

An Expression Analysis of *cry1Ac* IN BT Cotton Event MON531

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Abstract— The presence of unauthorized germplasm not only has a negative economic impact on cotton productivity but also indicates a rapid development of resistance in target insect pests due to sub-lethal expression of the transgene. Here we report an assay for a comparative expression analysis of *cry1Ac* and *ghtub1* by standard curve method involving $\Delta\Delta Ct$ values. The assay proves the validity of the Sybr Green I chemistry to be successfully used for the determination of transgene construct and expression. It can also be applied in sorting out the certified Bt cotton varieties (event MON531) from the un-authorized transgenic cotton germplasm.

Keywords—Bt cotton, expression analysis, *cry1Ac*; *ghtub1*, $\Delta\Delta Ct$.

I. INTRODUCTION

Cotton genotypes are mostly developed through agrobacterium mediated transformation method and a crossing of Bt cotton germplasm with conventional elite varieties to introduce the gene cassette in them. Random rearrangement in the transgene nucleotide sequence, the order of the affiliated regulatory sequences as well as number of integrations of the transgene per genome copy resulting from these random hybridizations may result in the loss of transgene expression or even its complete silencing. Thus there is an urgent need to develop an assay to validate the type/construct and especially the comparative expression of the transgene in Bt cotton genotypes.

In order to detect the nature and construct of these nucleotide sequences, conventional and real time PCR techniques are applied. These techniques aim to screen out the controlling sequences such as cauliflower mosaic virus promoter sequence and *Agrobacterium tumefaciens nopaline synthase* terminator (*T-Nos*) while construct-specific detection targets the junctions between the adjacent elements of the transgene construct, i.e. the region between the inserted gene and promoter/terminator sequences (Fig. 1).

This study aims to develop an efficient and rapid assay of determining the true character of a Bt cotton genotype for the event MON531 and expression analysis of the *cry1Ac* with *ghtub1*(*Gossypium hirsutum beta-tubulin*)- a standard endogenous reference gene for cotton.

II. MATERIALS AND METHODS

Five commonly grown Bt cotton genotypes viz. Bt666, Bt886, CM555, SL1, and CM996 were purchased from local agricultural market in Multan in 2013. For each analytic sample, multiple DNA/RNA extractions were made by using Purelink Genomic DNA/RNA extraction kit (Invitrogen) according to the manufacturer's instructions. The conventional PCR mixes per reaction included 0.5 μ M of each primer, 5 μ L of diluted DNA in a final volume of 50 μ L of 1x PCR buffer. PCR conditions include initial denaturation at 95°C for 3 minutes, followed by 40-50 cycles of denaturation for 30s at 94°C; 30s at 58°C (annealing) and for 30s at 72 °C (extension). The final extension was carried at 72 °C for 10 min. The PCR amplicons were analyzed on 2% agarose /ethidium bromide gel (Vivantis) along with 100bp DNA ladder (Invitrogen). SuperScript® VILO cDNA Synthesis Kit and PowerUp™ SYBR® Green Master Mix (Thermo Scientific™) were used for RT qPCR. All the primers were synthesized by Vivantis Technologies. The primer sequences and their amplicon sizes are shown in Table 1.

The qPCR for *ghtub1* and *cry1Ac* was performed in iCycler (Bio Rad) with a final volume of 50 μ L (20 μ L of water, 0.5 μ M of each primer, 3 μ L for each of five 10-fold DNA dilutions of certified reference material (CRM), and 25 μ L of Sybr Green PCR Super Mix Universal (Invitrogen). The thermocycler (Bio-Rad PTC-06) was programmed with initial denaturation at 94°C for 3 min. The cycling conditions for 40 cycles were set for denaturing at 94 °C for 30 sec, annealing at 58 °C and extension at 72°C for 30 sec. The final extension was at 72°C for 10 min which was followed by melt peak curve analysis. The certified reference material (CRM) of MON531 event (AOCS 0804-C) which contains a single copy of the transgene *cry1Ac* was used as a control. Individual and independent runs for both the reference gene in CRM and the target gene in the sample variety were carried out in separate PCR vials in the same PCR run simultaneously.

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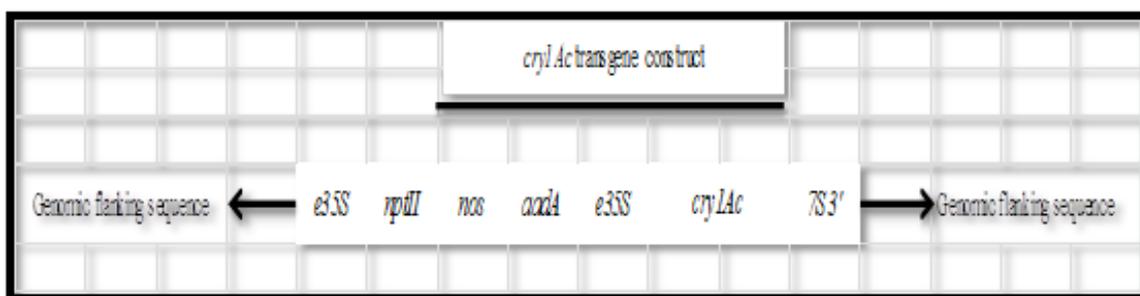


Fig. 1: The transgenic construct of MON531 and the target positions

TABLE 1. SEQUENCES AND AMPLICON SIZES OF THE PRIMERS

Sr.No	Primer Name	Seq. 5' to 3'	Amplicon size	Reference
	MON531 (<i>cryIAC</i> + <i>nos</i>)	F.GATACGGTGAGGGTTGCG R.AAGTCATAGTTAAATAGCCCGATA	577	[7]
1	<i>ghtub1</i>	F.AAATCTAATGGAATAATTTGGATGT R.ACTTAAGGTGTACTTGAAATTACT	220	[7]
2	<i>cryIAC</i>	F.GCCAATGCCTCGTGATTGTTCTCTGC R.GATTTGCGAGGCTGGCCAGCTCCACG	280	[1]

III. RESULTS AND DISCUSSION

MON531 event consists of *cryIAC* along with 35S promoter, and *nos* terminator. In order to confirm that the varieties under trial are true-to-transgenic event, a sequence of DNA spanning from *nos* to *cryIAC* was amplified (Fig.2).



Fig.2: The amplification of *cryIAC+nos* in cotton genome

The primer pair of *cryIAC+nos* amplified a 577 bp DNA fragment containing both the 3'-end of *cryIAC* and 5'-end of *nos*. The fragment of *cryIAC+nos* is specific for MON531 and its presence confirms the transgenic cotton event as MON531 [3].

3.1. Calculation of the Comparative Gene Expression

The relative expression ratio (R) of a target gene with respect to a reference gene is calculated by a range of mathematical models based upon $\Delta\Delta Ct$ values [6].

$$\text{Relative expression ratio (R)} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta Ct_{\text{reference}}(\text{control-sample})}}$$

The threshold cycle (CT, also known as Cq i.e. quantification cycle) is the cycle number at which the amount of amplified DNA sequence reaches a fixed threshold in a real-time PCR assay. Equation (1) describes the exponential amplification of the PCR:

$$X_T = X_0 \times (1 + EX)^{CT}, X \text{ -----(1)}$$

where X_T is the amount of target molecules at threshold cycles, X_0 is the initial amount of the target molecule, EX is the efficiency of the amplification and CT, X is the threshold cycle of the target DNA amplification [2].

Similar to equation (1), the equation for the reference gene (internal control gene) PCR reaction is

$$R_T = R_0 \times (1 + ER)^{CT}, R = KR \text{ -----(2)}$$

where R_T is the amount of reference molecules at threshold cycles, R_0 is the initial amount of the reference molecule, ER is the efficiency of the amplification and CT, R is the threshold cycle of the reference DNA amplification.

From the equation of PCR amplification, the PCR efficiency (EX, ER) can be obtained after the construction of the relative standard curve of the serial dilution of target and reference DNA. Standard curves for *ghtub1* and *cryIAC* were constructed for the CRM through serial dilution method (0.01ng, 0.1ng, 1.0ng, 10ng, 100ng) in three repeats for each dilution point. Average Ct values for each dilution point were plotted to yield standard curves (Fig. 2, 3, 4).

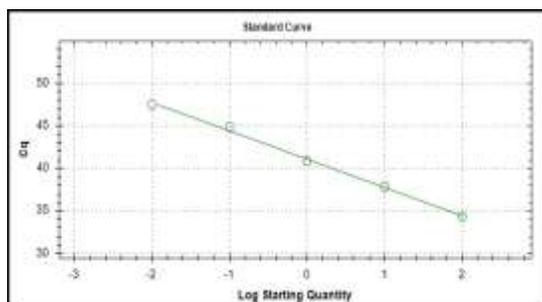


Fig. 2: Dilutions for *ghtub1* in CRM

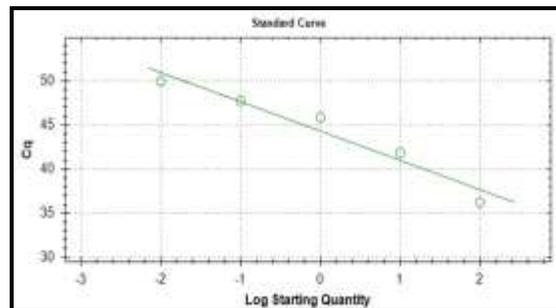


Fig. 3: Dilutions for *cryIAC* in CRM

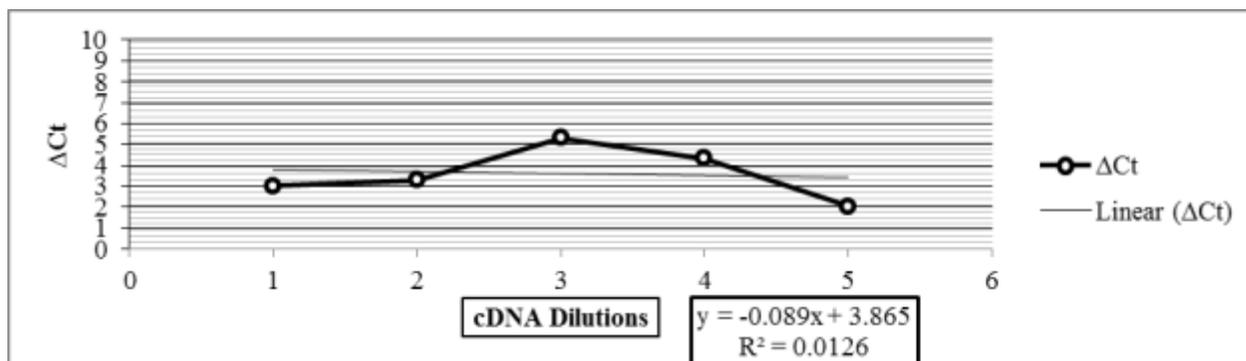


Fig. 4: ΔCt values over the range of dilutions

The Ct values from the standard curves shown above were used to calculate ΔCt which was later used to draw ΔCt curve and its respective R^2 value (table 2).

The expression ratio is calculated according to the following equation:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta Ct_{\text{target}} (\text{control-sample})}}{(E_{\text{ref}})^{\Delta Ct_{\text{reference}} (\text{control-sample})}}$$

The amplification of *cryIAC* and *ghtub1* was carried out in separate PCR vials in the same reaction set up in two repeats for each gene and the corrected Ct values were calculated based upon the respective standard curve as per equations 1 & 2. ΔCt values were used for determination of the expression ratio (table 3,4).

TABLE. II: CALCULATION OF THE ΔCt VALUES

Sr.No.	Dilution series	QTY as determined by applying the equation 1 (ng)	-----Ct Values-----		ΔCt
			<i>cryIAC</i>	<i>ghtub1</i>	
1	0.01ng	0.014	50.50	47.48	3.02
2	0.1 ng	0.07	48.19	44.91	3.28
3	1.0 ng	0.275	46.18	40.87	5.31
4	10 ng	4.36	42.18	37.85	4.33
5	100 ng	234.42	36.41	34.36	2.05

TABLE. III: CT VALUES OF THE TWO GENES FOR EACH TRANSGENIC COTTON VARIETY AND CRM ACCORDING TO THEIR CORRESPONDING STANDARD CURVES BY RT PCR

Sample variety	Bt666	Bt886	CM555	SL1	CRM
<i>cryIAC</i>	30.58	35.21	41.79	35.48	50.93
<i>ghtub1</i>	32.72	35.44	42.57	35.94	57.02

TABLE IV. THE COMPARATIVE EXPRESSION RATIO OF THE *CRYIAC* IN COTTON VARIETIES

Sr No	DI	SV	CIC	CIS	$\Delta Ct_{\text{cryIAC}}$	GIC	GIS	$\Delta Ct_{\text{ghtub1}}$	$(E_{\text{tgt}})^{\Delta Ct_{\text{tgt}}}$	$(E_{\text{ref}})^{\Delta Ct_{\text{ref}}}$	Ratio
1	100	Bt666	30.23	30.58	-0.35	31.34	32.72	-1.38	1.001	1.0040	0.997
2	100	Bt886	35.11	35.21	-0.1	35.39	35.44	-0.05	1.0004	1.0001	1.000
3	100	CM555	41.75	41.79	-0.04	42.24	42.57	-0.33	1.0001	1.0009	1.000
4	100	SL1	35.15	35.48	-0.33	36.20	35.94	0.26	1.0013	0.9992	1.002
5	100	CRM	48.92	50.93	-2.01	54.84	57.02	-2.18	0.92	1.022	0.90

Abbreviations used in the table denote as follows: DI=

DNA input (ng) SV= Sample variety

CIC= *cryIAC* in the Control (Mean Ct) CIS= *cryIAC* in the Sample (Mean Ct)

GIC= *ghtub1* in the control (Mean Ct) GIS= *ghtub1* in the Sample (Mean Ct)

$$(E_{\text{tgt}})_{\text{sample}}^{\Delta Ct_{\text{tgt}}} = (E_{\text{tgt}})_{\text{tgt}}^{\Delta Ct_{\text{tgt}} (\text{control-sample})} \quad (E_{\text{ref}})_{\text{ref}}^{\Delta Ct_{\text{ref}}} = (E_{\text{ref}})_{\text{ref}}^{\Delta Ct_{\text{ref}} (\text{control-sample})}$$

The transgene *cryIAC* has been introduced into a multitude of crop plants such as cotton, maize, soybean, poplar etc. for resistance against insect pests particularly *H. armigera*. A consistently optimum expression of the transgene is the main goal of transgenic crop breeding so that the subsequent progenies of the plants exhibit the similar level of introduced characteristics [8]. The expression profile of a transgene depends upon multiple factors such as DNA methylation, site of integration and the variable number of transgene copies [4] which depend upon the transgenic approach applied e.g. agrobacterium mediated method, particle bombardment. It leads to a variable production of the desired protein in transgenic plants [5]. In this study, the expression ratio of the transgene *cryIAC* in different varieties exhibits approximately an equal level as compared with the certified reference material. RT qPCR coupled with standard curve method yields a robust assay to sort out the transgenic cotton from the conventional non GM varieties/unauthorized varieties along with a determination of their expression profile.

IV. CONCLUSION

The expression level of *cryIAC* is found at par with *ghtub1* indicating the presence of a single copy of *cryIAC*. However whether the toxin expression is sufficient enough to ward off the damaging insects can only be determined through further proteomic quantification and determination of the LD₅₀ assays.

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