Polymerase Chain Reaction Detection of the Modified *cry1Ac* Gene in Transgenic Bt (Bollgard®) Cotton[1]

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Abstract Transgenic cotton (*Gossypium hirsutum* L.) containing a modified *cry1Ac* gene from the soil bacterium *Bacillus thuringiensis* Berliner has been widely adopted for suppression of lepidopterous pests. As of 2004, over 90% of the cotton acreage in the mid-southern United States contained this modified *cry1Ac* gene. We developed a technique using the polymerase chain reaction (PCR) for routine detection of the *cry1Ac* gene in transgenic cotton plants. A total of eight *cry1Ac* genes were aligned for the PCR primer design. A DNA fragment was amplified from transgenic cotton, sequenced, and confirmed to be a portion of the *cry1Ac* gene. A total of 150 cotton plants representing four cultivars were examined for the presence of the *cry1Ac* gene. Results demonstrated that all of these cotton plants harbored the *cry1Ac* gene (i.e., 100% purity). This PCR technique can be used for future studies involving the expression of *cry1Ac* gene as well as corresponding protein expression.

Key Words PCR, Bt, cotton, gene purity

Cotton (*Gossypium hirsutum* L.) varieties engineered to express the insecticidal protein of the modified *cry1Ac* transgene, isolated from *Bacillus thuringiensis* Berliner (Bt), suppress lepidopteran pests of cotton in the United States. Cotton varieties containing the modified *cry1Ac* gene have been commercially available since 1996 (John 1997, Zhang et al. 2000). Bt toxin expression levels in cotton were correlated to seasonal change (Adamczyk and Sumerford 2001, Adamczyk et al. 2001) or different environmental conditions (i.e., such as plant nitrate levels) (Pierce et al. 1999). Enzyme-linked-immunosorbent assays (ELISA) have been developed and commercialized for detecting expression levels of Cry-protein(s) within cotton tissues.

Because ELISA exclusively relies on protein levels in the cotton, factors resulting in reduced toxin levels cannot be properly reflected by ELISA data. Absence of Bt gene in cotton seed is a potential problem of seed contamination. Many environmental parameters adversely affect gene transcription, protein translation, and toxin stability. Identifying potential factors relies not only on ELISA data, but confirmation of the transgene and measurement of gene transcripts as well. Routine methods to determine the purity of transgenic *B. thuringiensis* or Bollgard® cotton (Monsanto Co., St. Louis, MO) are limited, because no published methods are available to determine if an individual plant contains the modified *cry1Ac* gene. Therefore, without a routine DNA assay to detect the modified *cry*-gene, it is difficult to differentiate between plants

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that do not contain the *cry1Ac* gene (i.e., Bt cotton seed contaminated with conventional seed) and transgenic plants which are improperly expressing the protein product. We developed a simple, robust polymerase chain reaction (PCR) protocol that selectively amplifies the modified *cry1Ac* gene in transgenic Bt cotton. This technique will be used to address purity issues with certain insect-resistance management strategies.

Materials and Methods

DNA extraction. Cotton genomic DNA was prepared using a Qiagen DNeasy plant mini kit (Cat.No. 69106, Qiagen, Valencia, CA). A fresh leaf disk (9 mm diam) from an individual plant was ground in lysis buffer using a motorized homogenizer (Glas-Col, Terre Haute, IN) at room temperature. DNA precipitation and wash were conducted following manufacturer's instructions. DNA was eluted with 100 µl buffer.

Primer design and PCR amplification. The DNA sequence (Adang et al. 1985, GenBank accession M11068) for *cry1Ac* and seven synthetic or modified *cry1Ac* DNA sequences were downloaded from the GenBank of the National Center for Biotechnology Information (*http://www.ncbi.nlm.nih.gov/*). These modified Bt sequences included M60856 (Perlak et al. 1991), Y09787 (Nayak et al. 1997), AY126450 (Park et al., unpubl.), AF177675 (Kemp and Sutton, unpubl.), U63372 (Adang et al. 1985), AF023672 (De Rocher et al. 1998), and AF537267 (Quanhong et al., unpubl.). CLUSTALW multiple alignment protocol (Thompson et al. 1994, http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/n psa_clustalwan.html) was used to align seven modified *cry1Ac* DNA sequences with unmodified *cry1Ac* sequence. Conserved regions were revealed and used for primer design. Four primer regions were selected (Fig. 1) to synthesize two forward primers, BtF1 and BtF2, and two reverse primers, BtR1 and BtR2. These primers covered more than 62% of the N terminal coding region for the synthetic *cry1Ac* genes (Fig. 1).

Polymerase chain reaction (PCR) was performed to amplify the *cry1Ac* gene fragment from cotton DNA. Annealing temperature and DNA concentration were opti-

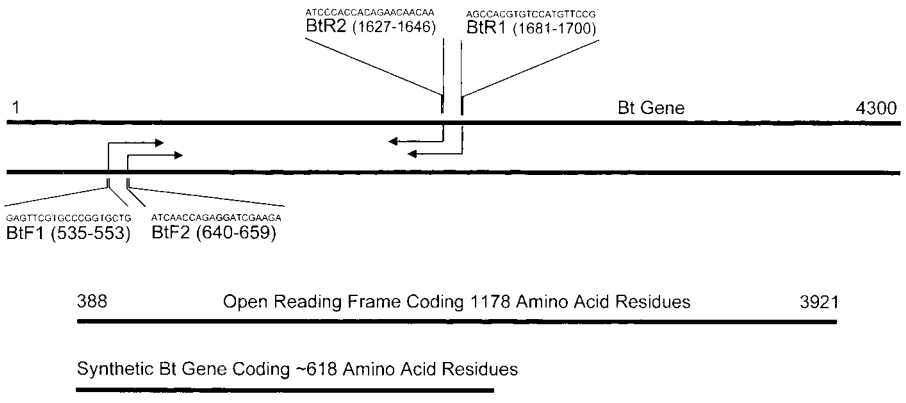

Fig. 1. Bt gene and primer design. Diagram of approximate primer locality on *cry1Ac* Bt gene (Adang et al. 1985; GenBank accession M11068) and coding regions.

mized during pretrail experiments. Initial PCR reactions (50 µl) contained 10 mM Tris-HCl at pH 9, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 50 mM KCl, 0.1 mM of each dNTP, 0.05 unit/µl of *Taq* DNA polymerase (Promega), and 1 µl of cotton tDNA template (approximately 44 ng), and were performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). DNA was initially denatured for 2 min at 94°C, and the PCR amplification was conducted for 40 cycles, with 30 s denaturing at 94°C, 30 s annealing at 56°C, and 1 min extension at 72°C. PCR products were separated on 1% low-melting point agarose gel containing 0.5 µg/ml ethidium bromide. The DNA fragment with expected size was excised from the gel and extracted using a Wizard PCR Preps DNA purification system (Promega, Madison, WI). The isolated DNA fragments were ligated into a pGEM-T vector (Promega) at 22°C for 1 h, then at 4°C overnight. Transformed *Escherichia coli* cells with vectors were plated on LB/ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. PCR-confirmed colonies were inoculated into 4 ml LB/ampicillin cultures and plasmid DNA was extracted using a Qiagen Plasmid Mini kit (Qiagen, Valencia, CA). DNA inserts were sequenced using an automated sequencer (ABI Prism 3700). The *cry1Ac* DNA sequence was confirmed by homology searching of GenBank using the Blastn protocol (Altschul et al. 1997).

cry1Ac detection in cotton plants. Cotton fields were located in Washington Co., MS, which were planted with Delta and Pineland Co. cultivars {DP 444BRR, DP555BRR (2 fields), NuCOTN 33B, and SG125 BRR}. Thirty young cotton leaves (approximately 6×6 cm) were randomly collected from each of five fields (total 150 cotton plants). Two primers, BtF2 and BtR1, were used to amplify the above mentioned 1061 bp *cry1Ac* gene from cotton DNA.

Results and Discussion

Cotton DNA preparation contains components which might be inhibitory to the PCR amplification. By lowering DNA content in the PCR reaction and adjustment of annealing temperature, we were able to successfully amplify target DNA fragments (Figs. 2, 3).

In the first attempt of PCR amplification, four combinations of primers were used with Bt cotton and conventional cotton DNA preparations. PCR amplification using combinations of forward primer BtF1 with other two reverse primers, BtR1 and BtR2, generated a major band with size at approximately 900 bp (lanes 1-4, Fig. 2). This fragment appeared to be from non-target fragment because it was amplified from both Bt cotton and non-Bt cotton DNA preparations with the same size lower than the expected size (1165 bp for BtF1+BtR1, and 1115 bp for BtF1+BtR2). PCR amplification using forward primer BtF2 with the reverse primers, BtR1 and BtR2, resulted in a distinct band with expected fragment size, 1061 bp for BtF2+BtR1 or 1007 bp for BtF2+BtR2 (lanes 5 and 7, Fig. 2). These fragments also were amplified from Bt cotton DNA, and no corresponding fragments were amplified from non-Bt cotton DNA. The 1061-bp fragment was selected for cloning and sequencing. By using Blastx homology search of GenBank database, the 1061-bp cotton DNA fragment was perfectly matched to *cry1Ac* genes. The translated protein sequence (436 a.a. residues) was nearly identical to many Bt δ-endotoxins. By using the Blastn homology search protocol, DNA sequence identity reached almost 100% to the *cry1Ac* gene (GenBank: Y09787, Nayak et al. 1997) and an artificial *cry1Aa* insect control gene (GenBank:

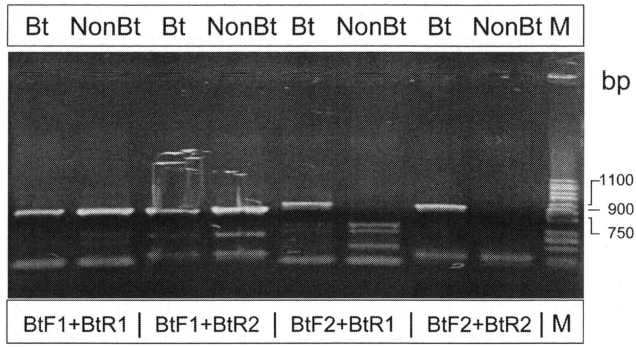

Fig. 2. PCR amplification of Bt cotton and non-Bt cotton DNAs using four combinations of primers. M = DNA size standard.

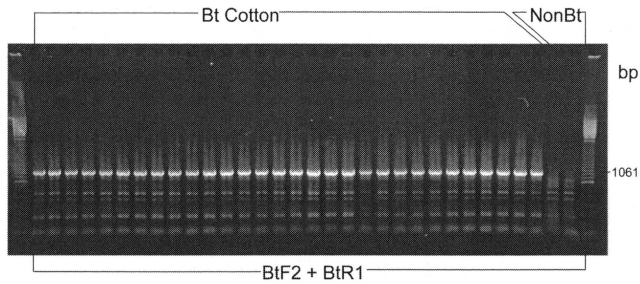

Fig. 3. Representative PCR amplifications of 1061-bp *cry1Ac* DNA from cotton plants using forward primer BtF2 and reverse primer BtR1.

M60856, Perlak et al. 1991). Other highly match genes included a synthetic construct insecticidal protein Cry1Ac1 (*cry1Ac1*) gene (GenBank: AY126450, deposited by Park et al., unpubl.) with 97% sequence identity, and to a synthetic construct *cry1Ac* insecticidal endotoxin gene (GenBank: AF177675, deposited by Kemp and Sutton, unpubl.) with 91% sequence identity. The DNA sequence identity to wild type Bt *cry1Ac* genes was 81%. Based on sequence analysis, we concluded that the 1061-bp fragment was amplified from an artificial *cry1Ac* gene inserted into the cotton genome.

By conducting PCR amplification with forward primer BtF2 and reverse primer BtR1, we surveyed a total of 150 cotton plants from five fields. A 1061-bp DNA fragment was specifically amplified from all Bt cotton plants (100%) (representatives from lane 1 to 30, Fig. 3).

In conclusion, we developed a specific DNA marker for surveying the *cry1Ac* gene within transgenic cotton genome. In future studies, we will use this artificial fragment from the *cry1Ac* gene to develop a probe to study gene expression levels and insecticidal function under different environment conditions. This PCR technique will be used in conjunction with ELISA to detect the Cry1Ac protein (Adamczyk and Sumer-

ford 2001), and issues regarding gene purity and the possibility of gene silencing can be addressed. In addition, the amount of non-expressing or non-*cry1Ac* plants in a given field can be estimated using our technique which is crucial for certain insect-resistance management strategies (i.e., mixtures) (Agi et al. 2001).

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