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Research Article

Quantification of *Cry1Ac* Protein in Bt Eggplant Fruits

M.M. Khatun, M.K. Hasan[1*], M.K. Jamil and D. Khanam

Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur 1701, Bangladesh.

Abstract

The cultivation of genetically engineered crops has been expanded rapidly in worldwide in a very short span of time. Bt eggplant is a transgenic eggplant created by inserting a crystal protein gene *Cry1Ac* from the soil bacterium *Bacillus thuringiensis*. Four Bt eggplant varieties - BARI Bt Eggplant-1, BARI Bt Eggplant-2, BARI Bt Eggplant-3 and BARI Bt Eggplant-4 are started to cultivate in Bangladesh in 2014 as first genetically engineered crop in the country. In the present study, ELISA technique was adopted to quantify the *Cry1Ac* proteins in the fruits of newly released four Bt eggplant varieties. The expression of *Cry1Ac* was found among the fruits of the varieties varied from 29.53 to 33.99 µg g^{-1}.

Key words: *Cry1Ac* Protein; Bt: eggplant

Introduction

From the initial planting of 1.7 million hectares in 1996 when the first biotech crop was commercialized, the 185.1 million hectares planted in 2016 indicates 110-fold increase, thus, biotech crops are considered as the fastest adopted crop technology in the history of modern agriculture (ISAAA, 2016). The increasing adoption rate admits its good impact. Eggplant is one of the popular vegetables throughout the Asia. The crop is damaged severely by the notorious insect eggplant fruit and shoot borer (EFSB) and the damage may cause up to 90% losses in yield (Parimi & Zehr, 2009). For controlling this pest, farmers frequently apply large quantities of insecticides, but the success is very poor. *Bacillus thuringiensis* (Bt) *is* a gram positive, rod-shaped soil borne bacterium. As a commercial insecticide of *B. thuringiensis* subspecies *kurstaki* (which produces *Cry1Ac* among other *Cry* proteins) firs used in in France in 1938 has been registered with US EPA since 1961 (Schnepf *et al.,* 1998). Commercial release of insect resistant transgenic crops transformed with genes from the bacterium *Bacillus thuringiensis,* a successful developments in the area of genetic engineering during 1990s (Ferry *et al.*, 2006). Bt Eggplant is a genetically engineered eggplant developed by inserting a crystal protein gene, *Cry1Ac* from the bacterium into the genome of eggplant cultivars. Eggplant fruit and shoot borer larvae ingest the *Cry1Ac* when they feed on Bt eggplant. The insect gut is alkaline (pH >9.5) where the

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[1]*Corresponding author
M.K. Hasan,
Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur 1701, Bangladesh.
Email: kamrulnk@yahoo.com

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protein is activated and disrupt the gut and finally the borer becomes to die. The government of Bangladesh approved the cultivation of Bt Eggplant at farmers' field in limited scale on October 30, 2013. Bangladesh becomes the 29th country in the world cultivating GM crop commercially since 2014. From a study it was found that Bt eggplant can increased yield 30 % and reduced 71-90% number and cost of insecticide (Choudhary *et al*., 2014). As a new transgenic crop, it is need to find out the concentration of Bt proteins in eggplant for its efficacy and risk assessment. A rapid, selective, and sensitive method for monitoring protein levels in plant and related products is of significance for product quality control, environmental risk assessment, and other relevant studies (Stave, 2002 and Grothaus *et al*., 2006). Enzyme Linked Immunosorbent Assay (ELISA) is one of the methods for identification of proteins produced by the introduced trait gene through the detection of its specific antibody. ELISA is the most widely used method for detection of specific proteins as they are much more sensitive than diffusion and agglutination methods, use less antibody and can be employed for simultaneous handling of a large number of samples in routine testing (Chalam and Khetarpal, 2012). In the present study, ELISA method was validated for the quantitation of the *Cry1Ac* protein in the fruit tissues of the newly released transgenic eggplant plants.

Materials and Methods

The experiment was conducted at the Molecular Genetics and Genetic Engineering Laboratory of Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh. Mature fruits of four Bt Eggplant varieties - BARI Bt Eggplant-1, BARI Bt Eggplant-2, BARI Bt Eggplant-3 and BARI Bt Eggplant-4 were used. The *Cry 1Ac* protein in brinjal fruits extracts was determined through Enzyme Linked Immunosorbent Assay (ELISA) method. About one kg of edible fruits for each variety was collected from four different Bt eggplant plants. Plant tissue samples were frozen in –80ºC freezer and subsequently lyophilized in a Christ Alpha 1-4 Lyo Display Freeze Dryer. The dried samples were ground into a fine powder after complete lyophilization using micro grinder and stored in containers (sealed) at –20ºC freezer. *Cry1Ac* content in fruit samples were quantified by commercially available DesiGen Quan-T ELISA kit. Five mg of lyophilized sample was kept in a 1.5 ml microcentrifuge tube. By adding 500 µl of ice-cold 1X sample extraction buffer (freshly prepared) in tube, the sample was macerated and homogenized. The lyophilized sample was macerated by hand using pestle in microcentrifuge tube for 30 second, chilled on ice for 30 second and macerated again for 30 second then centrifuged at 8000 rpm for 15 minutes. The supernatant was trypsinized by adding 3.5 µl of 5 mg ml^{-1} trypsin per 100 µl of extract following incubation at 37ºCfor 30 min. Then 2.5 µl of 500 mM Phenyl Methyl Sulfonyl Fluoride (PMSF) per 100 µl extract was added, mixed and used in assay. To make the positive and negative control, 500 µl of 1X Buffer A was added to the positive and negative seed samples provided with the kit. After crushing well with a disposable plastic pestle, it was spinned for 30 second and 100 µl of each supernatant per well was used. The *Cry1Ac* protein standard dilution series (range 20.000-0.625 ng ml^{-1}) was prepared in 1X diluent buffer and loaded in triplicate on each plate. Quality control (QC) positive and negative standards were also run in triplicate on each plate for validation purposes. The antibodies against the *Cry1Ac*, that is, goat anti-*Cry1Ac (Ab2)* was added to each well. Extracts were diluted 1:8 in 1X diluent buffer. Buffer blank, standards, positive and negative controls were added to each well and incubated at 37°C for 1.5 hour in a humid environment. The plates were washed with 1X wash buffer and an alkaline phosphatase conjugate detection antibody (*Ab3*) was then added to the wells. After a 45-min incubation at 37ºC, the plates were washed in 1X wash buffer in thrice. Finally, the buffered enzyme substrate pNPP was added and the enzyme reaction was carried out in dark at room temperature for 30 min. The absorbance of contents were measured at 405 nm (using Multiskan FC version 1.00.96, ThermoFisher Scientific Company) along with the positive and negative control well. SigmaPlot software was used to generate a 4-parameter sigmoidal standard curve and the *Cry1Ac* levels in the samples were derived from this plot.

Results and Discussion

The quantification result of *Cry1Ac* in eggplant fruit tissue sample are shown in Table 1 & 2 and Fig. 1 & 2. As per conditions of the protocol of the manufacturer, the assay was accepted. The mean absorbance of buffer blank should be ≤ 0.246. Our mean absorbance of buffer blank was 0.238 (Table 1) which was correct. The mean absorbance of the highest standard should be ≥ 1.305. In that case, our mean absorbance of the highest standard was 3.501 (Table 1) which was also correct. Percent of residuals were also fitted the prescribed value. Moreover, R^2 of the standard curve should be ≥ 0.98. In our case, we got the accepted R^2 value 0.99 (Fig. 1).

The mean absorbencies of BARI Bt Eggplant-1, BARI Bt Eggplant-2, BARI Bt Eggplant-3 and BARI Bt Eggplant-4 for *Cry1Ac* clearly shows the presence of Bt proteins in the samples. Moreover, the average absorbencies of the samples at 405 nm were higher than positive control (Table 2).

Table 1: Predicted concentration and residual against standard concentration

Standard concentration (ng/ml)	Mean absorbance at 405 nm	Standard Dev.	% C.V.	Predicted concentration (ng/ml)	% Residual	Buffer Blank
20.000	3.501	0.002	0.047	20.005	0.026	0.238
10.000	2.027	0.131	6.462	9.977	-0.229	
5.000	1.105	0.111	10.078	5.039	0.780	
2.500	0.557	0.051	9.167	2.484	-0.622	
1.250	0.265	0.017	6.511	1.223	-2.126	
0.625	0.125	0.005	3.838	0.646	3.350	

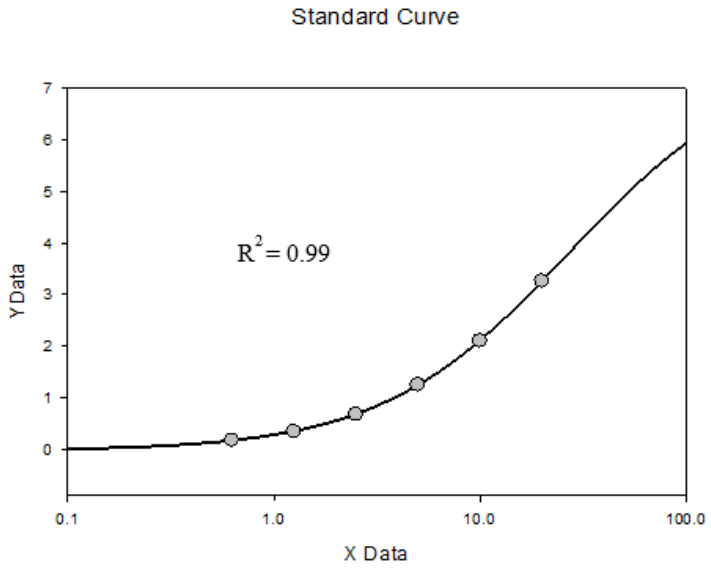

Fig 1: Standard curve of the standard concentrations

Table 2: Average absorbance value of different BARI Bt Eggplant varieties

Bt Eggplant varieties	Absorbance at 405 nm (average)	Standard Deviation	Standard Error
Positive control	3.075		
BARI Bt Eggplant-1	3.300	0.101	0.059
BARI Bt Eggplant-2	3.654	0.084	0.049
BARI Bt Eggplant-3	3.477	0.271	0.156
BARI Bt Eggplant-4	3.511	0.084	0.049

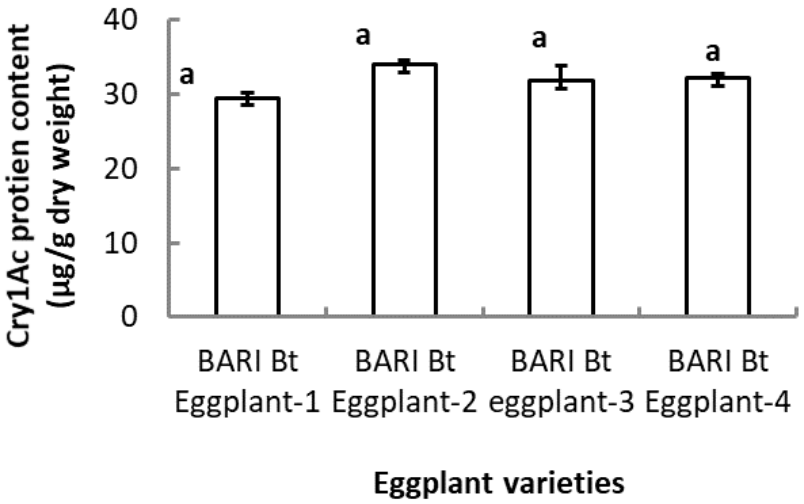

Fig. 2: *Cry1Ac* protien in the fruits of four Bt eggplant varieties

The highest amount of Bt protein (33.99 µg g^{-1} dry weight) was found in BARI Bt Eggplant-2 followed by BARI Bt Eggplant- 4 (32.14 µg g^{-1} dry weight) and BARI Bt Eggplant-3 (31.78 µg g^{-1} dry weight) (Fig. 2). The lower amount of Bt protein was in BARI Bt Eggplant-1 (29.53 µg g^{-1} dry weight). The quantity was very close to each other and no significant variation among the varieties. Although genetical variation of Bt protein was reported some authosrs. Adamczyk and Sumerford (2001) noticed that genetic background has a major effect on *Cry1Ac* expression in Bollgard cotton cultivars. Adamczyk and Meredith (2004) also reported that the differences in *Cry1Ac* protein expression between cotton cultivars were largely due to the genetic background of parents. Furthermore, Kranthi *et al.* (2005) observed variability amongst the cotton hybrids in case of *Cry1Ac* expression.

Although the critical value was not determined to control BFSB, the varieties were found free from the attack of eggplant fruit and shoot borer whereas the counterparts were affected by the insects at the practical field. This indicated that the prevailing levels of Bt protein is effective to control the insect. On an average of different locations in India, *Cry1Ac* protein varied 16.78 to 40.34 µg g^{-1} and found effective to control fruit and shoot borer (Mahyco, 2007). In case of cotton, (in India), Kranthi *et al.* (2005) had calculated a critical level of Cry1Ab/c (1.9 µg g^{-1}) for effective control of *Helicoverpa armigera*, below which the target pest had higher chances of survival. Ullah *et al*. (2014) found the critical value of *Cry1Ab/c* at any given time is found to be 770 ± 25 ng g^{-1} for the control of *H. armigera* population in Pakistan.

Conclusion and recommendation

ELISA method was found effective to quantify the *Cry1Ac* protein in transgenic Bt eggplant varieties. This information may be used for further research. But it is time consuming, laborious, costly and also requires technical know-how.

Four Bt eggplant varieties released in 2014 and five are in pipeline to release. As a new transgenic crop in Bangladesh, post release monitoring as well as extensive research should be carried out on Bt eggplant considering influence of external environmental factors on the expression of Bt protein, optimization of minimum standard value of Bt protein, quantification of Bt protein is not only the fruit tissue but also the other plant parts, study of Bt protein at molecular levels etc.

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