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Kisan B
Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Narasimlu
Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Tilak IS
Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Nidagundi
Main Agricultural Research station, University of Agricultural Sciences, Raichur, Karnataka, India

Bheemanna
Main Agricultural Research station, University of Agricultural Sciences, Raichur, Karnataka, India

Shankergoud I
Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

Correspondence
Kisan B
Department of Molecular Biology and Agricultural Biotechnology, College of Agriculture, University of Agricultural Sciences, Raichur, Karnataka, India

DNA based methods for detection of transgenes in cotton hybrids

Kisan B, Narasimlu, Tilak IS, Nidagundi, Bheemanna and Shankergoud I

Abstract
The rapid adoption of Bt. transgenic cotton hybrids in India have un-doubtedly been the most significant event. Efficient detection strategies for genetically modified crops need to be in compliance with regulatory frameworks and address consumer concerns need fully. There is a need for GM testing and detection to enhance GMOs and inhibit the spurious infiltration in the present market. The DNA based techniques using PCR, RT-PCR, LAMP, RT-LAMP and Multiplex PCR are currently the major detection methods that are widely used due to their ease and accuracy in detection of GM and Non GM crops. The present investigation was envisaged to differentiate transgenic and non transgenic plant, Six cotton hybrids were obtained from Main agriculture research station, University of agricultural sciences Raichur, Karnataka, India. Among six hybrids three of them are transgenic *viz.*, 201KSSCH, 203KSSCH and 207KSSCH, other three hybrids are non transgenic namely 202KSSCH, RAHH455 and SHH188. To differentiate these samples at molecular level, they have been screened using PCR based technology upon employing gene specific CRY1AC F/R primer for the detection of *Cry1AC* gene among six cotton hybrids. Results revealed that three transgenic cotton hybrids *viz.*, 201KSSCH, 203KSSCH and 203KSSCH have been amplified by expressing a gene specific amplicon of 280 bp indicating the presence of C*ry1AC* gene in hybrids. Non transgenic cotton hybrids namely 202KSSCH, RAHH455 and SHH188 failed to amplify gene specific fragment, due to absence of *Cry1AC* gene. It has been confirmed that gene specific CRY1AC F/R primer can be used to study presence of *Cry1AC* gene in cotton samples. Further it can be used in the breeding programmes such as marker assisted selection in cotton.

Keywords: cotton, cry1ac, transgene, PCR

Introduction
Cotton belongs to the genus Gossypium of family Malvaceae. Agriculture is the backbone of Indian economy, cotton is the major raw fiber for our textile industry. bOf the 50 species, only 4 are cultivated, namely G. arboretum L., G.herbaceum L. , G. hirsutum L.and G. barbadense L. Gossypium arboretum and G.herbaceum are diploids (2n = 26) and are popularlyknown as the desi cottons. Both G. hirsutum and G.barbadense (2n = 4x = 52; AD genome species), evolvedin the New World and are referred to as allelotetraploids oramphidiploids and are popularly known as American or Upland (G. hirsutum) and Egyptian or Pima (G.barbadense) cottons. India is the only country in the worldwhere all the four cultivated species of cotton are grownon a commercial scale besides hybrids (both inter and in-tra specific). The first genetically modified Bt cotton was developed by transferring the cry1Ac gene from the ubiquitous soil bacterium Bacillus thuringiensis. Insertion of these genes enables cotton (Gossypium sp.) plant cells to produce crystal insecticidal proteins, commonly termed cry proteins. The cry proteins are effective in controlling the lepidopteron pests. When the larva eatsthe cry proteins, its own digestive enzymes activate the toxic form of the protein. Toxic cry proteins then bind to specific receptors present on the intestinal walls and rup-ture midgut cells. Infected larvae stop feeding within a few hours after the first nibble, and, if they have consumed sufficient toxin, die within 48-72 hours. Bollgard-Bt cotton with cry1Ac was first cultivated in the US during 1996 and was released in China and Australia in 1997. Later, it was released in Mexico, Colombia, Indonesia, Argentina, South Africa, and India (D. Blaise *et al.*, 2014) [2]. GM crops, although are known to be beneficial in most cases, still consumers have the right to know and choose between GM and non GM products, they buy. Consumer concerns related to development and marketing of GMO and derived food products have resulted in increased awareness regarding the food labeling all over the world. As far as India is concerned no GM crop (food/products) is allowed in India to be marketed except *Bt*-cotton which is a feed crop. Under Jurisdiction of Supreme Court on May8, 2007 GEAC was instructed to lay down the protocols for ensuring 0.01% of contamination by GM crops in field trials. The development and commercialization of GM crops is increasing at a faster pace, to

develop qualitative and quantitative methods for detection of GM crops has become even more challenging.

The infiltration of unapproved seeds into the market has been the reason for controversies regarding the acceptance amongst the farmers. Hence there is need for GM detection and testing. Detection of a GMO or a derivative of a GMO can be done by detecting a molecule (DNA, RNA or protein) that is specifically associated with or derived from the genetic modification of interest. The majority of the methods developed for detection of GMO and GMO-derivatives focus on detecting DNA, while only a few methods have been developed for detecting proteins or RNA (Ahmed *et al*., 2002). The analytic techniques are often by the quantitative and qualitative of the target analyte and hence conventional PCR is widely accepted for this purpose because of its specificity, sensitivity and reliability (Holst *et al*., 2003) [5]. *Bt* cotton was among the first GM crops to be commercialized during the 1900s at the global level and was officially approved for sale in India in 2002. It consists of a gene from the soil bacterium *Bacillus thuringensis (Bt*), which provides resistance against different bollworm species, a major pest in cotton and helps cotton growers benefit through efficient pest control. In this study, we have used CRY1AC F/R gene specific primers for the screening of transgenic cotton using conventional PCR technology.

Materials and Methods

Plant materials

For the purpose of characterization and identification of Cry1AC gene in cotton hybrids at molecular level, six cotton hybrids were obtained, among them three of them are transgenic *viz.*, 201KSSCH, 203KSSCH and 207KSSCH, other three hybrids are non-transgenic namely 202KSSCH, RAHH455 and SHH188, obtained from Main Agriculture Research Station, University of Agricultural Sciences, Raichur, Karnataka, India.

Genomic DNA isolation from leaves

Genomic DNA was extracted from young cotton leaves during seedling stage by CTAB method (Doyle and Doyle, 1990) [4]. Two grams of leaves samples were weighed and homogenized using autoclaved mortar and pestle. The homogenized tissue was transferred to 1.5ml centrifuge tube containing1.2ml of preheated extraction buffer. Tubes containing ground tissue were placed in water bath (with gentle shaking) for 60 minutes at 65ºC with periodical shaking at an interval of five minutes. Later, the tubes with leaf tissue extract were incubated at room temperature for 15 minutes. Ten ml of Chloroform: Isoamyl alcohol (CIA) mixture (24:1) was added to tissue extract and the contents were mixed by shaking then the tubes were centrifuged for 10 minutes at 10,000 rpm at room temperature. Equal quantity of chilled isoproponal was added to each tube and mixed by inverting and incubated at -20ºC for overnight. The content was centrifuged for ten minutes at 10,000 rpm at 40ºC. The supernatant was discarded. The DNA pellet obtained was washed with 70 per cent ethanol and the tubes were inverted on blotter paper to dry the pellet. The DNA was dissolved in 100µl T_{10} E_1 buffer and stored at -20ºC for further study.

DNA quality and quantity estimation

The quality and quantity of DNA were estimated using a spectrophotometer based on the 260/280-nm and 260/230-nm UV absorption ratios and analyzed by 0.8% agarose gel electrophoresis.

PCR Analysis

PCR analysis of transgenic and non transgenic cotton was carried out by using the primer CRY1AC F/R. The primer sequence and their amplicon size are shown in Table.1. Primers were synthesized by Sigma Aldrich Pvt. Ltd, Bangalore. The plants were thus screened initially for the presence and absence of Cry1Ac gene using PCR. The PCR amplification was carried out using 25 µl reaction mixtures containing Taq DNA polymerase (Sigma) 50 µM of each forward and reverse primers, 200 mM of each dNTP and 3 µl DNA. The master mix of 25 µl was added to PCR tubes and was given a short spin to mix the contents. The tubes were placed in the thermal cycler for amplification. The PCR reaction was carried out using master cycler gradient (Eppendorf thermal cycler gradient 5331) with the following thermal cycling conditions, Initial denaturation : 94°C for 4 min, Final denaturation : 1cycle of 94°C for 30 s, Annealing: 50°C for 50 s, Primer extension :72°C for 30 sec. There were 35 cycles. However, the final extension was 2 min at 72°C. buffer, 0.2 µM dNTP's, 0.5 pmol of each primer (forward & reverse) and 0.2-0.5. The PCR product was subjected to gel electrophoresis containing 3% agarose gel which was stained with Nucleic Acid Staining Solution. It was carried out for one hour in 1X TAE buffer at 75V. Later amplified bands were visualized and captured under UV light (254-366 nm) by UV- Gel Documentation System (Alpha Innotech).

Table 1: Primer used for detection of Cry1AC gene in cotton hybrids.

Primer Name	Sequence (5'-----3')
CRY1AC F/R	F:GCCAATGCCTCGTGATTGTTCTCTGC R:GATTTGCGAGGCTGGCCAGCTCCACG

Results and Discussion

The introduction of the polymerase chain reaction (PCR) has enabled the development of powerful genetic markers. Genetically modified (GM) cotton was developed in a view to reduce the heavy reliance on pesticides. The bacterium *Bacillus thuringiensis (*Bt) naturally produces a chemical harmful only to a small fraction of insects, most notably the larvae of moths and butterflies, beetles, and flies, and harmless to other forms of life. The gene coding for Bt toxin has been inserted into cotton, called Bt cotton, to produce this natural insecticide in its tissues. In many regions, the main pests in commercial cotton are lepidopteran larvae, which are killed by the Bt protein in the transgenic cotton they eat. Identifying cotton hybrids for CRY1AC gene content, is one of the way to distinguish the transgenic and non transgenic cotton hybrid. Singh *et al*., 2007 developed PCR based approach for detection, identification and gene stability confirmation of *cry1Ac* transgene construct in *Bt* cotton. They conducted single standard PCR assays to amplify predominant GM DNA sequences (CaMV 35S promoter, nos terminator, and *npt-II* marker gene) a housekeeping gene. The resultant amplicons were excised, eluted, and purified. Studies conducted by Randhawa *et al*., 2010 showed the presence of 195bp specific amplicon for 35S promoter, 180bp presence of Nos terminator and npt-II gene having 215bp. Subsequently Dolhare and Tank (2014) [3] developed and standardized different detection of transgenes *cry1Ac*, *cry2Ab*, *npt-II*, 35S Promoter & NOS terminator for *Bt* -cotton using PCR based strategies and applied to detect transgene in *Bt*-cotton. Kamle *et al*., 2011 [6] reported qualitative assay for detection for *cry2Ab* and also confirmed the amplification compatibility with promoter, p35S (195 bp), terminator, t-nos (180 bp) and

marker gene, npt II (215 bp) using *Bt* cotton event MON15985. Hence, established a comprehensive multiplex PCR method for detection of cry2Ab gene in a GM crop/products. Similar studies were conducted by ShreeVidhya *et al.* (2012) [10] using P-35S, T-nos, *nptII* and cry1Ac primers, with the PCR conditions of denaturation-94ºC for 30sec, annealing 55ºC (*nptII*, T-Nos and Cry1Ac) and 54ºC (P-35S) for 40sec, extension at 72ºC for 30sec, final extension 72^0C for 10min.The PCR amplicons were analyzed on 3%agarose gel and the images were recorded using gel documentation. Subsequently Dolhare and Tank (2014) [3] developed and standardized different detection of transgenes *cry1Ac*, *cry2Ab*, *npt-II*, 35S Promoter & NOS terminator for *Bt* - cotton using PCR based strategies and applied to detect transgene in Bt-cotton. Shahid *et al*., 2015 [9] conducted PCR to detect transgenes in Cotton (*Gossypium hirsutum* L.). They amplified cry2Ab using specific oligo-s and the bands were clearly visible at the product size of 162 bp and the samples were run with 100 bp ladder on 2% agarose gel. Keeping this in view, in our study, Six cotton hybrids were evaluated for molecular diversity and molecular characterization using CRY1AC F/R gene specific primer.

The DNA was isolated from the young and fresh emerging leaves and evaluated for suitability in quantitative analysis. Isolated DNA was checked on 0.8% agarose gel electrophoresis. After isolation and quantification of DNA from different *Bt* and non - *Bt* cotton samples. PCR was conducted using specific set of primer CRY1AC F/R. PCR products obtained were checked on 3% agarose gel electrophoresis with a 1kb ladder loaded parallel to check the size of amplicon. A product of 280bp was observed for Cry1AC gene presence in three transgenic *Bt*-cotton cotton hybrids *viz.*, 201KSSCH, 203KSSCH and 207KSSCH, remaining three samples namely 202KSSCH, RAHH455 and SHH188 were not amplified, because of absence of Cry1AV gene in non-Bt cotton samples (Figure 1). Cotton with insect resistance (Bt) has been planted in South Africa since 1998, and in 2016, there were 9,000 hectares planted to IR/HT cotton, a 25% decrease in planting due to drought and low global cotton price. All cotton is GM with Bt-Bt stack and glyphosate tolerance. It is expected that cotton prices will increase as global prices stabilize, leading to increased prospects for cotton in the 2017-2018 season (Global Status of Commercialized Biotech/GM Crops: 2016) [12].

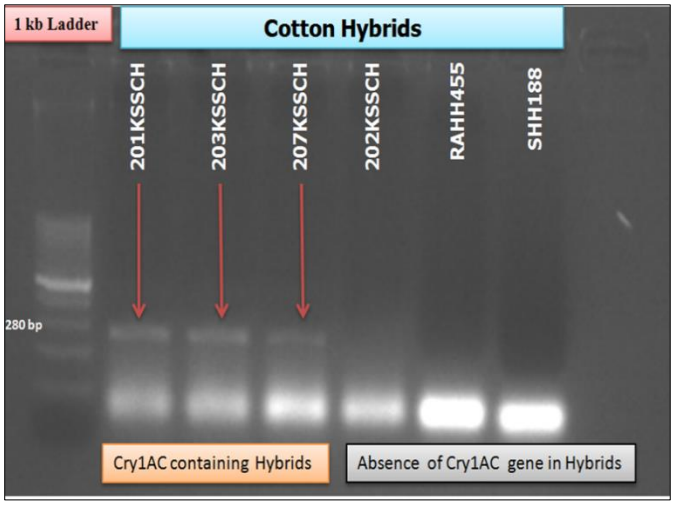

Fig 1: PCR amplicon patterns for bt-cotton and non bt-cotton, generated by marker CRY1AC F/R. Construct-specific PCR using forward primer of promoter and reverse primer of transgene, i.e Cry1AC gene.

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