Research Article

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RP BT GENE AND BT PROTEIN STUDIES IN TULASI COTTON HYBRIDES BY PCR AMPLIFICATION.

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Abstract:

Cotton, the most prevalent fiber used in the clothing today, often referred as the White Gold. It contributing up to 75% of total raw material needs of textile industry. But, cotton crop is highly susceptible to insects; especially to the larvae of lepidopteron pests, which is impacting cotton production. Of these pests, the American boll worms alone cause yield reduction up to 40 – 70 % under severe incidence. it has been argued that adoption of Bt cotton could help in protecting the crop against potentially the most damaging bollworms and thus reduce the risk of crop failures and the use of insecticides leading to create eco-friendly environment without compromising on profitable yield. a transgenic plant, produces an insect controlling protein Cry1A(c), the gene for which has been derived from the naturally occurring bacterium, Bacillus thuringiensis subsp. Kurstaki. The Present studies aims at identifying the Bt gene and Quantifying the Bt protein in three different Tulasi Bt cotton hybrids TCHH-4, TCHH-9, TCHH-17.This involve isolating the DNA from the Hybrids and amplifying the Bt gene by PCR amplification and Testing the Zygosity of the Bt gene in the plant hybrid using Gel electrophoresis

Key Words:

Bacillus thuringiensis, Cotton hybrids, PCR amplification, Gel electrophoresis.

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Introduction:

Cotton is often a cross pollinated crop and has tap root system, Leaves are simple and petiolate. Phyllotaxy is 1/3 in Asiatic cotton and 3/8 in American and Egyptian cotton. Cotton belongs to the genus Gossypium,

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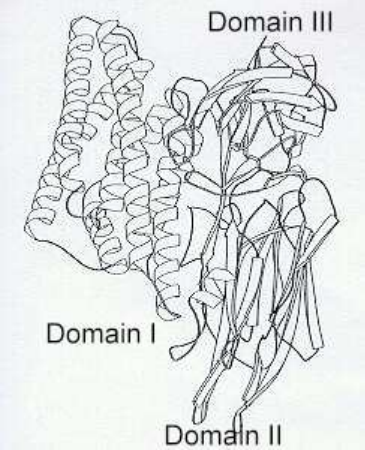
which is one among the eight genera under the tribe Gossypieae in the family Malvaceae. There are about 42 species of Gossypium out of which four species namely *G.hirsutum* L. and *G.barbadens* L.(New world, Tetraploid with 2n=4x=56) and the old world species *G.arboreum* L and *G.herbaceum* L.(Diploid with 2n=26)are cultivated for their spinnable fiber called lint. And of the remaining 38 of them are wild, out of which eight are Tetraploids and the remaining are diploids.



Structure of BT protein

Cotton is an important crop grown in more than 70 countries and it plays an increasing role in global economy in the 21st century. Despite the advent of a multitude of other fibers, Cotton rules the world of textiles. Even today it is unchallenged as a natural textile fiber and gained more global importance in recent times as the affluent public; both in developed and in developing nations has renewed liking for the pure fabrics. In addition to fibers for human clothing which is the chief purpose for which cotton is grown in several countries such as India, US, Russia, China, Pakistan, and Egypt. Its seed a byproduct also plays an important role in the economics cum industrial development.

The American Bollworm is a major pest of agriculture and horticulture in Australia, Asia, Africa and Southern Europe. In Australia it is called the cotton bollworm. In India it is called the American Bollworm. Scientists call it *Helicoverpa armigera*. This moth attacks more than 100 different commercial crops including cotton, maize, wheat, sorghum, sunflower, chickpeas, soybeans, tobacco, tomato, lettuce, sweet corn, capsicum and beans and flowers including chrysanthemums, gladioli and roses. It is the single biggest pest for global plant agriculture. *Bacillus thuringiensis* (or Bt) is a naturally occurring bacteria, common in soils. Several strains of these bacteria can infect and kill insects. Because of this property, *Bacillus thuringiensis* was developed as a type of microbial insecticide, used for control of a variety of insects.

The insecticidal activity of Bt was first discovered in 1911. However, it was not commercially available until the 1950's. In recent years, there has been tremendous renewed interest in Bt and several new products are available. Bt acts as a stomach poison. It must be eaten to affect the susceptible insect. It has no contact activity, so it cannot act by being absorbed through

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the cuticle of the insect. Most Bt products are designed to be mixed with water. It is then sprayed on leaves to be eaten by pest insects, released into water, or used as a soil drench, depending on the intended use. The insecticidally active ingredients of *Bacillus thurgingiensis* are various proteins produced by the bacteria. In particular, compounds known as the delta-endotoxins, which form crystals inside the bacteria, kill the insect. In the gut of susceptible insects, the delta-endotoxin becomes activated. If activated, it disrupts the cell along the lining of the insect gut within minutes after contacting them. Usually within hours, cells lining the gut are killed. The gut is paralyzed and the insect stops feeding by this time.

Materials and methods:

Cotton seed extract is taken from the plants. All the reagents, chemicals are of hogh purity and were purchased from DesiGen Diagnostics Company. All the glassware was obtained from Matrix technologies and was sterilized prior to the use.

Master cycler gradient (PCR) is used for the DNA amplification. Agarose gel electrophoresis is used to separate DNA strands by size.

Preparation of Stock solutions:

The solutions are prepared according to their concentrations or percentages mentioned in the procedure. The solutions are diluted with sterile distill water.

Procedure:

Qualitative ELISA:

The leaf sample is collected by punching 2-3 discs using mcf tube by placing a leaf between the lid and the tube opening and closing the lid onto the leaf. 500 µl of extraction buffer is added and the sample is crushed with a pestle for 30 seconds and 100 µl of this extract is used for sampling.

To the pre-coated plate, add 100ul/well of anti-Cry1Ac conjugate. Add 100 µl of the sample extract and 0.5pbb positive controls provided, to the appropriate wells and incubate the plate at room temperature for 45min.Wash the plate four times with 1xwash buffer [refer to recipe].pat the plates dry (wells down) on blotting paper to remove excess buffer. Add 100 µl of freshly prepared 1x substrate per well, Incubate the plate at room temperature in dark for 15-min.Add 100ul/min stop solution after 15 min of incubation. Measure the absorbance at 450 nm using an ELISA plate reader.The absorbance of a blank well must be subtracted from absorbance values of samples and controls.

DNA Isolation:

Add 3 mm glass beads, or equivalent, per tube containing approximately 100 200 mg ground seeds. Add 550 µl of extraction buffer.Beat in a paint shaker, or equivalent, until the ground seed material is saturated with extraction buffer. Incubate for 1hr at 65^{o}

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C; invert, or vortex, after 30 minutes.Centrifuge for 1minute at 1,500 rpm. This step helps reduce contamination by forcing the lysate and/or condensation to the bottom of each tube. Add 150 µl of 5M sodium acetate. Beat in a paint shaker, or equivalent, until the sodium acetate is thoroughly mixed with the lysate. Incubate for at least 20 minutes at 20 ^{o} C. Centrifuge for 20 minutes at a minimum of 3,000 rpm at 4 ^{o} C.Transfer approximately 300 µl of DNA supernatant into a new tube or well containing 400 µl of isopropanol. A layer of particular matter may be present on the surface; be careful to transfer a minimal amount of solids with the supernatant. Invert 20 minutes, or gently vortex, and incubate for at least 30 minutes at 20 ^{o} C to precipitate the DNA.Centrifuge for 20 minutes at a minimum of 3,000 rpm at 4 ^{o} C.Pour off the supernatant and blot tubes upside down on a paper towel, or equivalent, to drain excess liquid. Dry the DNA pellet at 65 ^{o} C for 15minutes. Do not over dry the DNA pellet. Re-suspend (by pipetting up and down) the DNA pellet in 200µl of 1X TE buffer.

Add 25µl of 3M sodium acetate. Add 450 µl isopropanol. Invert 20 times, or gently vortex, and incubate for at least 30 minutes at -20 ^{o} C to precipitate the DNA. Centrifuge for 20 minutes at a minimum of 3,000 rpm at 4 ^{o} C.Pour off the supernatant and blot tubes upside down on a paper towel, or equivalent, to drain excess liquid. Dry the DNA pellet at 65 ^{o} C for at least 30 minutes, or until dry. Do not over dry the DNA pellet.Re-suspend (by thoroughly mixing) the DNA pellets in 200µl of 1X TE buffer and Store at 4 ^{o} C.

Polymerase Chain Reaction:

Polymerase chain reaction, or PCR, is a powerful tool for amplifying a stretch of DNA. From as little as a single piece of target DNA, you can make enough copies for sequencing, cloning or gel electrophoresis. A PCR reaction is typically carried out in a small tube placed in a machine called a thermocycler. The DNA amplification in PCR precedes the fallowing steps.

Step 1: Denaturation

Each cycle consists of three steps: denaturation, annealing, and extension. In the denaturation step, the temperature of the reaction is raised to above 90 degrees C in order to break the hydrogen bonds between strands and produce single-stranded DNA.

Step 2: Annealing

In the annealing step, the temperature is lowered in order to allow the oligonucleotide to anneal to the target DNA using complementary base pairing. The exact temperature needed for the annealing step is a function of the length and sequence of the oligonucleotide.

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Step 3: Extension

In the extension step of the cycle, the annealed oligonucleotide is used as the primer for DNA synthesis. As you should recall, all DNA polymerases synthesize in a 5-prime to 3-prime direction starting from double stranded DNA. The extension step makes the complimentary DNA of the template sequence. The polymerases used for PCR differ from normal DNA polymerases in that they are able to survive the extreme temperatures without being inactivated due to denaturation. Typically they are cloned from bacteria which live in high temperature environments such as hydrothermal vents.

Preparation of master mix for Cry1Ac

Reagent	Amount	comments
18 mega ohm water	Add to a final volume of 20 µl	
10x reaction buffer with	2 µl	1x final concentration buffer, 1.5 mM final conc. of mgcl2
10mM solution of dATP, dCTP, dGTP, and dTTP	0.4 µl	200 µM final conc. of each dNTP
Primer 1(re-suspended in 1x TE buffer or 18mega ohm water to a conc. of 10 µM	0.4 µl	0.2 µM final Conc.
Primer 2 (re-suspended in 1x TE buffer or 18mega ohm water to a conc. of 10 µM)	0.2 µl	0.1 µM final Conc.
Primer 3 (re-suspended in 1x TE buffer or 18mega ohm water to a conc. of 10 µM)	0.4 µl	0.2 µM final Conc.
RED*Taq* DNA polymerase (1 unit/µl)	1.0 µl	1 unit/reaction
Extract DNA (template):		
• Samples to be analyzed (individual leaves)	10-200 ng of genomic DNA	
• Negative control	50 ng of non transgenic genomic DNA	
• Negative control	No DNA template	
• Positive control	50 ng of Cry 1 Ac heterozygous genomic DNA	
• Positive control	50 ng of Cry 1 Ac homozygous genomic DNA	

Preparation of master mix for Cry2Ab

Step	Reagent	Amount	comments
1	18 mega ohm water	Add to a final volume of 20 µl	
2	10x reaction buffer with	2 µl	1x final concentration buffer, 1.5 mM final conc. of mgcl2
3	10mM solution of dATP, dCTP, dGTP, and dTTP	0.4 µl	200 µM final conc. of each dNTP
4	Primer 1(re-suspended in 1x TE buffer or 18mega ohm water to a conc. of 10 µM	0.4 µl	0.2 µM final Conc.
5	Primer 2 (re-suspended in 1x TE buffer or 18mega ohm water to a conc. of 10 µM)	0.2 µl	0.1 µM final Conc.
6	Primer 3 (re-suspended in 1x TE buffer or 18mega ohm water to a conc.of 10 µM)	0.4 µl	0.2 µM final Conc.
7	RED*Taq* DNA polymerase (1 unit/µl)	1.0 µl	1 unit/reaction
8	Extract DNA(template):		
	• Samples to be analyzed(individual leaves)	10-200 ng of genomic DNA	
	• Negative control	50 ng of non transgenic genomic DNA.	
	• Negative control	No DNA template	
	• Positive control	50 ng of mon15985 heterozygous genomic DNA	
	• Positive control	50 ng of Mon 15985 homozygous genomic DNA	

Quantitative ELISA

The lyophilized tissue samples were extracted with buffer by vigorous shaking in a homogenizer. The extracts were clarified for centrifugation and the supernatants were stored overnight in a -20C freezer prior to ELISA analysis or in a -70c freezer for long term storage.

ELISA- Cry1Ac:

The validated method for quantification of Cry1Ac in cotton tissues is as follows Ninety-six-well micro titer plates were coated with mouse monoclonal antibody specific to Cry1Ac. A series of six Cry1Ac

protein standard dilutions(0.625 to 20 mg/ml) were prepared for each ELISA plate

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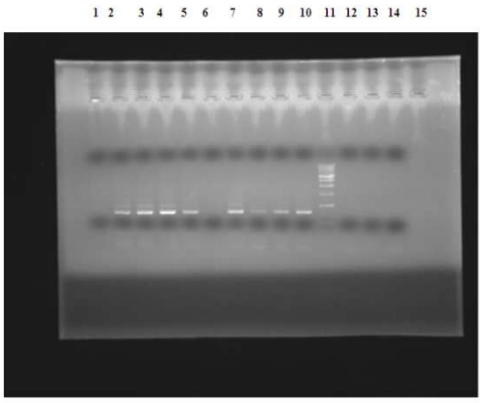
in phosphate-buffered saline with tween-20(PBST: Sodium chloride-0.14M; Sodium phosphate di basic - 0.008M; potassium chloride -0.003 M; Potassium di-hydrogen phosphate - 0.002 M; Tween 20-0.05% v/v) containing Ovalbumin (PBSTO: 5gm/L of buffer). All tissue extracts were trypsinized (Bovine pancreas trypsin, at 5mg/ mL in 50mM sodium carbonate buffer) and diluted 1:4 in PBSTO. A series of Cry1Ac protein samples were loaded in triplicate in each ELISA plate. Immediately following the addition of sample or standard, A polyclonal antibody specific to Cry1Ac is added to the entire plate and incubated at 37c for 1hr. After the incubation was completed, the plates were washed with PBST buffer and a secondary alkaline phospahtase - conjugated detection body was added to the entire plate and incubated at 37c for 45 min. The plates were to be washed as described earlier and the substrate para-nitrophenyl phosphate (1 mg/mL) was added to the well and to incubate in the dark for 30 min. The reaction was to be stopped by the addition of 0.15N NAOH. The absorbance of the resulting yellow color was read at 405nm using a micro plate reader.

ELISA- Cry2AB:

The validated method for quantification of Cry2Ab in cotton tissues is as follows Ninety-six-well micro titer plates were coated with mouse monoclonal antibody specific to cry2Ab2.All tissue extracts were diluted 1:75 in PBSTO. Precoated plates were washed with PBST. A series of six Cry2Ab2 protein dilutions (2.5 to 80 ng/mL) were prepared in PBSTO buffer for each ELISA plate. A series of Cry2Ab2 protein standards, Diluted samples, and positive and negative quality control samples were loaded in triplicate in each ELISA plate. Immediately following the addition of a sample or standard, a polyclonal antibody specific to Cry2Ab were to be added to the entire plate and incubated at 37c for 1hr. After the incubation was over the plates were washed with the PBST buffer and a secondary alkaline phosphatase conjugated detection antibody was added to the entire plate and incubated at 37c for 45 min. The plates were to be washed as described earlier and the substrate para-nitrophenyl phosphate (1 mg/mL) was added to the well and to incubate in the dark for 45 min at 30c.The reaction was to be stopped by the addition of 0.15N NAOH. The absorbance of the resulting yellow color was read at 405nm using a micro plate reader.

Results and Discussions:

Cry1Ac:



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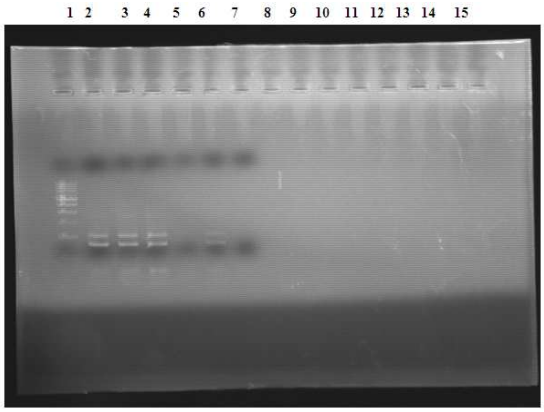
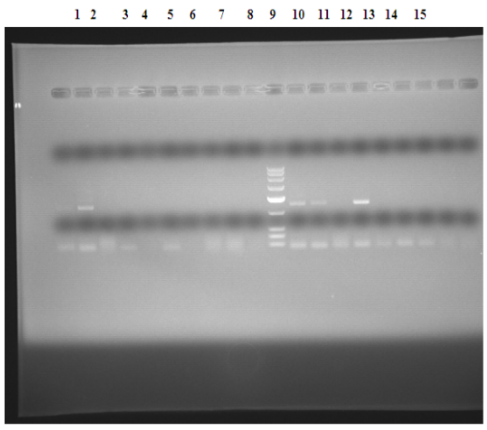
The first three bands and the fifth from the extreme left shows the presence of cry1Ac gene in heterozygous condition showing the presence of internal band at 1000 bps and also the cry1Ac band at 800 base pairs. The fourth sixth seventh and eighth bands shows a homozygosity for cry1Ac gene as it shows the band specific for cry1Ac gene at 800 bps with no internal band which demarcates the heterozygous condition.

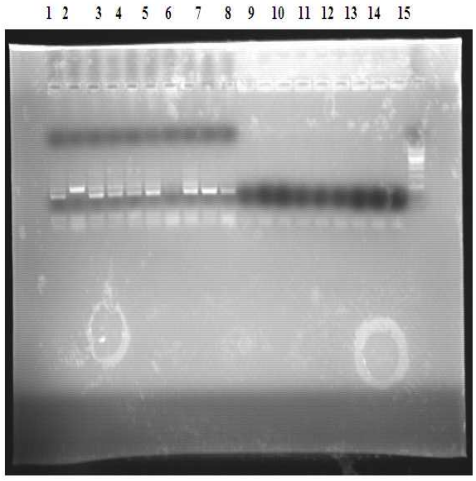


These bands show that all the samples here are heterozygous for cry1Ac condition. As all the samples show both the 800bps band specific for cry1Ac as well as the internal band 1080.

Cry2Ab:



The samples here show the homozygosity for cry2Ab at 1800bps (specific for cry1Ab gene) and internal band at 1000 bps for 5000 bps ladder.



Here we can observe that the sample have both heterozygosity homozygosity as well as the condition where the gene is entirely absent.

Homozygous- 2nd sample from left (1800bps)

Heterozygous- 1, 3,4,5,6, 7 from left (1000bps)

Not present - 8 and 9 from left only internal band.

Cry1Ac levels in various tissues of TCHH-4 Bt field tested in Guntur Tissue type/DAP	S1 (31 DAP)	S2 (45 DAP)	S3 (59 DAP)	S4 (77 DAP)	S5 (94 DAP)	S6 (117 DAP)	S7 (127 DAP)
Terminal leaf	15.68	12.92	12.80	14.24	9.85	8.15	11.98
Square	NT	7.65	6.75	8.09	8.75	4.25	5.59
Boll epicarp	NT	NT	NT	13.89	12.36	8.59	7.92

Table 1 A: Cry1Ac levels in various tissues of TCHH-4 Bt field tested in Guntur

Tissue type/DAP	S1 (31 DAP)	S2 (45 DAP)	S3 (59 DAP)	S4 (77 DAP)	S5 (94 DAP)	S6 (117 DAP)	S7 (127 DAP)
Terminal leaf	391.54	480.57	302.50	196.14	226.68	198.95	298.11
Square	NT	434.16	313.66	232.59	204.10	189.68	281.98
Boll epicarp	NT	NT	NT	211.56	189.56	214.25	237.19

Table 1 B: Cry2Ab levels in various tissues of TCHH-4 Bt field tested in Guntur

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Tissue type/DAP	S1 (31 DAP)	S2 (45 DAP)	S3 (59 DAP)	S4 (77 DAP)	S5 (94 DAP)	S6 (117 DAP)	S7 (127 DAP)
Terminal leaf	16.68	12.98	13.65	14.37	9.85	8.15	11.98
Square	NT	6.65	6.02	6.59	8.31	5.42	6.68
Boll epicarp	NT	NT	NT	13.89	11.01	6.92	6.31

Table 2 A: Cry1Ac levels in various tissues of TCHH-9 Bt field tested in Guntur.

Tissue type/DAP	S1 (31 DAP)	S2 (45 DAP)	S3 (59 DAP)	S4 (77 DAP)	S4 (94 DAP)	S6 (117 DAP)	S7 (127 DAP)
Terminal leaf	431.68	413.89	287.32	149.24	187.65	166.85	192.54
Square	NT	289.32	240.21	150.98	242.10	206.21	194.12
Boll epicarp	NT	NT	NT	124.62	132.89	124.25	199.19

Table 2 B:Cry2Ab levels in various tissues of TCHH-9 Bt field tested in Guntur

S-sampling points; DAP-days after planting, NT- No tissue.

Discussion:

cry1Ac

Terminal leaf

From table 1-3a we can see that in the terminal leaf the protein levels decrease with the sampling time periods and after the S6 qa the protein levels start increasing for the three hybrids

Square

From table 1-3a we can see that in the square the protein levels decrease with the sampling time periods and after the S6 sampling the protein levels start increasing for the three hybrids

Boll epicarp

From table 1-3a we can see that in the Boll epicarp the protein levels decrease with the sampling time periods.

cry2Ab

Terminal leaf

From table 1-3b we can see that in the terminal leaf the protein levels decrease with the sampling time periods and after the S6 sampling the protein levels start increasing for the three hybrids

Square

From table 1-3b we can see that in the square the protein levels decrease with the sampling time periods and after the S6 sampling the protein levels start increasing for the three hybrids.

Boll epicarp

From table 1-3b we can see that in the Boll epicarp the protein levels decreases with the sampling time periods.

Interpretation of results

In all the three hybrids, we can see the pattern of dwindling protein levels of cry1Ac and cry2Ab in same manner. The amount of protein present different tissues at the particular sampling period also varies disregarding the three hybrids.There is very insignificant variation of protein levels in the three hybrids at any given sampling.

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