination	Type of DNA used
1.	RBFl 4F_ 3R ARB	Bt DNA of Event No.78
2.	RBFl 1F_ 4R ARB	Bt DNA of Event No.78
3.	RBFl 1F_ 3R ARB	Bt DNA of Event No.78

Plate 7b: Confirmation of RBFl (P) primers with ARB (S) primers



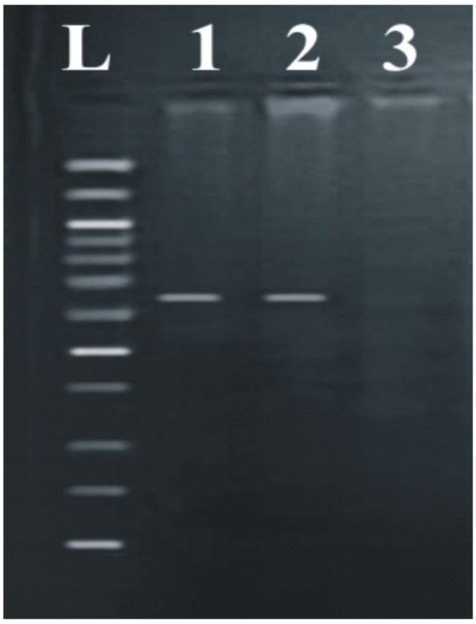
Sl. No.	Primer combination	Type of DNA used
L.	100 bp Ladder	
1.	RBFl 1F_5R LBFl	Bt DNA of Event No.78
2.	RBFl 1F_6R LBFl	Bt DNA of Event No.78
3.	RBFl 1F_5R LBFl	F1 Bt DNA of Event No.78
4.	RBFl 1F_6R LBFl	F1 Bt DNA of Event No.78
5.	RBFl 4F_5R LBFl	Bt DNA of Event No.78
6.	RBFl 4F_6R LBFl	Bt DNA of Event No.78
7.	RBFl 4F_5R LBFl	F1 Bt DNA of Event No.78
8.	RBFl 4F_6R LBFl	F1 Bt DNA of Event No.78

Plate 8: Evaluation of primers for RBFl (P) and LBFl (Q)



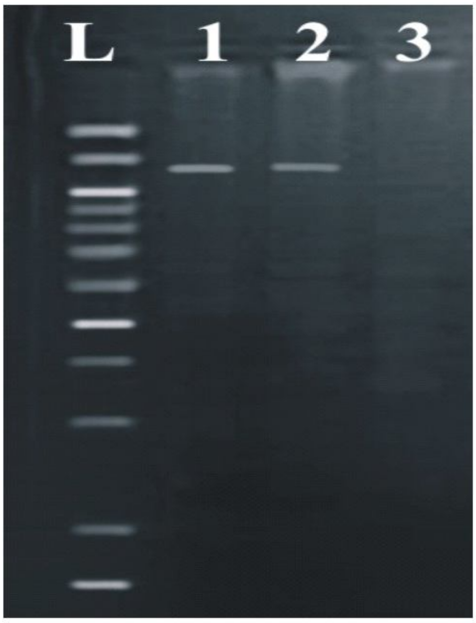
Sl. No.	Primer combination	Type of DNA used
1.	RBFl 4F_5R LBFl	F1 Bt DNA of Event No.78
2.	RBFl 4F_6R LBFl	F1 Bt DNA of Event No.78
3.	RBFl 4F_5R LBFl	F1 Bt DNA of Event No.78
4.	RBFl 4F-6R LBFl	F1 Bt DNA of Event No.78
5.	RBFl 1F_5R LBFl	F1 Bt DNA of Event No.78
6.	RBFl 1F_6R LBFl	F1 Bt DNA of Event No.78
7.	RBFl 1F_5R LBFl	F1 Bt DNA of Event No.78
8.	RBFl 1F_6R LBFl	F1 Bt DNA of Event No.78

Plate 9 : Confirmation of amplified primers of RBFl (P) with LBFl (Q)



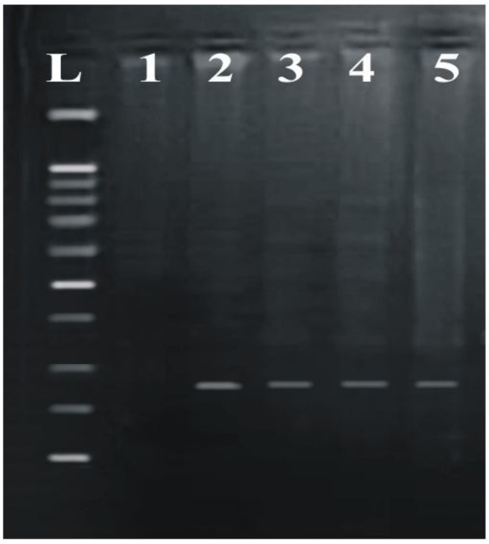
Sl. No.	Primer combination	Type of DNA used
L	100 b.p Ladder	
1.	RBFl 4F_4R NRB	Bt DNA of Event No.78
2.	RBFl 4F_4R NRB	F1 Bt DNA of Event No.78
3.	RBFl 4F_4R NRB	Non Bt DNA (RAH-100)

Plate 10a: Confirmation of RBFl (P) and ARB (S) set of primers in UASD Bt Cotton Event No.78 & F1



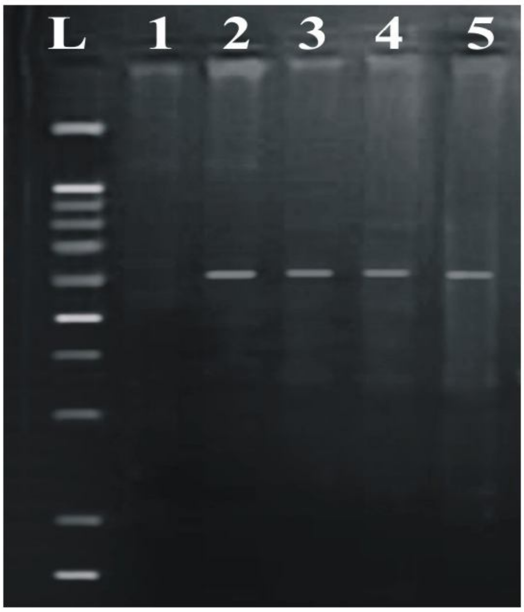
Si.no	Primer combination	Type of DNA used
L	100 b.p Ladder	
1.	RBFl 1F_3R ARB	Bt DNA of Event No.78
2.	RBFl 1F_3R ARB	F1 Bt DNA of Event No.78
3.	RBFl 1F_3R ARB	Non Bt DNA (RAH-100)

Plate 10b: Confirmation of RBFl (P) and ARB (S) set of primers in UASD Bt Cotton Event No.78 & F1



Sl.No.	Primer combination	Type of DNA used
L	100 b.p Ladder	
1.	RBFl 4F_6R LBFl	Bt DNA of Event No.78
2.	RBFl 4F_6R LBFl	F1 Bt DNAof Event No.78
3.	RBFl 4F_6R LBFl	Non Bt DNA (RAH-100)
4.	RBFl 4F_6R LBFl	Mon 531 (Ajeet-1190)
5.	RBFl 4F_6R LBFl	Mon 531 (Ajeet-1192)

Plate 11a: Confirmation of RBFl (P) and LBFl (Q) set of primers in UASD non-Bt Cotton Event No.78, F1, RAH-100 and commercial Bt check

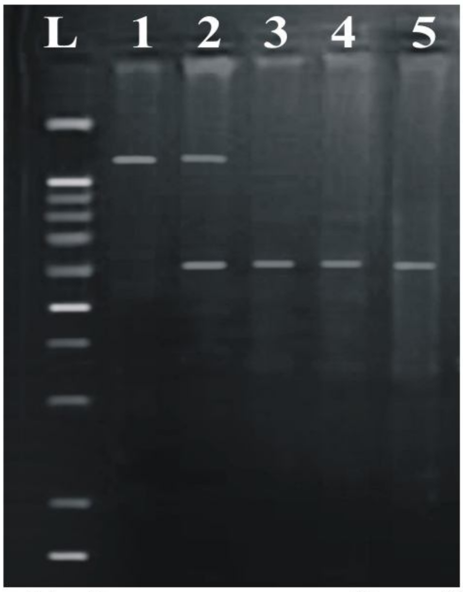


Sl. No.	Primer combination	Type of DNA used
	L 100 b.p Ladder	
1.	RBFl 1F_6R LBFl	Bt DNA of Event No.78
2.	RBFl 1F_6R LBFl	F1 Bt DNA of Event No.78
3.	RBFl 1F_6R LBFl	Non Bt DNA (RAH-100)
4.	RBFl 1F_6R LBFl	Mon 531 (Ajeet-1190)
5.	RBFl 1F_6R LBFl	Mon 531 (Ajeet-1192)

Plate 11b: Confirmation of RBFl (P) and LBFl (Q) set of primers in UASD non-Bt Cotton Event No.78, F1, RAH-100 and commercial Bt check

Sl. No.	Primer combination	Type of DNA used
	L 100 b.p Ladder	
1.	RBFl 4F_4R NRB _6R LBFl	Bt DNA of Event No.78
2.	RBFl 4F_4R NRB _6R LBFl	F1 Bt DNA of Event No.78
3.	RBFl 4F_4R NRB _6R LBFl	Non Bt DNA (RAH-100)
4.	RBFl 4F_4R NRB _6R LBFl	Mon 531 (Ajeet-1190)
5.	RBFl 4F_4R NRB _6R LBFl	Mon 531 (Ajeet-1192)

Plate 12: Confirmation of RBFl (P), NRB (S) and LBFl (Q) set of primers in UASD non-Bt Cotton Event No.78, F1, RAH-100 and commercial Bt check



Sl. No.	Primer combination	Type of DNA used
	L.100 b.p Ladder	
1.	RBFl 1F_3R ARB 6R LBFl	Bt DNA of Event No.78
2.	RBFl 1F_3R ARB 6R LBFl	F1 Bt DNA of Event No.78
3.	RBFl 1F_3R ARB 6R LBFl	Non Bt DNA (RAH-100)
4.	RBFl 1F_3R ARB 6R LBFl	Mon 531 (Ajeet-1190)
5.	RBFl 1F_3R ARB 6R LBFl	Mon 531 (Ajeet-1192)

Plate 13: Confirmation of RBFl (P), ARB (S) and LBFl (Q) set of primers in UASD non-Bt Cotton Event No.78, F1, RAH-100 and commercial Bt check

5. DISCUSSION

Bt strains contain *Cry* genes that produce endotoxins specific to some of the major pests of important crop plants (Gatehouse, 2008). Many *Cry* genes have been characterized and tested against major insect pests (Hofte *et al.,* 1989 and Crickmore *et al.,* 2016). Expression of a *Cry* gene in cotton was first reported in 1987 by Monsanto and Delta & Pine Land Co. Ltd (Falck *et al.*, 1997) Subsequently, transgenic cotton containing a *Cry*1Ac gene (event Mon 531) was released for commercial use in 1996. However, from the first testing of Bt crops in the United States to the present, the development of resistance to *Cry* toxins in insect pests has remained a major concern (Tabashnik *et al.,* 2008). Diversification of events/genes, gene pyramiding and events with higher toxin levels can combat the insect resistance for sustainable use of Bt technology in farmer's fields.

Efforts were made at UASD to develop transgenic Bt cotton with *Cry* 1Ac (with double promoter) gene obtained from ICGEB, New Delhi. Among the many positive events developed, "UASD Bt Cotton Event No. 78", has been identified as potential one owing to significantly higher *Cry* protein expression with corresponding bioefficacy throughout the crop duration and all the plant parts studied in comparison to Monsanto's BGII (Manjunath Swamy, 2014). This Event No. 78 can successfully be promoted from the public sector for commercial cultivation which needs all the regulatory procedures including event characterization to be conducted.

Current regulations on crops bio-safety put great emphasis on the regulation of transgenic crops because of concerns such as co-introduction of selectable markers (particularly antibiotic resistance genes) and microorganism originated DNA sequences or unintended effects caused by random insertion (Li *et al.,* 2017).

Molecular analysis, as the key analytical tool to complementing field trials for event characterization at early stages of commercial transgenic trait development, has quickly shifted from traditional Southern blot analysis to high-throughput qPCR TAIL-PCR, Inverse PCR, LAMP-PCR *etc* for PCR based detection and Next-generation sequencing (NGS) for DNA-based detection.

The present study aimed to reconfirm the presence of *Cry* 1Ac gene in transgenic plants of UASD Bt cotton Event No.78, characterization of UASD Bt Cotton Event No.78 and zygosity studies of transgenic Event No.78. The results obtained during the course of investigation have been discussed below with relevant literature under following headings.

5.1 PCR confirmation of UASD Bt Cotton Event No.78

5.2 Event characterization of the *Cry* 1Ac UASD transgenic Event no.78 through TAIL-PCR.

5.3 Confirmation of zygosity status of plants with *Cry* 1Ac gene of event No.78

5.1 PCR confirmation of UASD Bt Cotton Event No.78 carrying *Cry* 1Ac gene

In the present study, "UASD Bt cotton Event, No.78" was used. Event-78 is transformed with *Cry* 1Ac gene with double promoter obtained from ICGEB (International Centre for Genetic Engineering and Biotechnology), New Delhi. PCR tests involved the amplification of the DNA with transgene specific primers (Table 5). The amplification of the genomic DNA to show the expected amplicon size indicates the presence of the transgene. Event-78 showed positive amplification of the corresponding *Cry* gene and confirmed the presence of *Cry* 1Ac gene.

5.2 Event characterization of the *Cry* 1Ac gene in UASD transgenic Event No.78 through TAIL-PCR

The information on the flanking DNA sequences in an event is the ultimate proof and identifies an independent event. The present study makes use of a powerful tool *i.e.* TAIL-PCR for the recovery of flanking sequences of T-DNA insert from the genomic DNA fragment.

5.2.1 Determination of the flanking sequence of the Right border of inserted T-DNA

An attempt was made to know the flanking sequences of the right border of the inserted T-DNA by TAIL-PCR amplification and verified by PCR. Nested target-specific primers combined with one of the four arbitrary degenerate primers (AD1, AD2, AD3, AD4) for sequencing the flanking sequence through TAIL-PCR was followed. One unique fragment of about 1 kb in size was amplified by using the combination of arbitrary primer AD4 and target-specific primer RB4R at the third TAIL-PCR. Along with 1kb, there was an amplification of another band of about 500bp owing to the possibility of arbitrary degenerate primers binding to a region in same amplified region with the same specific primer of T-DNA (Wu *et al.,* 2015 and Singer *et al.*, 2003) The amplicon of 1kb, was eluted and washed from the agarose gel by QIAGEN elution kit and used for sequencing following the BigDye™ Terminator v3.1 Cycle Sequencing Kit on the capillary Electrophoresis an Applied Biosystems (ABI)™ 3500/3500xL Genetic Analyzer with 3500 Series Sequencer. Sequencing results of 713 bp was obtained. The BLAST result of Right border flanking sequence of 713 bp showed homology to *Gossypium hirsutum* genome. From the BLAST analysis data, it was identified that out of 713 bp of right border flanking from 28 to 691 bp covered the *Gossypium hirsutum* species. The Right border flanking sequence of 713 bp when BLAST against pBI121, showed homology from 688 to 708 bp from the point of 2362 to 2343 of pBI121 (Genbank acc. No. AF485783.1)

Like TAIL-PCR many other PCR based techniques such as Inverse PCR (IPCR) (Ochman *et al*., 1988), gene walking PCR (Parker *et al.*, 1991) and single primer PCR (Parks *et al.,1991),* Ligation mediated PCR (LM-PCR) (Mueller and Wold, 1991) and Site finding PCR (Tan *et al*., 2005) can be used, but these employ special steps before PCR, such as southern analysis, restriction digestion and ligation, making the task laborious, and they tend to generate non-specific products. TAIL-PCR is only a PCR based method and is thus especially suitable for manipulating a large number of samples in manual or automation with the advantages of simplicity and high efficiency, TAIL-PCR and its modified procedures have been widely used in a variety of biological research in various organisms to identify the flanking sequence of inserted T-DNA (Liu, 2007). However, a different approach was used in transgenic *Rosaceace* species to find a flanking sequence of inserted T-DNA by using Fusion primer and Nested Integrated PCR (FPNI-PCR) (Wang *et al.*, 2011) which is a modification of the amplified fragment length polymorphism technique.

5.2.2 Confirmation of left border flanking sequence of T-DNA through PCR

The PCR method was adopted to confirm the flanking sequence of the left border terminus of the inserted T-DNA. Based on the known genomic sequence of the right border terminus beside the inserted T-DNA, the partial DNA sequence of the *Gossypium hirsutum* genome was downloaded from the NCBI database. The cotton genomic sequence after the insertion point corresponds to the left flanking sequence after the T-DNA. This left border flanking sequence was used to design two primers approximately 100bp after the T-DNA insertion point taking care of the probability of loss of few base pairs at the site of T-DNA integration. Forward primers were designed for the left terminal boundary of the T-DNA.

Two independent standard PCR reactions were performed using UASD Bt Cotton Event No.78 genomic DNA as the template with the above described primers. Gel electrophoresis showed expected amplicons of 500bp and 600bp in the two reactions depending on the position of reverse primer.

Amplification was not observed in RAH-100 (non Bt) in both the reactions. As in both the reactions the forward primer is specific to T-DNA, the results obtained confirms the T-DNA is in the same chromosome number on which right border flanking sequence is present.

In a similar study conducted by Zhang *et al.* (2016) the following method was adopted to analyze the flanking sequence of the 3’ terminus of the inserted DNA. Based on the known sequence of the 5’ terminus of the inserted sequence, the partial DNA sequence of the *Gossypium raimondii* genome from 61,252,000 to 61,253,972 of chromosome 11 were downloaded from Phytozome. Upstream primers were designed for the 30 -terminal boundary of the exogenous DNA and downstream primers were designed for the downloaded cotton genome sequence. Standard PCR was then performed using BG2-7 genomic DNA as the template. According to gel electrophoresis, there was a production of the amplicon of expected size among the obtained electrophoresis strips. The corresponding bands were recovered, ligated to the T-vector, transformed into E. coli, sequenced, and aligned. Ultimately, the 3’terminal flanking sequence was identified.

5.2.3 Reconfirmation of the flanking sequence of the Right border of inserted T-DNA

The PCR amplicon with RBFl 1F & NRB 4R primers of 550bp was sequenced through outsourcing. The sequence data obtained used for blast analysis hit the same accession number of *G. hirsutum* as in TAIL-PCR sequence. The BLAST result of the sequence obtained with pBI 121 again aligned to pBI 121 (Ac No. AF485783.1), from 2362 to 2343 as in TAIL-PCR sequence data analysis. This reconfirmed the sequence data obtained through TAIL-PCR and sequencing in this study.

5.3 Zygosity study by using 3 primer mutiplexing

Two sets of zygosity testing primers were identified each set consisting of three primers RBFl 4F_4R NRB _6R LBFl in one set and RBFl 1F_3R ARB _6R LBFl in another set. These sets of primer combination (primer multiplexing) were used to differentiate homozygous UASD Bt Cotton Event No.78 plant, F_1 UASD Bt Cotton Event No.78 (hemizygous), wild (Non-Bt RAH-100) and commercial Bt hybrid developed {Ajeet 1990 and Ajeet 1192 (MON 531)}. These sets of primers can be used to identify Bt cotton plants based on the Event. Further. In a similar study conducted by Glowacka *et al.* (2016), it was found that three primers used to differentiate homozygous transgenic Tobacco, hemizygous transgenic Tobacco and wild tobacco (non-transgenic Tobacco).

Even though, the literature on the effect of zygosity status of transgenic by TAIL-PCR is limited but has encouraging results. So, in future days, technologies like rtPCR, digital-PCR can be used to know the status of the transgene in transgenic plants. However, a different method called New generation sequencing (NGS) method was used to characterize soybean transgenic Event 1 and Event 2 (Guttikonda *et al.,* 2016) which is novel, rapid, and cost-effective options for molecular characterization of transgenic crops, from a single method analysis have identified copy number/ number of insertions, presence of backbone, T-DNA stability across generation, T-DNA integrity-qualitative, Transgene location in genome, T-DNA integrity at nucleotide level insertion or deletion at parental locus and NGS methods have advantages of high throughput and deep sequencing for accurate genome and T-DNA sequence.

5.4 Study of UASD Bt Cotton Event No.78 specific primer amplification in commercial Bt hybrids of Cotton developed based on Monsanto's transgenic Event MON 531

Flanking sequence is crucial in the transgenic event as they differentiate two transgenic events with the same T-DNA insertion also and hence is the ultimate tag of an Event.

In this study, two sets of zygosity evaluation primers, each set consisting of 3 primers named as P (for Right border flanking), S (For T-DNA) and Q (for Left border flanking) were identified.

Each of these two sets of zygosity testing primers identified contains 3 primers in each set, Set-I {(RBFl (P) 4F_4R NRB (S) _6R LBFl(Q)} and another set-II {(RBFl (P) 1F_3R ARB (S) _6R LBFl (Q)} were used for PCR amplification in UASD Bt Cotton Event No.78 and MON 531 (Ajeet 1900 and Ajeet 1922). The 550bp and 1.1kb amplicon in Set-I and II respectively with Right border flanking forward primer and T-DNA specific reverse primer was observed only in Event No.78 and not in MON 531 event. This confirms the flanking sequence identify to Event No.78. The 500bp and 210bp amplicon expected in Set-I and II respectively by a forward primer on Right border flanking sequence and reverse primer on Left border flanking sequence was observed in MON 531.

This confirms Event No.78 is unique and different from MON 531 Event.

Future line of work

1. The characterization of UASD Bt Cotton Event No.78 forms a platform for proceeding further for completion of regulatory requirements and conduct of field trails. The flanking sequence information will be submitted for the patenting of UASD Bt Cotton Event No.78

3. The identified combination of 3 primers in the present study can be utilized in breeding programmes to identify homozygous *Cry*1 Ac plants to advance the generation. The primers will also help in maintenance breeding.

4. The zygosity primers can be used to safeguard the UASD Bt Cotton No.78 as it will be patented.

6. SUMMARY AND CONCLUSIONS

Results obtained on the characterization of UASD Bt cotton Event No.78 and zygosity testing are summarized below.

1. UASD Bt cotton Event No.78 consist of *Cry* 1AC gene confirmed by PCR analysis using gene-specific primers.

2. Right border (RB) TAIL-PCR produced an amplicon of 1kb and 550bp with RB4R and AD primers.

3. 1kb amplicon of RB TAIL-PCR was eluted from the gel and purified.

4. Gel purified product of 1kb was sequenced by using BigDye™ Terminator v3.1 Cycle Sequencing Kit on the capillary Electrophoresis an Applied Biosystems (ABI)™ 3500/3500xL Genetic Analyzer with 3500 Series Sequencer at IABT, UASD.

5. The sequence of 713bp was obtained from the 1kb amplicon obtained by RB TAIL-PCR

6. The BLAST result of Right border flanking sequence of 713 bp showed homology to *Gossypium hirsutum* genome. From the BLAST analysis data, it was identified that out of 713 bp of right border flanking from 28 to 691 bp covered the *Gossypium hirsutum* species.

7. The Right border flanking sequence of 713 bp when BLAST against pBI121, showed homology from 688 to 708 bp from the point of 2362 to 2343 of pBI121 (Genbank acc. No. AF485783.1).

8. PCR (two reactions) with Near to left border (NLB 3F) specific primer on T-DNA (as a forward primer) and Left border flanking sequence (LBFl 5R and LBFl 6R) primers (as a reverse primers) gave an expected amplicon of 500bp and 600bp when DNA from Event No.78 was used as a template. Left border flanking primers were designed 100bp away from the point of insertion of T-DNA in Cotton genome.

9. PCR with Near to right border (NRB) specific primer on T-DNA (as reverse) of inserted T-DNA and right border flanking primers (as forward), designed using

sequence information obtained by sequencing TAIL-PCR product, gave an expected base pair of 550bp in Event No. 78. This Right border flanking sequence was sequenced by RBFl 1F and NRB4R primers by outsourcing. The sequence data of 550bp was reported by the outsourced company,

10. The BLAST analysis of event No.78 sequence data of 550bp of right border flanking ssequence, again aligned with same Accession number as obtained in TAIL-PCR sequence data BLAST. This confirms the flanking sequence data obtained by TAIL-PCR method.

11. The BLAST analysis of 550bp sequence data against pBI121 again aligned at bp No from 2362- 2343 which is in the coordination of the sequence data obtained through TAIL-PCR. This reconfirms the point of insertion

12. The alignment of 550bp sequence reported by Pvt. company with the 713bp sequence obtained by TAIL-PCR sequenced at IABT-UASD. Showed 84.85 % homology which again confirmed the sequence data of TAIL-PCR and hence the RB cotton sequence of Event No.78

13. PCR with P (RBFl 4F), S (4R NRB) and Q (6R LBFl) set of primers in single PCR reaction amplified the size of the expected amplicon to differentiate the Event No. 78 600bp(P and S), F_1 Bt Event No.78 of 600bp (P and S), and 210bp (P and Q) and in RAH-100 and MON 531 (Ajeet 1192 and Ajeet 1190) of 210bp (P and Q)

14. PCR with P (RBFl 1F), S (3R RB) and Q (6R LBFl) set of primers in single PCR reaction amplified the size of the expected amplicon to differentiate the Event No. 78 with 1.1KB (P and S), F_1 Bt Event No.78 with 1.1kb (P and S) and 550 (P and Q) and in RAH-100 (Non Bt) and MON 531 (Ajeet 1192 and Ajeet 1190) with only 550bp (P and Q)

15. This study completes the characterization of UASD Bt Cotton Event No.78. The flanking sequence data is unique to Event No.78 and can differentiate it from other events including MON 531, using the identified set of zygosity primers.

16. The sequence data and zygosity primers need to be used for completion of regulatory procedures for the release of Event No.78 and patenting of the Event.

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Appendix I: DNA Extraction Buffer

0.5 M glucose.

0.2 M Tris-Hcl

5 mM Ethylenediaminetetraacetic acid (EDTA)

2% Polyvinylpyrrolidone (PVP)

0.2% Mercaptoethanol

Appendix II: DNA Lysis Buffer

0.2% Mercaptoethanol

1.4 M NaCl

25 mM Ethylenediaminetetraacetic acid (EDTA)

2% Hexadecyltrimethylammonium bromide (CTAB)

2% Polyvinylpyrrolidone (PVP)

Appendix III: Loading dye (6x)

0.25% Bromophenol blue

40% (w/v) sucrose in water Stored at 4°C

Appendix IV: T10 E1 Buffer

10Mm Tris (pH-8.0)

1mM of EDTA To make 100ml solution of T10E1 – Put 1ml of 1M Tris-HCl (pH-8.0) and

0.2ml of EDTA (0.5mM), make up the volume to 100ml using double distilled water

Appendix V: 50X TAE composition

Tris base : 242 g

Glacial acetic acid : 57.1 ml

0.5 M EDTA (pH 8.0) : 100 ml Make up the volume to 1000 ml with double distilled water

VALIDATION AND EVENT CHARACTERIZATION OF *Cry* 1Ac UASD Bt COTTON EVENT NO. 78

BASAVANAGOUDA PATIL **2019** **Dr. MANULA S. MARALAPPANAVAR**
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ABSTRACT

UASD Bt *Cry* 1Ac Cotton Event No.78 has been developed by agrobacterium mediated transformation using pBI121 vector. Event 78 is superior to Mon531 event for *cry* protein expression. The present study was done for molecular characterization of this event after reconfirmation of the presence of transgene. The presence of the gene was confirmed through PCR with gene-specific primers. Molecular characterization was done through TAIL-PCR. Right border (RB) TAIL-PCR produced an amplicon of 1kb with RB4R and AD primers. Gel purified product of 1kb was sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit on Capillary Electrophoresis of Applied Biosystems (ABI-3500/3500) at IABT, UASD. The sequence data of 713 bp was obtained and the BLAST result showed homology to *Gossypium hirsutum* genome. Of this 713 bp right border flanking sequence, 28 to 691 bp showed homology with the *Gossypium hirsutum* species. Upon the BLAST of 713 bp sequence data against pBI121, 688 to 708 bp of query showed homology to pBI121 (Genbank acc. No. AF485783.1).

The sequence of *Gossypium* genome with highest homology was used as template to design specific primers on right and left flanking borders. An amplicon of 700 bp was obtained with cotton specific forward primer (RBFl 1F) and T-DNA specific reverse primer (NRB 4R). This amplicon was sequenced at Agri genome Pvt. Ltd. The BLAST analysis of 550 bp data of this amplicon showed homology with the same accession of *Gossypiym hirsutum* as obtained with TAIL-PCR sequence data which confirmed the RB flanking sequence.

PCR studies using the RB forward primer and LB reverse primer in the flanking sequence along with one T-DNA reverse primer conducted in Event-78, F1(Non Bt X Event-78), RAH-100 (Non Bt) and Mon 531 showed that these primers can confirm the presence of *Cry* gene specific to Event-78 and its zygosity status.