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Promoterless *gus* gene shows leaky β-glucuronidase activity during transformation of tomato with *bspA* gene for drought tolerance

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Abstract

Transformation of tomato (*Lycopersicon esculentum* Mill.) was carried out using disarmed *Agrobacterium tumefaciens* strain EHA 105 harboring a binary vector pBIG-HYG-*bspA*. The plasmid contains the *bspA* (boiling stable protein of aspen) gene under the control of a CaMV35S promoter and nopaline synthase (*NOS*) terminator, hygromycin phosphotransferase gene (*hpt*) driven by nopaline synthase promoter and polyadenylation signal of *Agrobacterium* gene7 as terminator and a promoterless *gus* gene. Very strong β-glucuronidase (GUS) expression was observed in transformed tomato plants but never in non-transformed (control). Since GUS expression was observed only in transformed plants, the possibility of the presence of endogenous GUS enzymes was ruled out. Possibility of false GUS positives was also ruled out because the GUS positive explants reacted positively to polymerase chain reaction (PCR) and PCR-Southern tests carried out for the presence of *bspA* gene, which indicated the integration of T-DNA in tomato genome. The promoterless GUS expression was hypothesized either due to leaky *NOS* termination signal of *bspA* gene or due to different cryptic promoters of plant origin. It was concluded that GUS expression was observed in the putative transgenics either due to the read through mechanism by the strong CaMV35S promoter or due to several cryptic promoters driving the *gus* gene in different transgenic lines.

Additional key words: cryptic promoter, leaky GUS expression, *Lycopersicon esculentum*.

Introduction

Agrobacterium mediated genetic transformation has become routine in plant science (McCormik *et al.* 1986, Foster *et al*. 1999, Wang *et al.* 2004) and *gus* reporter gene has been extensively used during these studies (Plegt and Bino 1989, Hu *et al*. 1990, Fobert *et al.* 1994, Foster *et al*. 1999, Mitić *et al*. 2004). The *gus* gene originates from *Escherichia coli* and is encoded by *uidA* locus. The encoded protein (GUS) is a hydrolase that catalyzes the cleavage of a range of glucuronides. GUS enzyme activity can easily be localised using the histochemical substrate X-gluc following enzyme mediated hydrolysis. It is known that transformed plants show GUS leaking and false GUS expression in transformed plants is observed very routinely. It has been reported earlier that false positive GUS expression is caused by either endophytic bacteria (Tor *et al.* 1992) or endogenous GUS enzymes (Hodal *et al.* 1992).

Endophytic bacteria exist in the micropropagated plants which show GUS expression. The expression of β-glucuronidase enzyme is induced by a variety of glucuronide substrates in these organisms. Earlier, intrinsic GUS activity in higher plants was reported to be negligible but now there are various reports on endogenous GUS activity. In all these cases enzyme activity was of plant origin (Plegt and Bino 1989, Hu *et al*. 1990).

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Abbreviations: *bspA* - boiling stable protein gene of aspen; DIG - digoxigenin; EDTA - ethylenediamine tetra acetic acid; EtBr - ethidium bromide; GUS - β-glucuronidase enzyme; *hpt* - hygromycin phosphotransferase gene; IAA - indole-3-acetic acid; LB - Luria-Bertani; MS - Murashige and Skoog; PCR - polymerase chain reaction.
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In the present investigation, tomato was transformed for enhanced drought tolerance using binary vector pBIG-HYG-*bspA* having a promoterless *gus* gene. It was observed that putative transgenic plants showed strong β-glucuronidase activity in all plant parts except roots. Since untransformed plants did not show any activity, the possibility of endophytic organisms as well as endogenous enzymes was ruled out. The proposed hypothesis is that a promoterless *gus* gene is expressed either by the read through behaviour of a strong promoter like CaMV35S because of its leaky *NOS* termination signal or it is driven by cryptic promoters of plant origin which get activated upon T-DNA integration.

Materials and methods

Bacterial strain and plasmid: The disarmed *Agrobacterium tumefaciens* strain EHA 105 harboring a binary vector pBIG-HYG-*bspA* was generously provided by Prof. Arie Altman (Hebrew University of Jerusalem, Rehovot, Israel). This plasmid contains the *bspA* gene under the control of CaMV35S promoter and hygromycin phosphotransferase (*hpt*) gene driven by nopaline synthase (*NOS*) promoter (Fig. 1). The pBIG-HYG-*bspA* was constructed as follows: The plasmid pJD330 bears a *gusA* gene driven by a CaMV35S promoter fused to the tobacco mosaic virus (TMV) translational enhancer Ω down-stream to the 35S promoter and linked to a *NOS* polyadenylation signal. The *bspA* gene was inserted into pJD330 at *Sal* I and *Sac* I, replacing the *gus* gene there. The above cassette containing: CaMV35S-*bspA*-NOSter was then cut out by *Xba* I and transferred to pBIG-HYG at *Xba* I, without taking the GUS out of pBIG-HYG (Fig. 2). The *GUS* gene present in the vector is promoterless. Bacterial cultures were grown overnight in LB broth containing 60 mg dm^{-3} hygromycin and 30 mg dm^{-3} rifampicin at 28 °C and maintained on a gyratory water bath shaker (*G76D*, *New Brunswick Scientific Co*., New Brunswick, NJ, USA; 250 rpm).

Plants: The seeds of *Lycopersicon esculentum* Mill. cultivar Pusa Ruby were procured from the National Seed Centre, Pusa Complex, New Delhi. They were surface sterilized by washing them in water for 30 min and then treating with 1 % Polysan for 10 min, 0.1 % mercuric chloride for 2 min and 95 % ethanol for 30 s. Seeds were germinated on Murashige and Skoog (MS) basal medium and leaf explants were excised from 6-week-old seedlings.

Co-cultivation of *Agrobacterium tumefaciens* and tomato leaf explants: Transformation was carried out according to the protocol of McCormik *et al.* (1986) with the modification that no feeder layer was used in the whole procedure. Approximately 2 cm^2 leaf explants excised from 6-week-old *in vitro* raised seedlings were pre-conditioned for 2 d on MS basal medium supplemented with 0.5 mg dm^{-3} zeatin, 0.5 mg dm^{-3} indole-3-acetic acid (IAA), 3 % sucrose and 0.8 % agar (regeneration medium) with abaxial side touching the medium. They were then removed and gently immersed in cultures of *Agrobacterium* which were earlier grown overnight in Luria-Bertani (LB) broth and diluted in the ratio of 1:20 with MS liquid medium. The leaf explants were dipped for 3 min in dark in *Agrobacterium* culture with constant shaking at 30 rpm until the leaf edges looked slightly wet. The leaf pieces were blotted dry between layers of sterile filter papers and returned to the same plates for a two-day co-culti-vation. Subsequently, the leaf explants were washed in MS liquid medium containing 500 mg dm^{-3} cefotaxime, blotted dry and transferred to selection medium (regeneration medium containing 40 mg dm^{-3} hygro-mycin and 500 mg dm^{-3} cefotaxime). Leaf explants with developing calli were sub-cultured after every 3 weeks on selection pressure medium till the shoots were formed. For inducing roots, shoots were separated from surrounding callus and

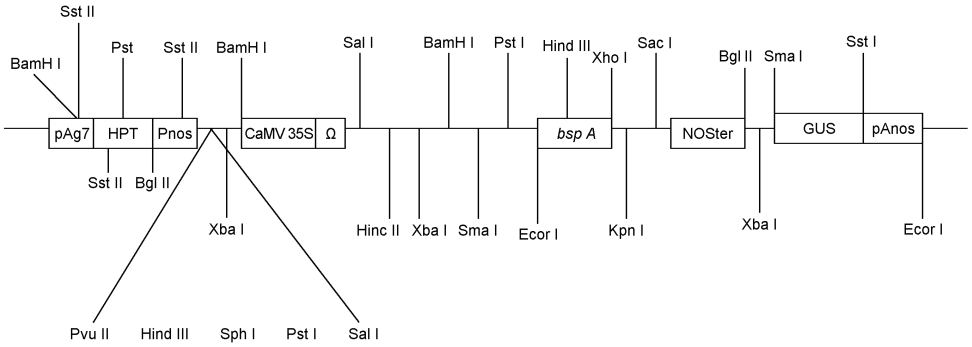

Fig. 1. Map of the binary vector plasmid pBIG-HYG carrying the *bspA* cDNA (not drawn to scale). The vector contains *bspA* (boiling stable protein) gene under the control of CaMV35S promoter (linked with TMV translational enhancer Ω downstream to the 35S promoter) and *NOS* terminator; HPT (hygromycin phosphotransferase) gene driven by *NOS* promoter and polyadenylation signal of *Agrobacterium* gene 7 as terminator. The *GUS* gene in the pBIG-HYG-*bspA* vector is not the reporter gene for a lack of promoter.

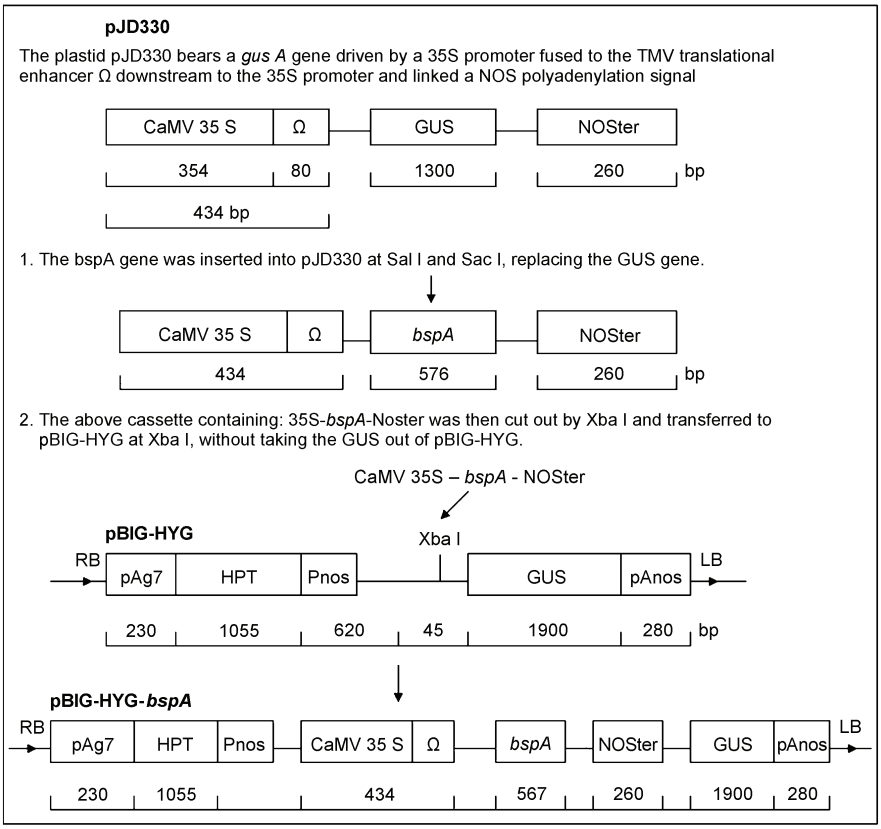

Fig. 2. Detailed construction of the binary vector pBIG-HYG-*bspA*.

transferred to rooting medium (MS + 0.5 mg dm^{-3} IAA) containing 40 mg dm^{-3} hygro-mycin. Shoots that rooted in the presence of hygromycin were transferred to soil.

GUS expression assay: GUS assay was carried out using histochemical staining by β-glucuronidase enzyme (GUS) following the protocol of Jefferson (1987). Transverse sections of stems and leaves as well as whole mounts of callus, leaf, root and stem were incubated at 37 °C for 2 d in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 100 mM EDTA, 0.1 % Triton X-100 and 0.1 mM each of potassium ferrocyanide and ferricyanide.

Polymerase chain reaction: Total genomic DNA was extracted from leaves of tomato plants using the SDS-extraction method (Dellapotra *et al.* 1983). The two primers for *bspA* gene were designed from both ends of the coding region. The forward primer sequence was 5'-AGAAAGGGAAGACATGGCAAC-3' and the reverse primer sequence was 5'-CAGCATTTATTGAACATTACA-3'. The PCR reaction was carried out in a total volume of 0.025 cm^3 comprising 100 ng of genomic DNA, 1× Taq buffer, 2 mM $MgCl_2$, 200 µM dNTPs mix, 0.5 unit Taq DNA polymerase and 90 pmol of each primer. The DNA was denatured at 94 °C for 2 min (hot start), followed by 36 amplification cycles (94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min). The amplified products were separated on 1.2 % agarose gel.

Plasmid isolation and probe preparation: Plasmids carrying *bspA* gene were isolated from *E. coli* strain XL-1 Blue using the protocol of Birnboim and Doly (1979). Isolated plasmids were then analyzed on 1 % agarose gel stained with 0.5 µg dm^{-3} EtBr. The probe used for PCR-Southern hybridization was the 496 bp PCR amplified fragment of plasmid DNA labelled with DIG-dUTP *via* random primer DNA labelling system. DIG labelling was carried out by the standard protocol of the manufacturer (*Roche Molecular Biochemicals*, Mannheim, Germany).

Southern blotting: Following the capillary method (Southern 1975), about 10 µg PCR amplified products of transgenic plants were transferred to nylon membrane. This was followed by fixation for 7 min under UV cross-linker (120 mJ s^{-1}). Hybridization of the blots to digoxigenin (DIG)-dUTP labelled probe was carried out at 37 °C following the manufacturer's instructions (*Roche Molecular Biochemicals*). Using the DIG luminescent detection kit (*Boehringer*, Mannheim, Germany) it was detected and the hybridization signals were visualized by exposing Kodak X-ray film (X-OmatTM XK-5) at 37 °C for 30 min.

Results

Lethal dose estimation: Before attempting transformation, lethal dose of hygromycin for the control leaf explants was determined to check their intrinsic resistance. Hygromycin at 40 mg dm^{-3} was lethal, as the explants started turning brown within one week of culture and no regeneration was observed even after a month.

Regeneration and selection of transgenic shoots: Among the 200 co-cultivated leaf discs, 52 began to differentiate green translucent callus at the cut ends in 8 - 9 weeks on selection medium. Three months after selection, multiple shoots differentiated *via* calli. When the shoots elongated to 2 - 3 cm, they were excised and transplanted to rooting medium containing 40 mg dm^{-3} hygromycin. Nearly 80 % of the putative transgenic shoots survived and rooted normally with well-branched roots after 5 - 8 d of transfer. The non-transformed leaf explants turned brown and did not show any morphogenic response even after six months of culture. The plants were later transferred to soil where they flowered normally.

GUS expression assay: The GUS expression in transformed tomato plants was assayed by histochemical staining method of β-glucuronidase enzyme (GUS). Callus, leaves, stem and roots of putative transgenic as well as non-transformed plants were kept individually in separate Eppendorf tubes. After staining for 2 d, the callus cells, stem and leaves (Fig. 3) of transgenic plants turned blue, confirming GUS expression after transformation but roots did not show positive assay in any of the transgenic lines tested (Fig. 3*F*). Non-transformed plants did not show GUS expression in any explant tested even after keeping for a month or two. Except roots, GUS expression was observed in every part of plants uniquely of all the transgenic lines.

PCR analysis: PCR analysis of hygromycin resistant and GUS positive plants was carried out. As expected, it detected a 496 bp band in 6 transgenic plants developed on root induction medium while no band appeared in control plant (Fig. 4). This showed the integration of *bspA* gene in tomato genome and thus confirmed the

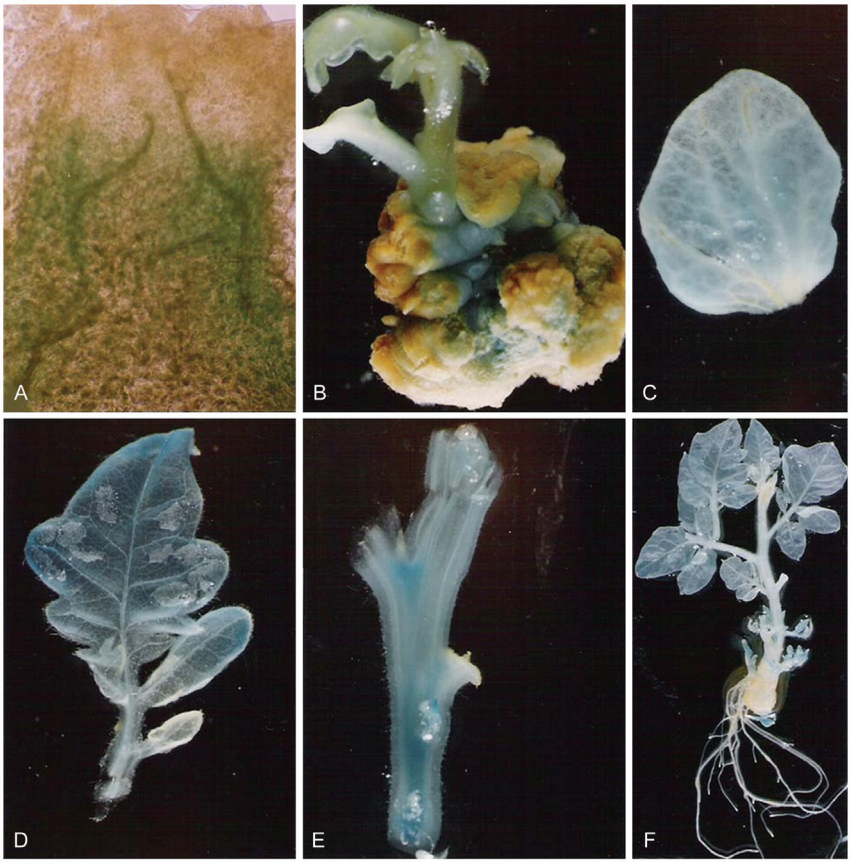

Fig. 3. Transformed tomato showing leaky GUS activity: *A* - Transient expression in callus cells; *B* - Feeble expression in a regenerated shoot from a GUS expressing sector of a chimeric callus; *C* - High expression in a single leaf of a two-month-old transgenic shoot; *D* - Stable expression in a leaf of a three-month-old transformed shoot; *E*, *F* - GUS expression in stem and whole plant (except roots), respectively.

transformation event. This also indicated that GUS positive plants were true transformants which had *bspA* gene integrated in their genome.

PCR-Southern blot analysis: Before attempting Southern blot hybridization, dot blot hybridization was carried out with DIG-dUTP labelled probe to test the integration of *bspA* gene in tomato genome. DNA extracted from 9 transgenic plants was dot blotted on positively charged nylon membrane (*Boehringer*) following the protocol described by Sambrook *et al.* (1989). Very strong signals were obtained in dots, which hybridized with transgenic plants while none was detected in the control plant. Thus, dot blot analysis further confirmed the integration of *bspA* gene in transgenic plants (Fig. 5*A*).

PCR-Southern analysis with DIG-dUTP labelled probe showed excellent consistency. Southern hybridization of PCR amplified DNA from transgenic plants with DIG-dUTP labelled probe confirmed the stable integration of *bspA* gene. PCR-Southern blot analysis was performed on 10 GUS and PCR positive hygromycin resistant plants with a non-transformed plant as control. As expected, the blot containing PCR amplified products of transgenic plants showed a signal of 496 bp band in positive control as well as transgenic plants after overnight hybridization with DIG labelled probe while none in the control plant (Fig. 5*B*).

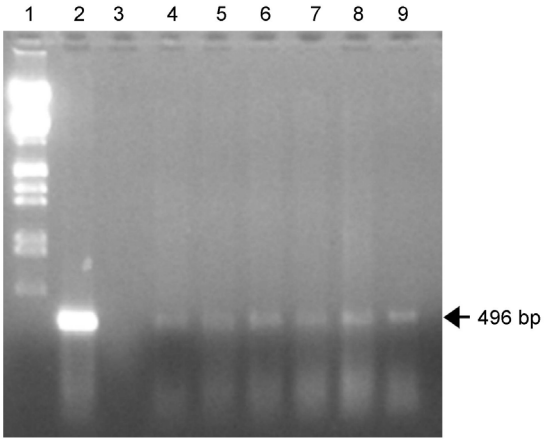

Fig. 4. DNA separation through agarose gel electrophoresis after PCR amplification of hygromycin resistant, *bspA* gene harboring transgenic tomato plants. The 496 bp DNA fragment was amplified using gene specific primers. *Lane 1*: λ DNA/EcoR1+ Hind III marker, *lane 2*: positive control (*bspA* gene in bacteria), *lane 3*: untransformed plant, *lanes 4 - 9*: 6 plants representing independent transformed lines.

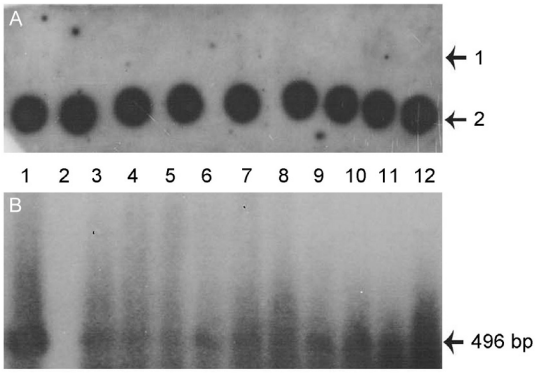

Fig. 5. Dot blot and Southern blot hybridizations in tomato. *A* - Dot blot showing intense dots of transgenic plants (second row) while no signal is seen in control plant (first row) after overnight hybridization with DIG labelled probe. *B* - Southern blot analysis of 10 transgenic lines of tomato transformed with pBIG-HYG-*bspA*. *Lane 1*: positive control (*bspA* gene in bacteria), *lane 2*: untransformed plant, *lanes 3 - 12*: 10 plants representing individual transformed lines.

The results of the three separate analyses, *i.e.* PCR, dot blot and PCR-Southern blot demonstrate that *bspA* gene has been integrated into the genome of the transgenic lines investigated presently. Since these three analyses were conducted in GUS positive plants, it also indicates that GUS positives obtained in the present investigation were not false positives but true transformants.

Discussion

In the present investigation, promoterless GUS expression was observed in transgenic plants. Transformation was carried out using disarmed *Agrobacterium tumefaciens* strain EHA 105 harbouring a binary vector pBIG-HYG-*bspA*. The *gus* gene in the vector is promoterless. In the absence of promoter, there could be GUS activity in plants transformed by this vector due to leakage.

Since GUS positive plants were also positive for PCR and PCR-Southern hybridization tests, it was concluded that GUS results are not false positive. Four possibilities were considered for explaining GUS staining: *1*) GUS staining was observed due to endogenous enzymes, *2*) GUS staining was due to endophytic micro-organisms, *3*) promoter driving the *bspA* gene is also driving *gus* gene due to weak/leaky termination signal, or *4*) *gus* gene is driven by cryptic promoters of plant origin.

Potassium ferrocyanide and potassium ferricyanide were added in the histochemical buffer to prevent diffusion of soluble-indoxyl reaction intermediate (dye) to surrounding non-transformed cells. GUS expression was not observed in any of the non-transformed explants but it was strongly expressed in hygromycin resistant transgenic plants. Experiment was repeated a number of times and no GUS expression was ever observed in non-transformed plants. Therefore, the possibility of endogenous GUS enzymes was ruled out. If the staining had been due to these enzymes, the non-transformed

explants would have also shown the GUS expression.

To eliminate the potential of false positives arising from persistent *Agrobacterium*, the cultures were later transferred to cefotaxime-free medium. No *Agrobacterium* appeared on this medium even after 30 d or longer. GUS expression was observed in these explants too. Later, these elicitated positive PCR and PCR-Southern hybridization tests while non-transformed plants neither showed GUS expression nor any positive signal for PCR or PCR-Southern hybridization. Thus, the second possibility was also ruled out that the transgenic plants showed GUS expression due to endophytic micro-organisms.

It was then considered that since only transgenic plants showed strong GUS expression and the GUS positive plants were also positive for PCR and PCR-Southern hybridization, possibly some promoter is driving *gus* gene.

Two hypotheses were proposed. First, that GUS expression is due to "readthrough" mechanism. In the plasmid vector used for transformation, CaMV35S promoter is driving the *bspA* gene and *NOS* is the terminator signal (Fig. 1). Therefore, it was proposed that this strong constitutive promoter is also driving the *gus* gene due to leaky *NOS* termination signal, by "readthrough" mechanism. "Readthrough" can be defined as transcription or translation beyond the normal termination signals in DNA or mRNA, respectively. It is a process by which a stop codon is misread as sense by the translational apparatus, allowing the synthesis of an extended polypeptide, which carries novel activities. Translational "readthrough" can be seen as "programmed translational error" occurring at specific sequences on the mRNA. In particular, it has been demonstrated that the nucleotide following the stop codon (defined as +4) is non-random, with purines over-expressed for the three stop codons. Thus +4 nucleotide in fact plays a key role in termination efficiency, leading to the proposition that termination is directed by a "four base signal". More generally, it has been demonstrated in numerous experimental systems that the nucleotide at position +4 plays an important role in suppression efficiency (Cassan and Rousset 2001). The pBIG-HYG-*bspA* construct for the present study was received from Israel but in the absence of whole sequence of pBIG-HYG-*bspA*, we were unable to compare the NOSter sequence with its sequence present in the gene construct to confirm our hypothesis. Experiments are underway to confirm it.

The second proposed hypothesis is that some cryptic promoter is driving the *gus* gene. Sequences which normally do not function as gene regulatory elements are referred to as cryptic (Irniger *et al.* 1992, Fobert *et al.* 1994, Foster *et al*. 1999). Such cryptic regulatory elements are inactive, but become active as promoters or regulatory elements when placed in new contexts. Cryptic elements predominantly have been found within or adjacent to genes, but they have also been discovered in untranscribed regions of the genome. Foster *et al*. (1999) reported the activation and isolation of a cryptic, constitutive promoter *tCUP* by T-DNA tagging in tobacco genome that controlled the expression of a promoterless *gus* gene. They found that tobacco genomic sequences adjacent to the integrated promoterless *gus* coding region supplied the *cis*-regultory elements needed for constitutive expression of *gus*. They believed that the cryptic elements, capable of activating plant gene expression, are abundant and are also preferential targets for T-DNA integration. They proposed that since higher plant genomes consist mostly non-coding or intergenic sequences which do not encode expressed genes, and as the traditional approaches for the isolation of gene regulatory elements concentrated on the isolation and characterization of expressed genes and their associated sequences, many such cryptic regulatory sequences have been overlooked. In the present investigation also, may be many such cryptic promoters are driving *gus* gene expression in different transgenic lines, which needs to be discovered. Since GUS expression was presently observed in many transgenic lines, probably different cryptic promoters are involved for driving GUS expression in different transgenic lines investigated presently. T-DNA tagging is an important tool by which a greater variety and a range of useful gene regulatory elements can be isolated. It is a tedious task to isolate and characterize such cryptic elements but it would be interesting to study as to which of the two hypotheses, we have proposed, is correct. What we are proposing is just a hypothesis because we were perplexed to observe promoterless GUS expression in a number of transgenic lines.

Thus, in the present investigation, the promoterless GUS expression in the transgenic plants can be explained either due to leaky *NOS* termination signal or due to cryptic promoters present in the plant genome. GUS expression was observed in every part of the plants except root. This can be because of difference in uptake of X-gluc in different cell types. Evidently, it would be interesting to study the molecular mechanism controlling the leaky behaviour of the *gus* gene in the construct.

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