CHAPTER 7: \textit{GUS reporter system as an alternative to detect Cry2Ab gene in BT cotton}

Introduction

Jefferson \textit{et al} (1986; 1987) exerted the existing possibilities for the localization of \(\beta\)-glucuronidase (GUS) in transgenic plants bearing the \textit{gusA} reporter gene from \textit{Escherichia coli}. Infact, in plant tissues GUS is present very rarely, even though its activity can be distinguished easily (Plegt and Bino, 1989; Hu \textit{et al}, 1990). This has been used by many researchers to study various aspects of gene regulation and cell biology. However, it should be stressed that such \textit{in situ} assay of enzymes including GUS must follow appropriate steps to avoid false positive or false negative results as well as false localization (Holt, 1959; Van, 1973).

Reporter Genes:

Using recombinant DNA methods, the normal promoter of the reporter gene is removed and replaced by the gene promoter of interest. The new chimeric gene is introduced into an organism, and the expression of the gene of interest is monitored by the assay for the reporter gene product. A reporter gene allows the study of expression of a gene for which the gene product is not known or is not easy to identify. In bacteria, the \textit{lacZ} gene from \textit{E. coli}, that encodes \(\beta\)-galactosidase is used as a reporter gene. Other reporter genes often used in bacteria and animals include \textit{cat} (encoding the enzyme chloramphenical acetyl transferase), \textit{fus} (encoding the jellyfish green fluorescent protein), and \textit{lux} (encoding the enzyme firefly luciferase). Plants contain endogenous \(\beta\)-galactosidase activity, so \textit{lacZ} is not generally a useful reporter gene for plants. A widely used reporter gene in plants is the \textit{gusA}, gene that encodes the enzyme \(\beta\)-glucuronidase (GUS) (Jefferson \textit{et al}, 1987).
Transgenic Plants:

There are several ways to generate transgenic plants. For many plant species, the easiest method is Agrobacterium-mediated gene transfer. Using recombinant DNA methods, the DNA of interest with gusA reporter gene is put between the T-DNA borders. In addition to the gene of interest, the gene for a selectable marker is put between the T-DNA borders. The modified T-DNA is also put back into Agrobacterium and is transferred to the plant by the normal infection process to produce transgenic plant. This gusA reporter gene encodes the enzyme β-glucuronidase (GUS) which cleave the chromogenic (color generating) substrate X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), resulting in the production of an insoluble blue color in those plant cells displaying GUS activity (Fig. 57). This fusion gene expresses GUS activity in appropriate cell types under the appropriate environmental conditions (Karcher, 2002).

Plant cells themselves do not contain any GUS activity, so the production of a blue color with X-Gluc indicates the activity of the promoter that drives the transcription of the gusA-chimeric gene in that particular cells.

This reporter gene (gusA) is widely used to follow gene transfer and monitor the genetic transformation of plant species (Wijayanto and Mc Hughen, 1999) as the gene expression patterns can be quantified by fluorometric and spectrophotometric analysis. Additional advantages of the GUS assay include being very straightforward and requires no expensive equipment. The major disadvantage of the GUS assay is that the chemicals necessary for this assay are expensive. Also the GUS assay is
lethal for the plant tissues. With these limitations, however, the GUS assay is still one of the most effective reporter gene systems used by the scientists in plant gene expression studies (Jefferson, 1987; Basu et al, 2004).

**Fig. 57:** X-Gluc, a substrate of β-glucuronidase, is cleaved to produce glucuronic acid and chloro-bromoindigo. When oxidized, chloro-bromoindigo dimerizes to produce the insoluble blue precipitate dichloro-dibromoindigo.

Introduction of GUS in Cotton Bollgard-II:

Bollgard II cotton event 15985 was developed by Monsanto to produce the Cry2Ab protein which provides effective season-long control of lepidopteran insect pests to which additional reporter gene that is gusA was added. This cotton event was produced by re-transformation of Bollgard cotton event 531, which contains the CrylAc insect-control protein, selectable markers like NPTII (neomycin
phosphotransferase type II) and ADD (aminoglycoside adenyltransferase). Therefore, Bollgard II cotton produces two proteins (*Cry1Ac* and *Cry2Ab*) for effective control of the major lepidopteran insect pests of cotton, including the cotton bollworm, tobacco budworm, pink bollworm, and armyworm (Anonymous, 2003).

Bollgard-II cotton event 15985 was generated through plasmid vector, PV-GHBKI l (8.7Kb) containing well-characterized DNA elements for selection and replication of the plasmid in bacteria. The purified, linear DNA was inserted into the cotton genome. The linear plasmid fragment contains two plant gene expression cassettes. The first cassette contains a copy of the *Cry2Ab* gene encoding the BT insecticidal *Cry2Ab* protein and the second cassette contains the *gus* A gene encoding the β-D-glucuronidase (GUS) marker protein to facilitate selection of *Cry2Ab*-producing plants (Fig. 58). The GUS protein serves no other purpose and has no known insect control properties. The expression of *Cry2Ab* and *gus* A gene is regulated by the enhanced cauliflower mosaic virus 35S promoter (e35S) (Odell *et al*, 1985).

Therefore, confirmation of *Cry2Ab* is easily possible by detection of GUS reporter gene through modified histochemical assay (Jefferson, 1987). GusA gene present in Bollgard-II cotton was used as a marker to facilitate the selection of *Cry2Ab* producing plants. This reporter gene served no other purpose and has no known insect control properties. This technique is easy to perform, sensitive, relatively inexpensive, highly reliable, safe, requires no specialized equipment, and is highly visual (Jefferson 1987; Jefferson *et al* 1987; Wilson *et al*, 1991).
**Fig. 58:** PV-GHBK11 plasmid used to generate insect-resistant cotton plant event-15985 (Bollgard-II)

**Review of literature**

In the past few years, genetically modified cotton carrying insect- and herbicide-resistant genes have been commercialized successfully. Transgenic cottons play an important role in cotton production worldwide by conferring useful agronomic and fiber traits. Typically, the beneficial gene is expressed in transgenic plants under control of a regulatory DNA sequence known as a promoter (Song et al, 2000).

The cauliflower mosaic virus (CaMV) 35S is the most commonly used viral-based promoter to drive transgene expression in plants. The CaMV 35S promoter, its derivatives CaMV 35ST and CaMV 35ST/AMV were compared to evaluate their effects on the expression of the gus reporter gene in transgenic hairy roots of
cucumber (*Cucumis sativus*). Histochemical staining showed over-expression of the *gus* gene when driven with the CaMV 35S compared to CaMV 35ST/AMV and CaMV 35ST promoter in which, the reduced activity of the CaMV 35ST was observed (Mohammad et al, 2011).

Basu *et al* (2003) also studied the GUS expression in tissues (young leaves, mature leaves and roots) of creeping bentgrass (*Agrostis palustris*) driven by four different promoters (ubiquitin 3-potato, ubiquitin corn, ubiquitin rice and CaMV 35S) and suggested that the ubiquitin rice promoter is most active in cells. The reporter gene activity was dramatically high by all the four promoters used except ubiquitin 3-potato in mature leaves. The relative efficiency of each promoter was almost same in roots and leaves. This information describing promoter functionality in bentgrass will be important when designing gene constructs.

The choice of a good reporter gene is an integral component for the success of plant transformation studies. Studies on chimeric GUS reporter gene fusion systems have been conducted for various purposes like: (i) to understand developmental patterns of gene expression; (ii) to optimize the particle bombardment parameters; (iii) to compare different transformation methods; (iv) to identify the ideal promoter for plant species transformation and (v) to compare the best explant for plant transformation and regeneration. A wealth of literature is already available on in-depth analysis of GUS gene expression studies in many different types of plant species (Basu *et al*, 2004).
A variety of reporter gene systems are available and used at present. The most widely used systems employ chloramphenicol acetyltransferase (CAT) from *Escherichia coli* (Gorman, 1985), β-galactosidase (LacZ) from *E. coli* (An *et al*, 1982), bacterial and firefly luciferases (Lux) (Ow *et al*, 1986; Olsson *et al*, 1988), β-glucuronidase (GUS) from *E. coli* (Jefferson *et al*, 1986, 1987), and the green fluorescent protein (GFP) from *Aequorea victoria* (jellyfish) (Chalfie *et al*, 1994; Gholam and Irina, 2005).

A number of reporter genes have been used as convenient markers to visualize gene expression and protein localization in a wide spectrum of prokaryotic and eukaryotic systems. Each gene has specific, inherent characteristics that define both its limitations and the applications for which it will be useful. The *Escherichia coli gusA* reporter gene, which encodes β-glucuronidase (GUS), has been extensively used in plants (Martin *et al*, 1992). The β-glucuronidase assay is very sensitive, and it is possible to obtain both qualitative (histochemical) and quantitative (fluorometric) data. This reporter gene is routinely used for promoter analysis, to study protein targeting and is a valuable tool to follow gene transfer and monitor the genetic transformation of plant species. However, the histochemical GUS assay is destructive for tissue and therefore not suitable for direct visual selection of transformed plants (Jefferson *et al*, 1987).

The expression patterns of two tissue-specific promoters were analyzed in transgenic cotton plants. These promoters were *Gh-sp*, derived from a seed protein gene, and *Gh-rbcS*, obtained from a leaf chloroplast gene. These promoters were
attached to a β-glucuronidase (GUS) reporter gene. These tissues were incubated with X-Gluc to determine the expression patterns by each promoter. As expected, GUS expression under control of the Gh-sp promoter was detected only in maturing seeds while GUS expression controlled by the Gh-rbcS promoter was seen predominately in leaves. These results indicate that two promoters can be used to target the tissue-specific expression of important genes in transgenic cotton plants (Song et al, 2000). This similar result was reported for endogenous cotton Lea genes (Hughes and Galau, 1991).

Various factors like explant type, medium and bacterial strains were studied for transformation of gusA reporter gene in Alfalfa (Medicago sativa L) for which two different Agrobacterium tumefaciens strains like LBA4404 and AGL1 containing pBl121 plasmid were used. The plasmid contained the neomycin phosphotransferase gene, as the selectable marker under control of the nopaline synthesis promotor and the gusA as reporter gene under control of the CaMV 35S promotor. The highest percentage of transformation frequencies was obtained from AGL1 strain. Moreover, meristem explant is more suitable for transformation compared to cotyledon explant (Sobhanian et al, 2012).

Zhong et al (1993) analyzed transgenic creeping bentgrass for GUS expression after microprojectile bombardment under the control of rice actin promoter. They observed differential GUS activities in different tissue types where highest expression was observed in the stem node and lowest in the root hair zone.
Ha et al (1992) electroporated hygromycin-resistant gene with GUS reporter gene (both driven by the CaMV 35S promoter) and observed GUS activity in most of the transformed cells. Penmetsa and Ha (1994) also worked on factors and parameters of electroporation that influence transient gene (GUS) expression in protoplasts and observed highest GUS activity 24 h after electroporation.

**Materials and Methods**

Materials required (Fig. 59):

a) ELISA washer  
b) ELISA reader  
c) Centrifuge machine  
d) Pliers  
e) 96 deep well block  
f) 96 well microtiter plate  
g) Seed/ leaf sample  
h) 96 rod metal block  
i) Micropipettes (Volume 30-300 µl, 100-100 µl)

Chemicals required:

a) Na$_2$HPO$_4$ (dibasic sodium phosphate anhydrous) (AR, Ranbaxy Fine Chemicals Ltd. India)  
b) NaH$_2$PO$_4$.H$_2$O (monobasic sodium dihydrogen phosphate) (AR, Ranbaxy Fine Chemicals Ltd. India)  
c) H$_3$PO$_4$(Phosphoric acid) (AR, Ranbaxy Fine Chemicals Ltd. India)  
d) X-Gluc (Molecular Grade, Fermentas, Inc. USA)  
e) NaOH (Sodium hydroxide) (HiMedia, Mumbai. India)
Fig. 59: Materials required a. ELISA washer; b. ELISA reader; c. Centrifuge machine d. Pliers; e. 96 deep well block; f. 96 well microtiter plate; g. Leaf/seed sample; h. 96 rod metal block; i. Miropipettes
f) Triton X-100 (Sigma Aldrich, USA)
g) NaCl (Sodium chloride) (AR, Ranbaxy Fine Chemicals Ltd. India)
h) KH₂PO₄ (Potassium dihydrogen phosphate) (AR, Ranbaxy Fine Chemicals Ltd. India)
i) KCl (Potassium chloride) (AR, Ranbaxy Fine Chemicals Ltd. India)
j) Tween-20 (Sigma Aldrich, USA)

Chemical preparation:

- **1M sodium phosphate stock solution (100 ml):**
  
  Dissolve 14.2 gm of Na₂HPO₄ and 13.8 gm of NaH₂PO₄·H₂O in approximately 70 ml of sterilized distilled water. Adjust the 7.0 pH using NaOH and H₃PO₄. Finally, makeup the volume to 100 ml with sterilized distilled water.

- **1 mM X-Gluc solution (100 ml):**
  
  Weigh about 0.052 gm of X-Gluc and dissolve in 50 ml of distilled water. Add 5 ml of 1 M sodium phosphate stock solution and 0.1 ml of the detergent Triton X-100. Mix it properly and makeup the volume (100 ml). Store in the dark condition at low temperature (4°C).

- **Protein extraction buffer:**
  
  Dissolve 8.0 gm of NaCl, 1.15 gm of NaH₂PO₄·H₂O, 0.2 gm of KH₂PO₄, 0.2 gm of KCl and 0.5ml of Tween-20 in 800 ml of distilled water. Adjust the pH 7.4 with KCl. Makeup the volume to 1000 ml.

Method:

1. Seed sample was used for the detection of Cry2Ab protein. The seed samples were crushed with the help of pliers. The powder was
transferred in to the 96 deep well block to which 900 µl protein extraction buffer was added. This was mixed properly with the help of 96 rod metal block and centrifuged (1 minute; 3000 rpm) at room temperature. The supernatant was taken for further experimentation.

2. 50µl of blank, Cry2Ab positive control and Cry2Ab negative control were added to the empty 96 well microtiter plate (Fig. 60), while, all the other wells were loaded by the seed sample extracts.

Fig. 60: Micro titre plates demonstrating presence of Cry2Ab endotoxin in seed homogenates performed by two methods a. Proposed method; b. ELISA method B, Blank; P, Positive Control and N, Negative Control. Blue colour indicate the presence of Cry2Ab endotoxin whereas the colourless wells show the absence

3. 50 µl of the 1mM X- Gluc solution was added in all the wells.

4. The plate was incubated overnight in dark condition at room temperature.

5. To avoid evaporation, the plates were covered with the parafilm.

6. For leaf samples, leaf pieces were isolated with punching machine and incubated overnight in 1mM X-Gluc solution. Excess chlorophyll was removed by incubation it later in 70% alcohol (1-3hours).

7. The X-Gluc solution should be added to the plate in the dark and stored in the same condition to avoid false result as it is highly photosensitive.
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8. Use the supernatant after the cell debris have fully settled.

Results and Discussion

The samples that contain GUS reporter gene produces blue colour, which can be interpreted visually. 84 transgenic and 6 non-transgenic cotton forms were detected out of 90 samples in the assay. This result was also confirmed by ELISA method (Fig. 60). The presence of insoluble blue colour in the sample indicates the presence of GUS activity. Such samples can be declared as Cry2Ab positive because Bollgard II cotton event 15985 has Cry2Ab protein in association with GUS reporter gene.

Different homogenates of cotton tissues like seed, leaf, shoot and root were incubated with 1mM X-Gluc solution. The expression was determined in terms of absorbance (630nm) on ELISA reader. Among various plant parts tested for GUS activity, maximum absorbance noted was 1.480, 1.229, 1.020 and 0.828 respectively for seed, root, shoot and leaf (Fig. 61).

![GUS expression by different part of BT cotton](image)

**Fig. 61:** GUS expression by different part of BT cotton
The results indicate that, GUS expression was more prominent in seed than compared to other parts of the cotton plant. When preparing homogenates from plant organs, the number of cells that contribute to the extract including the protein contents of each cell and cell type will vary and this will reflect the expression of GUS activity (Labarca and Paigen, 1980; Jeferson 1987).

The absorbance was monitored for extended period to determine the stability of GUS expression in all the four samples. The rise was more sharper till 6 hours, after which the increase was gradual. The results demonstrated the maximum expression upto 48 hours and followed by linear trend (Fig. 62). The GUS activity remains linear (even in crude extracts) for a very long time, sometimes days (Jefferson et al, 1987). This assay is the most sensitive and versatile. This method is intrinsically 100 to 1000 times more sensitive than colorimetric methods (Reiss et al 1984).

![Graph showing GUS expression by different parts of BT cotton at different time intervals](image)

**Fig. 62:** GUS expression by different part of BT cotton at different time intervals
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The cost for 3 different methods namely molecular, ELISA and the proposed method were evaluated (Table-24). On comparison, it was noted that the maximum chemical cost was found to be ₹ 28,032/- for testing of 90 samples in molecular method due to costly consumables. The chemical cost for ELISA method was ₹ 900 while, in the proposed method it was the lowest (₹ 260) for 90 seed samples testing.

Table-24: Comparison of different methods for presence of transgene Cry2Ab in BT cotton seeds

<table>
<thead>
<tr>
<th>Components</th>
<th>Method</th>
<th>Chemicals 90 seeds testing</th>
<th>Instrument required</th>
<th>Time required</th>
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<td></td>
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<td>DNA Extraction</td>
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<td>PCR analysis</td>
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<td>Agarose gel electrophoresis</td>
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<td>Particulars</td>
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<td>Cost (₹)</td>
<td>Cost (₹)</td>
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<tr>
<td></td>
<td>Molecular</td>
<td>7,680</td>
<td>Testing kit including all consumables and 96 well antibody coated ELISA plate</td>
<td>900 X-Gluc and other chemicals</td>
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<tr>
<td></td>
<td>ELISA</td>
<td>19,200</td>
<td>96 well ELISA plate</td>
<td>40</td>
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<td>Histochemical</td>
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<td>Total</td>
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<td>28,032</td>
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<td>260</td>
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<td>Micropipettes</td>
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<td>12,000</td>
<td>38,000</td>
<td>38,000</td>
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<td></td>
<td></td>
<td>Micropipette</td>
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<td>ELISA reader</td>
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<td></td>
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<td>Temperature controlled micro centrifuge</td>
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<td>ELISA reader</td>
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<td></td>
<td>Waterbath</td>
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<td></td>
<td></td>
<td>Oven</td>
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<td></td>
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<td>PCR</td>
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<td>Agarose gel electrophoresis</td>
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<td>UV-transilluminator</td>
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<td>3,88,000</td>
<td>2,38,000</td>
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More expensive instruments are essential for molecular method as compared to other methods. Moreover, the proposed method is of short duration as compared to
the other methods, so that more samples can be tested in a given time. Hence, the proposed method is cheaper, requires less time and efficient.

For the GUS reporter in plants, the indigogenic reaction is used almost exclusively. The primary reaction product, 5-bromo-4-chloro-3-indolyl is colorless and soluble. This product is then oxidized and dimerized in the visualization reaction to form an insoluble, intensely blue, final reaction product -indigo. The presence of ferri- and ferrocyanide in the incubation medium accelerates the formation of the final reaction product. It also protects the formed indigo from further oxidation, which would convert it to otherwise colorless or yellowish product. The presence of ferro- and ferricyanide can minimize the diffusion of primary reaction product and provides more precise localization (Burstone, 1962). Song et al (2000) studied the expression of two tissue-specific promoters in transgenic cotton plants in which immature seeds from non-transferred and transgenic cotton containing reporter gene construct (gusA) with a promoter (Gh-sp) were stained to determine GUS activity. The embryo of transgenic seed showed intense blue colour with more stain in the cotyledon, indicating high levels of GUS activity in these organs. Histochemical method can be a powerful tool for resolving differences in gene expression between individual cells and cell types in the tissue. Various plant parts of transgenic and non-transgenic plants were stained with 1mM X-Gluc solution at room temperature for 12 hours. Microscopic evidences suggested that the plant section from transgenic plants showed blue colour development due to presence of GUS activity which was absent in non-transgenic plants. In leaf sample, all tissues exhibited blue
colour, being more in the veins. Transverse section of shoot, root and embryo, all indicated the blue colour expression all over the cells (Fig. 63).

**Fig. 63:** Histochemical assay for GUS activity in transgenic (Jay BG-II) and non-transgenic (Ajit-155) cotton a-b. leaf; c-d. T.S. of shoot; e-f. T.S. of root; g-h. Developing embryo. Horizontal bar=0.25mm (a-f) and 2mm (g-h)
The distinct, non-uniform distribution of GUS activity in the seed, leaf, shoot and root containing gusA gene was observed in transformed plant. Different cell types within the plants are expected to have different metabolic activity with corresponding differences in the rate of transcription and translation (Jefferson, 1987), which is reflected in our result. The promoter is preferentially active in cells during the S phase of the cell cycle. If this true, than the pattern of observed GUS staining may reflect intense cell division activity in these cells. Different cell-types present in each organ contribute differently to the patterns of gene expression and each organ consists of different proportions of these cell-types (Nagata et al, 1987).

The farmers, seed producers and traders use either molecular or ELISA testing which are expensive and time consuming. The method proposed here can be a clear alternative being convenient and sensitive for such seed testing before their plantation.