

FUNCTIONAL ANALYSIS OF INTRONS FOR THEIR EFFECT ON TRANSGENE EXPRESSION IN PLANT

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ABSTRACT

*Most plant genes contain intervening sequences known as introns that are found in nearly all plant genes and they are transcribed into pre-mRNA, the later will be removed by splicing machinery. Proteins are encoded by exons which separated by intron segments while the non-coding exons are conserved in the mature transcript. In this study, level of spliced and non-spliced introns was measured in transgenic *Nicotiana tabacum* lines expressing *gusint-gfpint* fusion gene controlled by the CaMV 35S promoter. Transcription analysis of the transgenes was performed by (RT)-PCR. All transcripts of transgenic lines generated two different length of fragments for *gfp* and *gus* genes.*

*The results indicated that the gene expression of transcripts (without intron) in which their intron was spliced out, was higher than those of non-spliced transcript (with intron) in which the intron was maintained i.e. the both introns inside *gus* and *gfp* were partially and not completely spliced out of the transcripts. Furthermore, the relative quantifications of band intensity of *gfp* transcripts revealed that the percentage of non-spliced transcripts compared to the housekeeping gene, elongation factor 1-alpha (*ef1 α*) using different amplification cycles (25, 30, 35 and 40) was 23.3 %, 28.3 %, 21.7 % and 20 % respectively and the percentage of spliced transcripts was 76.7 %, 71.7%, 78.3 % and 80 % in that order. While, the proportion of *gus* transcripts revealed that the non-spliced transcripts were 34.2 %, 15.7%, 12.8 % and 16.3 % and the spliced was 65.8 %, 84.3 %, 87.2 %, and 80 % at 25, 30, 35 and 40 cycles respectively.*

*Conclusively, in the present study, the expression of *gus* and *gfp* genes might linked to the process of splicing and not to a particular intron sequence or the partial splicing may due to splicing signals within the*

sequences of the both introns. In conclusion, in spite of the incomplete splicing of the IV2 second intron of the potato ST-LS-1 gene and fristintron of a castor bean gene for catalase, the expression of gus and gfp genes was not affected. Where, it was ranged from 71.7 % to 80 % and from 65.8 to 87.2 for the gus and gfp, respectively in the spliced introns. Therefore, the expression of the non-spliced gus and gfp introns was much lower than those of splicedones.

Keywords: Intron, Splicing, Transgene, Gene Expression, Transgenic Plants, Gus and GFP gene.

INTRODUCTION

Introns are intervening DNA sequences largely distributed throughout gene coding regions in eukaryotes. All the DNA coding for a protein would be continuous in the genes of prokaryotes. Whereas, in eukaryotic cells the encoding DNA is typically discontinuous: stretches of encoding DNA (exons) are interspersed with long stretches of non-encoding DNA (introns). After the DNA is transcribed into pre-mRNA the introns are edited out from the nascent mRNA in a multiple-step process collectively called splicing before its translation into protein. Although introns have sometimes been loosely called "junk DNA," the fact that they are so common and have been preserved during evolution leads many researchers to believe that they serve some function. Introns were incorporating into plasmid vectors to eliminate the expression of certain proteins in bacterial cells (Ohta *et al.*, 1990; Johnson *et al.*, 2005), to increase expression of transgene in transgenic plants (Bartlett *et al.*, 2009), to make a hairpin linker in RNAi vectors (Wesley *et al.*, 2001), or to study the splicing mechanisms of plants (Rose, 2004).

Many reporter genes used for plant transformation are expressed in bacterial cells (Vancanneyt *et al.*, 1990), and it could take several months to prevent *Agrobacterium* from plant tissues (Barghchi., 1995). Consequently, it is intricate to visualize the early stages of transgene expression with confidence that the observed signal derives from plant cells rather than from *Agrobacterium*. On the other hand, the expression of transgene in bacterial cell should be prevented by the presence of an intron whereas allowing the normal expression in plants. Preferably, the used intron should have a number of stop codons to avoid translation of unprocessed mRNA in bacterial cells, and it should be effectively removed by the splicing mechanism in plant nuclei. Similar splice junctions are excited in the plant and animal introns. Generally,

plant introns are rich in AT with an average length of 250 bp (McCullough *et al.*, 1993). The intron two of the potato ST-LS1 gene (Eckes *et al.*, 1986), is a model plant intron with an 80% AT content with a sequence length of 189 bp, typical splice junctions, and manifold stop codons in all translational reading frames. Vancanneyt *et al.* (1990) produced the intron PIV2 from the second intron of ST-LS1 by changing the internal splice borders to match the consensus plant intron sequence and cloning it into the bacterial *gus* gene. Transcripts of the produced *gus-int* gene are spliced efficiently in *Arabidopsis* (Vancanneyt *et al.*, 1990), tobacco (Rempel and Nelson., 1995), and maize (Narasimhulu *et al.*, 1996).

The modified castor bean catalase intron, which is derived from the first intron of the castor bean catalase gene CAT-1, is the most widely used plant introns. It was first characterized and used by Ohta *et al.*, (1990) as an insertion into the coding sequence of the *gus* reporter gene to prevent its expression in *Agrobacterium* cells. Expression level of transgene can be influenced by characteristics and sequence of the intron used, the location at which the transgene is inserted and orientation of the intron in the transcript, the nature of the other sequences in the construct, the cell-type and species in which the construct is expressed (Bourdon *et al.*, 2001; Vain *et al.*, 1996). Introns have been found to elevate mRNA accumulation in a wide range of eukaryotes. However, not all introns affect gene expression, and direct testing is currently the only way to identify stimulatory introns. Therefore, this work was conducted to investigate the effectiveness of the commonly used introns, the intron 1 of the catalase gene (*cat*) derived from the dicotyledonous species castor bean (Tanaka *et al.*, 1990) and IV2 second intron of the potato ST-LS1 gene (Vancanneyt *et al.*, 1990)] in the ability of their splicing in relation to transgene expression level in plant with the most commonly employed reporter genes (*gus* and *gfp*) in transformation experiments in plants.

MATERIALS AND METHODS

Construction of Gus-intron-Gus-GFP-intron-GFP fusion

The *Gusint* (*gus* gene with the second intron (IV2) of the potato ST-LS1 gene) was incised from the binary vector pPBgus (Fig. 1-A left) by digestion with restriction enzymes (fermentas) Sst I at the nucleotide 430 and Sma I at the nucleotide 2502 to generate a fragment of 2073 bps. The generated fragment was cut from the agarose gel and purified with MEGAquick-spin™ Total Fragment DNA Purification Kit as described by the manufacturer instruction manual, 5µl

of the purified fragment was loaded on 1% agarose gel (Fig. 1-C). DNA Pol I, Large (Klenow) fragment was used to remove the 3' overhangs of Sst I am cutting. The binary plasmid pKBgfp (Fig. 1-A right), was linearized with Nco I at 1148 site generating 5' overhangs. Generated 5' overhangs of the pKBgfp was degraded by Mung bean Nuclease. Then, the purified Gusint fragment was fused to CATgfp in the plasmid pKBgfp using T4 DNA ligase enzyme producing a new T-DNA containing Gus-int-Gus:GFP-int-GFP fusion gene driven by a double 35S promoter of Cauliflower mosaic virus (CaMV 35S) (Odell *et al.*, 1985) and terminated by the 35S of CaMV in addition to the bar gene regulated by nopaline synthase (nos) gene (Depicker *et al.*, 1982) and nos terminator (Fig. 1C). The new generated binary vector was introduced into *Agrobacterium tumefaciens*, LBA4404 strain using the freeze-thaw method described by An *et al.*, (1988).

Plant material and generation of transgenic plants

Plant transformation was performed as described by Khidr and Nasr (2012). In brief, Leaf discs of in vitro grown *Nicotiana tabacum* cv. Xanthi plants were incubated with *Agrobacterium* for 2 days at 25° C on MS medium (Murashige and Skoog, 1962) supplemented with 1mg/L BAP and 0.1 g/L NAA. The explants were transferred onto the same medium with 500 mg/L Carbenicillin, 5 mg/l phosphinothricin (PPT). The explants were sub-cultured every three to four weeks on fresh medium until callus and shoots began to form (6-8 weeks). Developed shoots were individually separated and transferred onto hormone free media. Regenerated plantlets with roots were transferred to soil.

RNA isolation and gene expression analysis by RT-PCR

Total RNA was extracted from 100 mg leaf tissues of putative transgenic lines using RNeasy Plant Mini Kit (Qiagen, Germany). The total RNA (1 µg) was used for reverse transcribed at 42 C° using RevertAid™ First Strand cDNA Synthesis Kit. The generated cDNA was used as a template for PCR. The cDNA-PCR was performed using the suitable primer pairs listed in Table 1. The PCR reaction was performed in 25 µl containing 50 ng of template DNA, 1× DreamTaq™ buffer, 0.2 mM dNTPs, 0.5 µM of each primer and 0.5-unit DreamTaq™ DNA polymerase. The PCR condition was performed as follows: Initial denaturation at 95 °C for 30 s, followed by 30 cycles of denaturation (94

GFPInt_F 5'-ggcttcctccggatctaagg-3'
GFPInt_R 5'-gatctggatacctgtaactatca-3'
GFPInt_R1 5'-catatgatctggatacctgaaaagc-3'

RESULT AND DISCUSSION

Molecular investigation of transgene-intron expression

1. RT-PCR analysis

Transcription analysis was examined on RNA of six putative transgenic lines by (RT)-PCR with primer pair designed to amplify the connection region of the exon–intron sites within the *gus* and *gfp* sequences to verify the transcription of these reporter genes and to evaluate the splicing percentage of these introns and their effect on the expression of both the *gfp* and *gus* genes. Results on *gus* gene sequences showed two transcript lengths, the first one was un-spliced fragment sized 300 bp in Fig. (2A) and the second with a spliced transcript of 112 bp in length without the IV2 intron Fig. (2B). Furthermore, the transcription level of the un-spliced *gus*-intron differed within the transgenic lines where, it was high with the samples 2 and 4 moderate in samples 3 and 6, and faint with samples 1 and 5 (Fig 2A). Moreover, the transcription of the *gus* gene varied within the spliced transcript in transgenic lines where, it was high in samples 1, 2, 3 and 4, and moderate in the samples 5 and 6 (Fig. 2B). On the other hand, transcription of the *gfp* gene followed the same pattern with two different lengths of transcripts.

The first one was un-spliced fragment (429 bp) in size (Fig. 3A) and the second with a spliced transcript of 244 bp in length without the Cat-intron (Fig. 3B). Despite the products were detected in all samples by RT-PCR, the transcript varied in their level within the un-spliced and within the spliced *gfp* gene where, it was high with the samples 2, 3 and 4 and faint with lines 1, 5 and 6 in the un-spliced *gfp* transcript (Fig. 3A). In addition, it was high in the samples 1, 2, 3 and 4, and moderate with the sample 5 and 6 in the spliced *gfp* transcript (Fig. 3B). In addition to the effect of introns in gene expression, these variations in the expression within and between spliced and un-spliced transgene might be also contributed to the site of integration, the transgene copy numbers, mutation of transgenes and epigenetic or gene silencing (Hobbs, *et al.*, 1990; Fladung, 1999; Maqbool and Christou, 1999). Individually or collectively, the transcription of spliced transcripts of the *gus* and *gfp* genes exhibited higher level than those of non-spliced one.

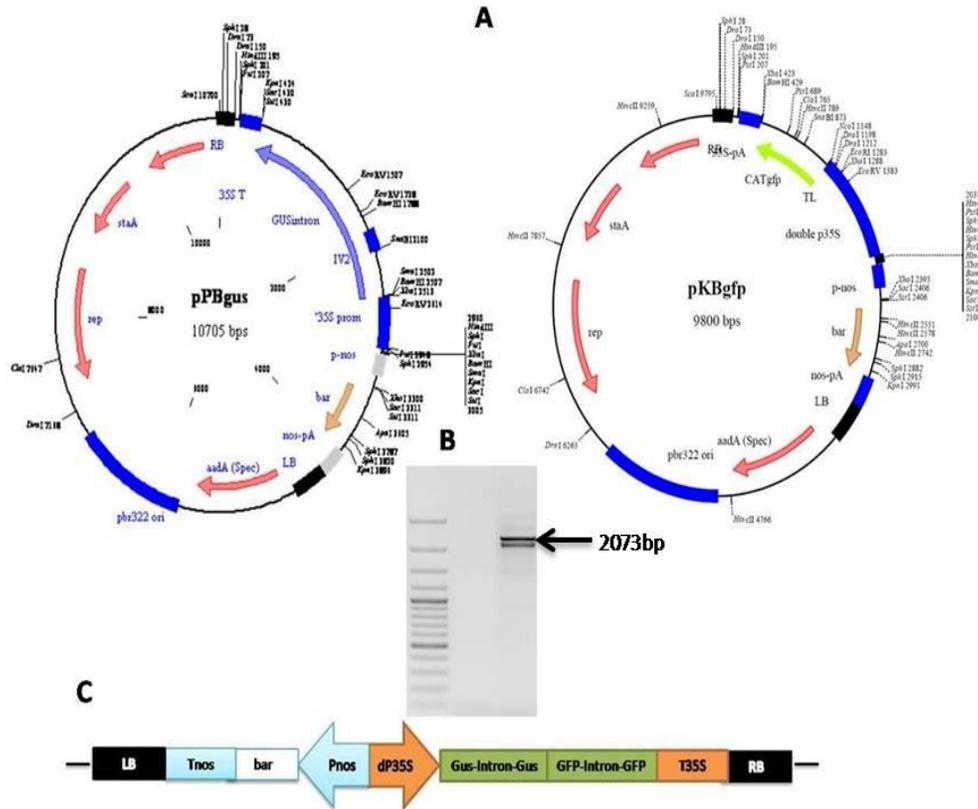


Figure 1:Construction steps of *gus-gfp* fusion genes containing intron. **A**, Binary plasmids used for the construction *gus-gfp* fusion; **B**, Digested and purified fragment (2073bps) of Gus gene containing IV2 intron with restriction enzymes SstI at position 430 bp and SmaI at position 2502 bp gene along with Gene Ruler™ 100 bp DNA Ladder Plus starting from 100 bp to 3000 bp; **C**, Schematic representation of T-DNA construction. LB and RB, T-DNA left and right border sequences; Pnos and T-nos, promoter and terminator sequences of the nopaline synthase gene; bar, *bar* selectable marker gene; P-35S and T-35S, promoter and terminator sequences of the 35S Cauliflower mosaic virus gene; Gus-Intron-Gus, reporter gene has IV2 second intron of the potato *ST-LSI* gene (Vancanneyet *et al.*, 1990; GFP-Intron- GFP, reporter gene of green florescent protein has Cat-int, first intron of a castor bean gene for catalase.

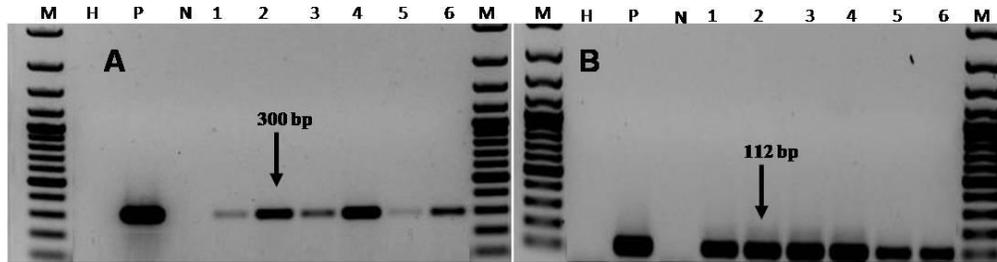


Figure 2: RT-PCR for detection of incomplete splicing of IV2-int, the second intron of the potato *ST-LSI* gene C, unspliced intron inside *gus* with a fragment size of 300bp. **A**, unspliced transcript of the *gus* gene with a fragment size of 300 bp. **B**, spliced *gus* gene with a fragment size of 112 bp. **M**, 100 bp Gene Ruler100 bp DNA Ladder Plus started with 100 bp and ended with 3000 bp; **H**, H₂O as negative control; **P**, plasmid DNA as positive control; **N**, non-transgenic plant; 1-6, transgenic lines.

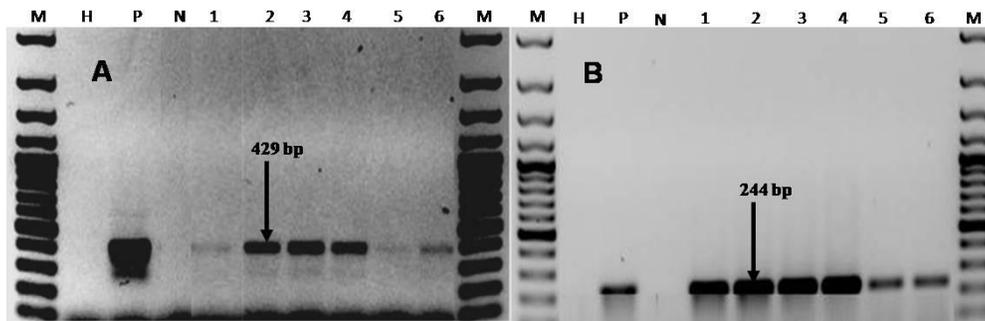


Figure 3: RT-PCR for detection of incomplete splicing of Cat-int, the first intron of a castor bean gene for catalase within the *gfp* transgene in transgenic *Nicotiana tabacum*. **A**, unspliced transcript of the *gfp* gene with a fragment size of 429 bp. **B**, spliced *gfp* gene with a fragment size of 244bp. **M**, 100 bp Gene Ruler100 bp DNA Ladder Plus started with 100 bp and ended with 3000 bp; **H**, H₂O as negative control; **P**, plasmid DNA as positive control; **N**, non-transgenic plant; 1-6, transgenic lines.

2. Semi-quantification RT-PCR of spliced and non-spliced transgenes

The transcript level of spliced and un-spliced transgenes (*gus* and *gfp*) was measured in comparison to the internal control of elongation factor 1- α (*EFl- α*) using semi-quantification RT-PCR at different amplification cycles, 25, 30, 35 and 40, and the band intensity was quantified using the gel Image Lab 3. The results of relative quantification for band intensity of the *gus* transcripts

Table 2. Relative quantification of transcripts of spliced intron and non-spliced intron within *gus* gene on cDNA of six transgenic lines relative to the housekeeping gene (*Efa1*) as a reference gene by different amplification cycles (25, 30, 35, and 40).

Cycles	Relative Quantity for <i>Gus</i> -intron plus						Mean	Relative Quantity for <i>Gus</i> -intron minus						Mean	Un-splicing %	Splicing %
	1	2	3	4	5	6		1	2	3	4	5	6			
25	0.24	0.11	0.59	0.21	0.44	0.46	0.342	0.74	0.91	0.39	0.81	0.54	0.56	0.658	34.2	65.8
30	0.41	0.19	0.11	0.19	0.03	0.01	0.157	0.61	0.79	0.91	0.79	0.99	0.98	0.843	15.7	84.3
35	0.29	0.21	0.09	0.11	0.01	0.06	0.128	0.69	0.81	0.89	0.91	0.97	0.95	0.872	12.8	87.2
40	0.36	0.19	0.26	0.14	0.02	0.01	0.163	0.66	0.79	0.76	0.84	0.99	0.98	0.837	16.3	83.7

Table 3. Relative quantification of transcripts of spliced intron and non-spliced intron within GFP gene on cDNA of six transgenic lines relative to the housekeeping gene (*EF1 α*) as a reference gene by different amplification cycles (25, 30, 35, and 40).

Cycles	Relative Quantity for <i>GFP</i> -intron plus						Mean	Relative Quantity for <i>GFP</i> -intron minus						Mean	Un-splicing %	Splicing %
	1	2	3	4	5	6		1	2	3	4	5	6			
25	0.34	0.06	0.29	0.21	0.09	0.41	0.233	0.64	0.96	0.69	0.81	0.89	0.61	0.767	23.3	76.7
30	0.41	0.34	0.11	0.19	0.11	0.54	0.283	0.61	0.64	0.91	0.79	0.91	0.44	0.717	28.3	71.7
35	0.44	0.11	0.01	0.11	0.02	0.61	0.217	0.54	0.91	0.97	0.91	0.96	0.41	0.783	21.7	78.3
40	0.36	0.24	0.21	0.14	0.11	0.14	0.20	0.66	0.74	0.81	0.84	0.91	0.84	0.80	20	80

revealed that the non-spliced transcripts were 34.2 %, 15.7 %, 12.8 % and 16.3 % whereas, the spliced transcripts were 65.8 %, 84.3 %, 87.2 %, and 80 % at 25, 30, 35 and 40 cycles respectively. While, the ratio of non-spliced *gfp* transcripts was 23.3 %, 28.3 %, 21.7 % and 20 % respectively and the percentage of spliced transcripts was 76.7 %, 71.7%, 78.3 % and 80 % at 25, 30, 35 and 40 cycles, respectively. The results pointed out that the gene expression of the transcripts (without intron) in which their intron was spliced out, was higher than those of non-spliced transcripts (with intron) *i.e.* the both introns inside *gus* and *gfp* were partially and not completely spliced out of the transcripts.

It has been reported that variation in expression levels can depend on many factors: (1) The characteristics and sequence of the intron used: In plants

enhancement of gene expression is observed only in the more GC-rich (monocot) genomes (Vain *et al.*, 1996). (2) The location and orientation in the transcript: Callist *et al.* (1987). Mascarenhas *et al.* (1990) and Clancy *et al.* (1994) reported that introns must be located within the transcribed sequences and in their normal orientation to stimulate expression. Bourdon *et al.* (2001) found that the position and sequence of an intron have remarked effects on expression levels. (3) The nature of other sequences in the construct (Bourdon *et al.*, 2001). (4) The cell-type and species in which the construct is expressed (Clancy and Hannah, 2002). However, the mechanism by which introns can enhance transgene expression remains enigma, and it has been suggested that more than one mechanism of intron-mediated enhancement may exist (Christie *et al.*, 2011). Insertion of the introns inside the open coding region of the reporter gene were exposed to improve gene expression (Tanaka *et al.*, 1990). However, increasing of gene expression by introns is not a common phenomenon since some naturally occurring genes do not include introns and are expressed powerfully.

In the present study, the expression of *gus* and *gfp* genes might linked to the process of splicing and not to a particular intron sequence or the partial splicing may due to splicing signals within the sequences of the both introns. In conclusion, in spite of the incomplete splicing of the IV2 second intron of the potato *ST-LS1* gene and first intron of a castor bean gene for catalase, the expression of *gus* and *gfp* genes was not affected. Where, it was ranged from 71.7 % to 80 % and from 65.8 to 87.2 for the *gus* and *gfp*, respectively in the spliced introns. Therefore, the expression of the non-spliced *gus* and *gfp* introns was much lower than those of spliced ones.

Conclusively, in the present study, the expression of *gus* and *gfp* genes might linked to the process of splicing and not to a particular intron sequence or the partial splicing may due to splicing signals within the sequences of the both introns. In conclusion, in spite of the incomplete splicing of the IV2 second intron of the potato *ST-LS-1* gene and first intron of a castor bean gene for catalase, the expression of *gus* and *gfp* genes was not affected. Where, it was ranged from 71.7 % to 80 % and from 65.8 to 87.2 for the *gus* and *gfp*, respectively in the spliced introns. Therefore, the expression of the non-spliced *gus* and *gfp* introns was much lower than those of spliced ones.

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التحليل الوظيفي للانترونات في تأثيرها على تعبير الجين المحول في النبات.

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⁽²⁾ قسم البيولوجيا الجزيئية ، معهد بحوث الهندسة الوراثية والتكنولوجيا الحيوية ، جامعة مدينة السادات، مصر

تحتوي معظم الجينات النباتية على تتابعات بينية تسمى انترونات والتي يتم نسخها إلى ال RNA الرسول الأولى (الغير ناضج) قبل إزالتها في وقت لاحق عن طريق عملية تسمى ال splicing. الانترونات تفصل القطع الجينية التي تسمى اكسونات والتي تحمل المعلومات الوراثية التي تشفر الى بروتينات ، او تكون غير مشفرة وتظل محفوظة في ال RNA المنسوخ النهائي (الناضج). في هذه الدراسة ،

تم قياس مستوى النسخ للانترونات التي تم ازلتها وكذلك التي ظلت باقية في منسوخات نباتات الدخان المحولة وراثياً المشفرة لجين *gusint-gfpint* المدمج والذي ينظم نسخه الـ *CaMV 35S* كقائد. تم تنفيذ تحليل عملية النسخ بواسطة تقنية تفاعل البوليميراز المتسلسل باستخدام إنزيم النسخ العكسي (RT)-PCR. كل منسوخات السلالات المحولة وراثياً أعطت طولين مختلفين للقطع المنسوخة لجين الـ *gfp* ، و جين الـ *gus* .

اوضحت النتائج ان التعبير الجيني للمنسوخات التي تمت ازاله نيتروناتها كان اعلى من تلك التي احتفظت بنيتروناتها مما يعنى ان كلا من الانترونات داخل تتابعات جين الـ *gfp* ، و جين الـ *gus* تم ازلتها جزئياً وليس كلياً من المنسوخ . علاوة على ذلك ، قياسات المقارنة لقوة وتركيز القطع الجينية للـ *gfp* أظهرت ان النسبة المئوية للمنسوخات التي لم يتم ازالة انتروناتها بالمقارنة مع جين عامل الاستطالة الداخلي 1-ألفا (*ef1α*) باستخدام دورات تضخيم مختلفة 25، 30، 35 ، 40 كانت 23.3%، 28.3%، 21.7% ، 20% على التوالي بينما كانت النسبة المئوية للمنسوخات التي تم ازالة انتروناتها 76.7%، 71.7%، 78.3% ، 80% على 25، 30، 35 ، 40 دورة تضخيم على التوالي. من ناحية اخرى، كانت النسبة المئوية لمنسوخات جين الـ *gus* التي احتفظت بانتروناتها 34.2% ، 15.7% ، 12.8% ، 16.3%، ونسبة التي تم ازالة انتروناتها 65.8%، 84.3%، 87.2% ، 80% على 25 ، 30 ، 35 ، 40 دورة على التوالي.

التوصية: في دراستنا، قد يكون التعبير الجيني لجين اتال *gus* و *gfp* مرتبط بعملية الـ *splicing* وليس بتتابع معين بالانترون *intron* أو انا لازالة الجزئية للانترون قد تعزى الى اشارات لازالة الـ *splicing* بداخل تتابعات كلا الانترونين . في الختام، على الرغم من عدم الازالة الكلية للانترون لكلا الانترونين، لم يتأثر التعبير الجينيل جينات *gus* و *gfp*. حيث تراوحت من 71.7% إلى 80% ومن 65.8 إلى 87.2 لـ *gus* و *gfp* على التوالي. لذلك، كان التعبير الجيني للـ *gus* و *gfp* التي لمتزالانترونات هم أقل بكثير من تعبيرات من تلك التي ازيلت.