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MOLECULAR CHARACTERIZATION OF RECOMBINANT AND NON RECOMBINANT COTTON VARITIES OF COMMERCIAL IMPORTANCE

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ABSTRACT : An extended multiplex PCR method was established to rapidly identify and classify *Bacillus thuringiensis* strains containing cry (crystal protein) genes toxic to species of Lepidoptera, Coleoptera, and Diptera. The technique enriches current strategies and simplifies the initial stages of large-scale screening of cry genes by pinpointing isolates that contain specific genes or unique combinations of interest with potential insecticidal activities, thus facilitating subsequent toxicity assays. For this assay we selected a pair of Cry 1 gene(npt II) and differentiated the BT-cotton and non Bt-cotton. And we identified that this Bt-cotton is rapidly infected or toxic to species of Lepidoptera, Coleoptera, and Diptera.

Key words **:** Cotton, Cry 1 gene(npt II), *Bacillus thuringiensis*, GMO seeds.

INTRODUCTION

Gossypium is the cotton genus

It belongs to the tribe *Gossypieae*, in the mallow family, Malvaceae, native to the tropical and subtropical regions from both the Old and New World. The genus *Gossypium* comprises around 50 species [1]. Making it the largest in species number in the tribe *Gossypioieae*.The name of the genus is derived from the Arabic word *goz*, which refers to a soft substance[2].

Cotton is the primary natural fibre used by modern humans. Cultivated cotton is also a major oilseed crop, as well as a main protein source for animal feed. Cotton plants thus have an enormous weight in the world economy and are of great importance for the agriculture, industry and trade of many tropical and subtropical countries in Africa, South America and Asia. Consequently, the genus *Gossypium*has long attracted the attention of scientists, on the other hand the naturally colored cotton is unique and exceptionally different from white cotton as it does not need to be dyed [3]. According to, agronomists the cost of dyeing could be up to half of the value, and also environmentally friendly.

Bacillus thuringiensis (or Bt) is a Gram-positive, soil-dwelling bacterium,was first isolated by the Japanese scientist S. Ishiwata, in1901, from silkworm larvae exhibiting the sotto disease [4]. Commonly used asa biological pesticide.

B. thuringiensis also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well on leaf surfaces, aquatic environments, animal feces, insect-rich environments, and flour mills and grain-storage facilities [5],[6]. It has also been observed to parasitize other moths such as *Cadra calidella*—in laboratory experiments working with *C. calidella*, many of the moths were diseased due to this parasite[7].

During sporulation, many Bt strains produce crystal proteins (proteinaceous inclusions), called ä-endotoxins, that have insecticidal action. This has led to their use as insecticides, and more recently to genetically modified crops using Bt genes, such as Bt corn[8]. Many crystal-producing Bt strains, though, do not have insecticidal properties[9].Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920s and are often applied as liquid sprays [10]. They are now used as specific insecticides under trade names such as DiPel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans,

wildlife, pollinators, and most other beneficial insects and are used in Organic farming[11]. However the manuals for these products do contain many environmental and human health warnings,[12][13] and a 2012 European regulatory peer review of 5 approved strains found that while there is data to support some claims of low toxicity to humans and the environment, there is insufficient data to justify many of these claims.[14]

BT cotton is geneticly modified (GM) to contain a natural toxin created by a bacteria whose initials are BT. The bacteria as well as its toxin have long been used in organic farming as it is only activated in a highly basic (as in not acidic) environment which is rather rare in nature except for the digestive system of several butterflies one of which is a major pest in cotton crops[15],[16].

Bacillus thuringiensis serovar israelensis, a strain of *B. thuringiensis* is widely used as a larvicide against mosquito larvae, where it is also considered an environmentally friendly method of mosquito control.

MATERIALS AND METHODS

Glassware and chemicals

In all the experiments acid washed (0.4N HCl) Pyrex glassware rinsed with double distilled water was used. For the preparation of culture media and other chemical reagents analytical grade chemicals (E Merk GR/BDH, Analar R) were used.

Sterilization

All the glassware used in the experiments were sterilized in hot air oven at 160ºC for 4 hours. The media used in the experiments were sterilized in an autoclave at 15 lbs per square inch for 15 minutes.

Germination of seeds

The seeds are placed on a paper towel on the bottom of a plastic bottle. A different plastic bottle will be used for each treatment. Each paper towel will be dampened by the liquid(water). The paper towels will be checked daily (except weekends) and moistened as needed. After the seeds germination collect the cotyledons for crushing.

Crushing the seed cotyledons

Collect the germinated seed cotyledons and place them a side. Wash the Mortar & pestle. After cleaning the mortar and pestle, crush the same size sample of the germinated cotyledons. Place the crushed, germinated cotyledons, in an appropriately marked eppendorf tube. Proceed the tubes for isolation of genomic DNA.

Isolation Of GenomicDNAFrom Seed Cotyledons

Bacteria from a saturated liquid culture were lysed and the proteins were removed by digestion with the proteinase K. Cell wall debris, polysaccharides, and remaining proteins were removed by selective precipitation with CTAB, and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Material

1. TE BUFFER: 1ml of 1M tris (pH 8) + 200micro/lit 0.5 M EDTA in 100ML of deionized water. Incubator or water bath at 50c, with shaker.
2. 10% sodium dodecyl sulfate(SDS) (10g of SDS to make 100ml of solution).
3. 20mg/ml proteinaseK (5micro/lit of 20mg/ml proteinase K per 1ml.
4. 5M Nacl(29.25g to make 100ml of solution).

5- CTAB/Nacl Solution(10% CTAB in 0.7M Nacl) : Dissolve 4.1 Nacl in 80ml water and slowly add 10g CTAB while heating and stirring. If necessary, heat to 65c to dissolve.Adjust final volume to 100ml withequal volume of .24:1 ratio of chloroform/ Isoamyl alcohol/Isopropanol, 70% ethanol (70ml Ethanol + 30ml deionized water).

Protocol

1. Take above crushed material in a 1.5mL microcentrifuge tube and centrifuge for 5min at 6000 rpm, or until a compact pellet forms. Discard the supernatant.
2. Resuspend the pellet in 567micro/lit TE buffer by repeated pipetting. Add 30micro/lit of 10%SDS and 3micro/lit of 20mg/ml proteinaseK to give a final concentration of 100mg/ml proteinase K in 0.5% SDS. Mix thoroughly and incubate 1hr at 37c.
3. Add 100micro/lit of 5M Nacl and mix thoroughly. Add 80micro/lit of CTAB/Nacl solution. Mix thoroughly and incubate 10min at 65c.
4. Add an approximately equal volume (0.7to0.8) of chloroform/isoamyl alcohol/Phenol(25:24:1), mix thoroughly, and spin for 8min at 8000rpm in a microcentrifuge.
5. Transfer the supernatant to a fresh tube. Add 0.6 volume isopropanol to precipitate nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. Spin at 8000rpm for 8min.
6. Wash the DNA with 70% ethanol to remove residual CTAB and respin 5min at room temperature to repellet it.
7. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer. Redisslove the pellet in 30-

50µl of Nuclease free water.

Quantification Of DNA

Isolated DNA was quantified by measuring absorbance at 260 and at 280nm. Ratio of absorbance 260/280 was used to determine the quality of isolated DNA .The concentration was calculated using the following formula

Concentration of the DNA = OD260 X 50 X dilution factor = mg of DNA /ml

Polymerase Chain Reaction (PCR)

The method of PCR will formed according to [17].

Components of PCR

DNA template: That contains the DNA region (target) to be amplified.

Deoxynucleoside triphosphates (dNTPs): Also very commonly and erroneously called deoxynucleotide triphosphates

Primers :

These are complementary to the DNA at 5' or 3' ends. DNA polymerase, such as Taq polymerase or another DNA only and erroneously called deoxynucleotide triphosphates, thebuilding blocks from which the DNA polymerases synthesizes a new DNA strand.

Buffer solution:Providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.

Divalent cations :Magnesium or manganese ions; generally Mg2+ is used, but Mn2+ can be utilized for PCR-mediated DNA mutagenesis, as higher Mn2+ concentration increases the error rate during DNA synthesis.

STEPS IN PCR

Initialisation: This step consists of heating the reaction to a temperature of 94-96°C, which is held for 1-9 minutes.

Denaturation: The reaction mixture was heated at 94-98°C for 20-30 seconds causes melting of DNA template and primers by disrupting hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing: Temperature is lowered to 50-65°C for 20-40 seconds for annealing of primers to SS-DNA template. Annealing temperature is about 3-5°C below the Tm of primers used. Stable DNA-DNA hydrogen bonds are only formed when primer sequence closely matches template sequence. Polymerase binds to primer-template hybrid and begins DNA synthesis.

Extension

Temperature at this step depends on DNA polymerase used; Taq polymerase has the optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used.

Final elongation

This single step is performed at 70-74°C temperature for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

Final hold :

This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

PCR is commonly carried out in a reaction volume of 20-150 ìl in small reaction tubes in a thermal cycler for about 20 t0 40 cycles.

Optimisation Of PCR

Initially, there was some variation from test to test when the same PCR program was used. Solving this reproducibility problem required adjustments of PCR components.

Amount of primer: Initially equimolar primer concentrations of each can be used in multiplex PCR, if there was uneven amplification, with some products barely visible even after the reaction was optimized for cycling conditions, changing the proportions of various primers in the reaction, with an increase in amount of primers for "weak" amplicons and a decrease in strong" amplicons amount.

$MgCl_2$ concentration

A recommended $MgCl_2$ concentration in a standard PCR is 1.5 mM at dNTP concentrations of around 200 mM each. If the amplification was not complete for all the primers varying concentrations of $MgCl_2$ ranging from 1.5mM to 3mM concentrations.

Amount of template

Template DNA concentrations for Normal PCR is 10 ng to 50 ng. For a multiplex PCR depending on the intensity of the amplicons template concentrations can vary between 50 ng to 200ng.

Amount of Taq DNA polymerase

Different concentrations of Taq DNA polymerase can be used, optimum concentration per reaction is 0.3 mL or 1U/25 mL reaction volume. Too much enzyme, possibly because of high glycerol concentration in stock solution, can result in an unbalanced amplification.

Use of adjuvants

Various authors recommend DMSO and glycerol to improve amplification efficiency (higher amount of

product) and specificity (no unspecific roducts) of PCR, when used in concentrations varying between 5%–10% (vol/vol).

Polymerase chain reaction was performed using primers that were designed from the specific gene of the ShigellaSps Primers were commercially obtained from Bio-serve India pvt, Ltd. All the primers designed were 20 base pairs in length with GC content in the range of 40-80%. Tm was calculated according to the formula. The annealing temperature for each pair of PCR primers was optimized experimentally. Tm(C) = 2(A+T) + 4(G+C) ± 5 .

Resolution ofDNAFragments on Agarose Gels:

Material

1. Electrophoresis buffer (1X TAE) .
2. Ethidium bromide solution 0.5 mg/ml.
3. Electrophoresis-grade agarose.
4. X loading buffer .
5. DNA molecular weight markers.
6. Horizontal gel electrophoresis apparatus.
7. Gel casting platform.
8. Gel combs (slot formers).
9. DC power supply.

Gel Preparation

Prepare an adequate volume of electrophoresis buffer fill the electrophoresis tank and prepare the gel.

Add 0.25g of the Agarose in 25 ml of 1XTAE to prepare 1%gel and dissolve it by heating it at 70°C in a micro-wave, cooled and add 1ml of the Ethidium bromide.

Pour it onto the gel-castner which has comb for wells creation and remove comb after gel solidification and Place the gel into gel loading tank and fill it with the 1 X TAE till the gel submerged into the buffer.

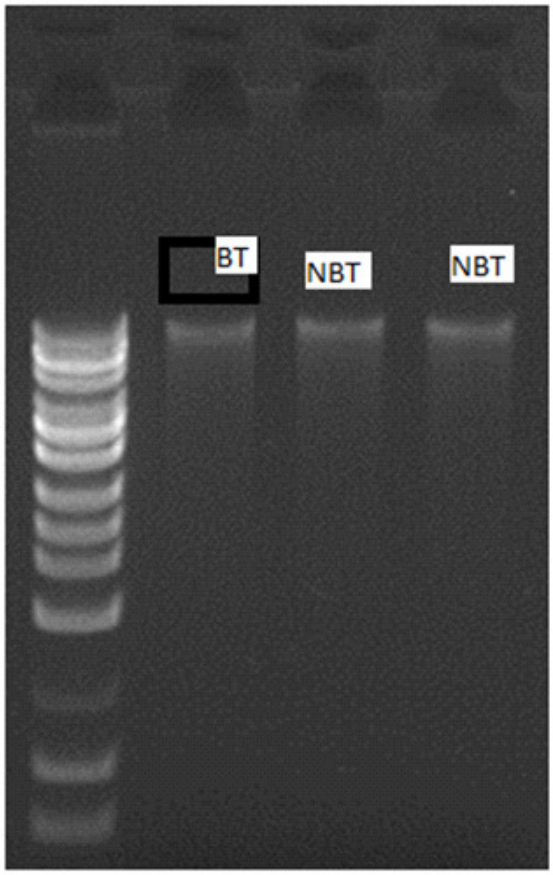
Load the samples mixed with gel loading dye which has bromophenol blue (Front dye) and Xylene cyanol (Tracking dye) and the Reference size standard (to know the size) in the gel and run at 75 volts till the time the bromophenol blue reaches 3/4th of the gel.

PCR amplification was carried out with following reaction parameters :

S.No.	Components	Concerntration	Volume
1	DNTPs	200µM	2.5µl
2	PCR reaction buffer	1X	2.5µl
3	Magnesium chloride	1.5	1.0µl
4	Primer (forward)	2.5 mM	1.0 µl
5	Primer (reverse)	10 pmoles	1.0 µl
6	Template DNA	100ng	2.0µl
7	Taq DNA polymerase	1U	1.0µl
8	MQ Water		14µl

The finalreaction volumewas 25µl.

DNA isolation result :



PCR result – PCR reaction was standardized at the following conditions :

Stage 01		Stage 02		Stage 03		Stage 04	
Hold (Initial Denaturation)		Three Temperature cycle (Repeat 40 times)		OneTemperature		Hold	
Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
95°C	3 min	95°C	30sec	72°C	5min	4°C	∞
		56°C	30sec				
		72°C	1.30min				

Visualize the DNA by placing on a UV light source and can be photographed directly by gel documentation unit.

RESULTS AND DISCUSSION

A lot of cotton seeds is taken, germinated and differentiated by using molecular technique i.e. PCR method (cry I gene (npt II) that GMO seeds are toxic to species of (Lepidoptera, Coleoptera, and Diptera) ,this result wasagreement with [18]who reported that the GMO seeds were toxic to (Lepidoptera, Coleoptera, and Diptera) when it Supported by(cry I) gene .And accordance with [19] who claimed that the (Cry1 toxins display activity against lepidopteran, dipteran, and coleopteran pests) ,also this result that coincidence with [20,21,22,and 23] whom documented thattheBacteria *B. thuringiensis* produce crystals comprised of Cry proteins active against insect pest, that which determine the synthesis proteins toxic for insects of (Lepidoptera, Coleoptera and Diptera).

Primer Sequence

npt II F 5'- CCGCCACACCAGCCGGCC-3'

npt II R 5'- CCGACCTGTCCGGTGCCC-3'

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