

## Stability of Luciferase Gene Expression in a Long Term Period in Transgenic Eggplant, *Solanum melongena*

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### Abstract

The *luc* gene, from firefly, was ligated into the multiple cloning site of the pBI121 plasmid and was then incorporated into *Agrobacterium tumefaciens* strain LBA4404. This vector containing a CaMV 35S promoter, a NOS terminator, a *NPTII* gene and a NOS promoter was used to transform 'Hibush' eggplant (*Solanum melongena*). Genomic DNA from the transformants was analyzed by assay of both PCR and southern blotting, which confirmed the presence of an expected *luc* fragment. Four transformants were chosen for an extended LUC assay. During the course of one year, LUC activity fluctuated, but was maintained at the initial activity even after 50 weeks. Expression of the *luc* gene in transgenic calli revealed that fluctuation of the LUC activity was due to changes in the environmental temperature. Luminescence from transgenic eggplant was detected using image analysis and light emission was found to be intense throughout the whole plant.

### Abbreviations

BA - benzyladenine, CaMV - cauliflower mosaic virus, 2,4-D-2,4-dichlorophenoxyacetic acid, DTT - dithiothreitol, GUS -  $\beta$ -glucuronidase, IAA - indole acetic acid, 2iP-N<sup>6</sup>-[isopentyl] adenine, MS - Murashige and Skoog, NAA -  $\beta$ -naphthaleneacetic acid, NOS - nopaline synthase, NPT-II - neomycin phosphotransferase - II, TDZ - thiodiazuron, LUC - luciferase

### 1. Introduction

To investigate and track the spatial and temporal activation of genes in plant cells, it is often convenient to use a good genetic marker. Historically, the luciferase gene, *luc*, isolated from the common North American firefly *Photinus pyralis* has been utilized as a powerful reporter gene for assessing gene expression. It has been expressed in *Escherichia coli* (de Wet *et al.*, 1985), *Nicotiana tabacum* (Ow *et al.*, 1986), and mammalian cells (de Wet *et al.*, 1987). These reports have shown that the *luc* gene can be used as a marker of gene expression in eucaryotic cells. However, since these studies focus on the transferring of *luc* gene, the gene expression is assessed transiently or within a short period of

time. In fact, it takes several months or years for a plant to grow.

This study is aimed at the investigation of gene expression from the transgenic eggplant during a one-year period. The *luc* gene was used to assess gene expression. We introduced the *luc* gene into eggplant through *Agrobacterium*-mediated genetic transformation according to the protocol reported by Billings *et al.* (1997). Using this protocol, the expression and the incorporation of *luc* gene and LUC activity in the transgenic eggplant was traced for over one year.

### 2. Materials and Methods

#### 2.1 Plant materials

Surface-sterilized seeds of 'Hibush' eggplant (*Solanum melongena*) were cultured on half-strength MS basal salts and vitamins containing 2% sucrose and 0.25% gellan gum (Wako Pure Chem. Ind. Tokyo, Japan). Leaves from shoots with six-eight leaves were excised and used for transformation experiments.

Callus was obtained by the culture of leaf segments (3 × 8 mm) on callus induction (CI) medium, containing MS salts and vitamins, 2% sucrose, 0.1 mg l<sup>-1</sup> 2,4-D, and 0.25% gellan gum (pH 6). These

embryogenic calli were maintained at 25 °C in the dark and proliferated by subculture on the same fresh medium.

### 2.2 Regeneration from leaf

Leaf segments (3 × 8 mm) were initially cultured on shoot regeneration (SR) medium, which contained MS salts and vitamins, 2% sucrose, and 0.25% gellan gum (pH 5.8) along with the plant growth regulators 2iP (10 μM) and TDZ (0.1 μM). After 4 weeks, shoot regenerants were then transferred to shoot elongation medium consisting of MS salts and vitamins, 1 μM zeatin, 2% sucrose, and 0.25% gellan gum.

### 2.3 Bacteria and plasmid

The pYS629 plasmid was constructed by first excising *GUS* gene from the pBI121 plasmid (Clontech, Palo Alto, CA) using *Bam*HI and *Sst*I restriction enzymes. Subsequently, the 1.7-kb *luc* gene, originating from the pGEM-luc plasmid (Promega, Madison, WI), was ligated into the pBI121 plasmid at the *Bam*HI and *Sst*I restriction sites between CaMV 35S promoter and *NOS* terminator instead of the *GUS* gene. This binary vector also contained a *NPTII* gene conferring kanamycin resistance. The construct was then transferred into *Agrobacterium tumefaciens* strain LBA4404 (Clontech, Palo Alto, CA). The new bacteria strain was named ESR712 and grown in YEB medium (1 g yeast extract, 5 g beef extract, 5 g peptone, 5 g sucrose, 0.5 g MgSO<sub>4</sub>, pH 7.2 in 1 liter). To select bacterial cells bearing the plasmid, 50 μg ml<sup>-1</sup> of kanamycin was added to the YEB medium. The culture was carried out at 28 °C and 250 rpm for 48 h.

### 2.4 Plant transformation

The leaf segments were inoculated for 1 min with *A. tumefaciens* strain ESR712 and cultured on the SR selection medium containing 300 μg ml<sup>-1</sup> augmentin (SmithKline Beecham, PA) and 50 μg ml<sup>-1</sup> kanamycin. To induce shoots the explants were cultured at 24 °C with a 16 h photoperiod under fluorescent lights at a photon flux of 50 μmol m<sup>-2</sup> s<sup>-1</sup>. After 6 to 8 weeks, the shoots with three leaves were excised to induce rooting.

The transformation of calli was performed according to the above method with some modification. The embryogenic calli were soaked in the *A. tumefaciens* ESR712 suspension containing 10 mg l<sup>-1</sup> acetosyringone for 1.5 min. The calli were transferred onto the CI medium supplemented with 100 mg l<sup>-1</sup> augmentin, 25 μg ml<sup>-1</sup> kanamycin. The culture was carried out at 25 °C for 2 weeks in the

dark.

### 2.5 Analysis of transformants by PCR and southern blotting

To analyze the presence of *luc* DNA, DNA from a putative transformed plant was extracted according to the method of Junghans and Metzlauff (1990). Polymerase chain reaction (PCR) was performed as described by Yoshioka *et al.* (1992) using the 5' primer 5' -GGCCTCGGAGGATTACAATA -3' (from 46-65 bp of the *luc* gene) and the 3' primer 5' -CTGGAGAGCAACTGCATAAG -3' (from 1671-1690 bp of the *luc* gene).

Five to ten micrograms DNA was restricted with *Bam*HI and *Sst*I and electrophoresed on a 0.9% agarose gel. The DNA was blotted onto a nylon membrane. Prehybridization, hybridization, detection, and randomly primed DIG-labeled probe preparation were carried out according to the Boehringer Mannheim Genius System (Indianapolis, USA). The DNA probe was 1.7-kb segment of the native *luc* gene random primed-labeled with digoxigenin-11-dUTP.

### 2.6 LUC Assay and imaging of *luc* gene expression

Primary transgenic plants and calli were subjected to the LUC assay according to LUC assay system (Promega, Madison WI). The photon from the extracted solution was counted by a luminometer (AB-2000, ATTO, Co. Ltd., Tokyo) and defines the specific LUC activity as the number of photons produced for a period of 10 seconds per fresh weight of leaves. All measurements were in triplicate and their average was taken.

To take an image of the expression of the *luc* gene, transgenic eggplant was sprayed with the LUC assay reagent (Promega, Madison WI) and wrapped in Saran film for 5 h. The luminescence from the transgenic eggplant was imaged in a dark room at room temperature using an image analysis system (Argus-50/Vim, Hamamatsu Photonics, Hamamatsu, Japan). The sensitivity range of this system is from 10<sup>2</sup>-10<sup>9</sup> quanta cm<sup>-2</sup> s<sup>-1</sup> at 550 nm and the resolution is 512 pixels x 483 pixels x 16 bits.

## 3. Results

### 3.1 Expression of *luc* gene in transgenic eggplant

One hundred shoots were obtained from 2,000 infected leaf-segments, of which 52 rooted and produced the primary transformants. The specific LUC activity from 13 transgenic eggplants was higher than 10<sup>4</sup> photons 10 s<sup>-1</sup> g fresh weight<sup>-1</sup>, which corresponds to a 0.1 nM LUC concentration and is noticeable to the naked eye. Thirty-four

transgenic eggplants had luminescence between  $10^2$ – $10^4$  photons  $10\text{ s}^{-1}\text{ g fresh weight}^{-1}$ . Only four eggplants expressed *luc* gene in the  $10$ – $10^2$  photons  $10\text{ s}^{-1}\text{ g fresh weight}^{-1}$  range. The specific activity of non-transformant eggplant was below 10 photons  $10\text{ s}^{-1}\text{ g fresh weight}^{-1}$ , which is the same as the noise level in the detection procedure. This indicates that all transgenic eggplant expressed the *luc* gene in the leaf.

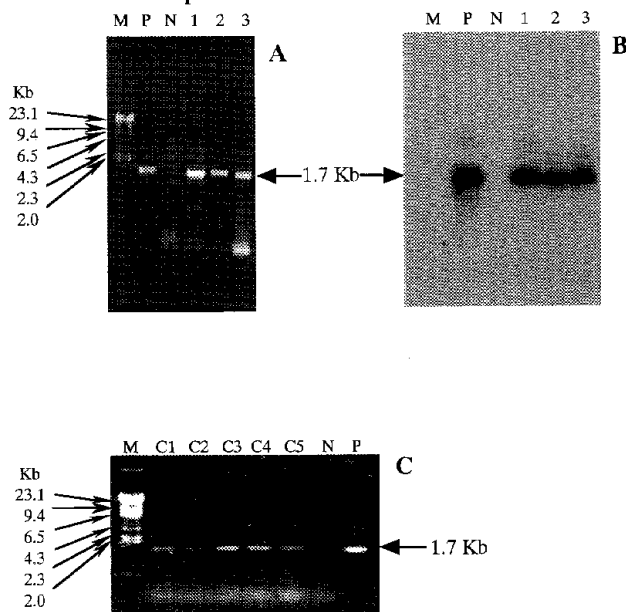
### 3.2 Analysis of transformants

The presence of *luc* gene in transgenic eggplant was analyzed using PCR with primers designed to amplify the internal sequence *luc* gene. Fig. 1 shows the analysis of the PCR amplification (Fig. 1A) of the *luc* gene using genomic DNA of four transgenic eggplants and the primary transgenic calli (Fig. 1C). Genomic DNA from these transgenic eggplants and primary transgenic calli contained a 1.7-kb band, which corresponds to the expected internal portion of the *luc* gene. The *luc* gene amplified from pGEM-*luc* is shown at the 1.7-kb band, and represents the positive *luc* control (P lane). Genomic *luc* gene was not present in the non-transformed control callus and transgenic plant (lane N). Southern blot analysis conducted on the same transgenic eggplants, which confirmed the integration of the *luc* gene into the genomes (Fig. 1B).

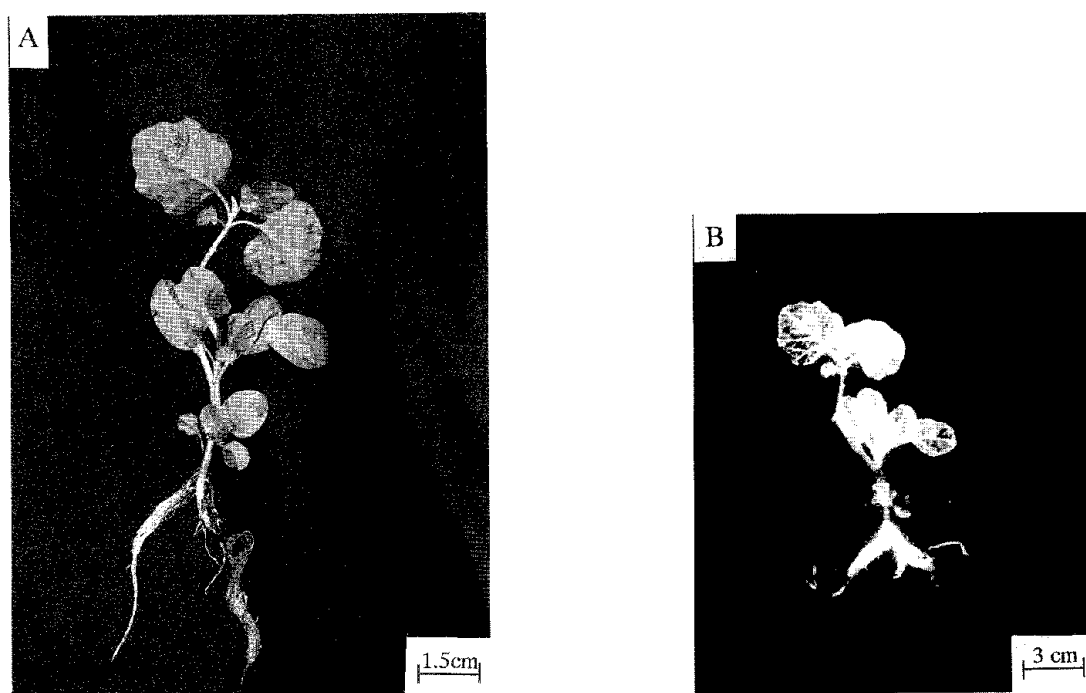
### 3.3 Luminescence of transgenic eggplant

Transgenic eggplant, 4 weeks after shoot formation (Fig. 2A) was investigated the luminescence

using an image analysis system. The pattern of light emission is expected to reflect the distribution of



**Fig. 1** Analysis of PCR (A and C) and southern blotting (B) of the *luc* gene amplified by using oligomers 20 bp at upstream position and 20 bp at downstream position. M, P and N denote  $\lambda$  *Hind*III marker, positive control from pGEM-*luc* and non-transformant, respectively. Panels of A and C denote *luc* genes originated from leaf (L1–L3) and callus (C1–C5) of transformant, respectively. The southern blots were probed with a 1.7-kb DIG-labeled *luc* gene. Washing and detection were according to the nonradioactive



**Fig. 2** Luminescence from the transgenic eggplant after 4 weeks germination. The eggplant was sprayed with the LUC detection reagent 5 h before it was imaged. Light emission of the plant was detected by an image analysis system in a dark room chamber. A and B denote photograph and image of luminescence from the eggplant after spraying the LUC detection reagent, respectively.

LUC activity in the transgenic eggplant. Light emission was intense throughout the whole plant (Fig. 2B).

### 3.4 Stability of *luc* gene expression in a long term period

Four transgenic eggplants (No. 4, 9, 25 and 29) were investigated for the stability of *luc* gene expression during 50 weeks in a greenhouse. Changes in the specific LUC activity are shown in Fig. 3. At 12 weeks after shoot formation, the specific LUC activity was at a high level, around  $10^6$  photons  $10\text{ s}^{-1}\text{ g fresh weight}^{-1}$ . However, the activity of all transgenic eggplants decreased drastically from 17 weeks and maintained at low activity level until 33 weeks, and after increased to the initial level (Fig. 3B). The activity measured for other transgenic plant fluctuated similarly, but the final levels were equal to those of the initially transformed eggplants. Temperature changes in the green house during the experimental period, are shown in Fig. 3A. For the first 18 weeks, the temperature was around  $22^\circ\text{C}$ , but increased to  $28^\circ\text{C}$  during weeks 19–30. After week 35 the temperature returned to  $23^\circ\text{C}$ . This indicates that the *luc* gene was stable and maintained in the transgenic egg-

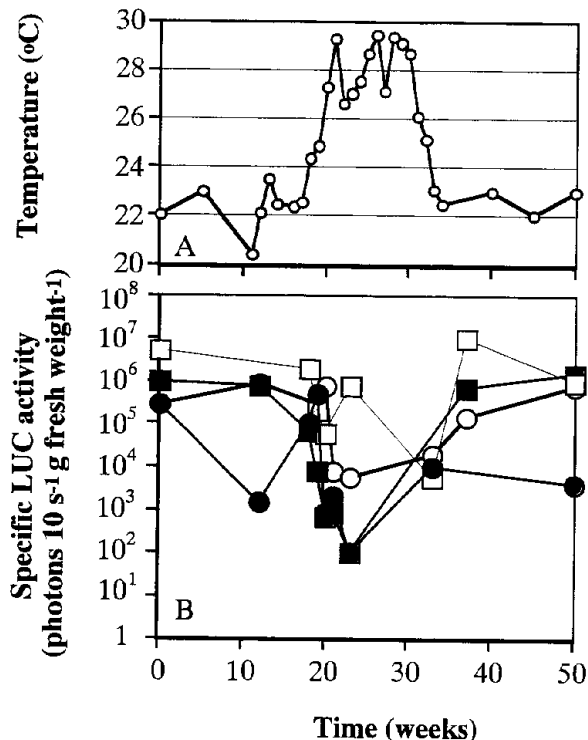


Fig. 3 Stability of LUC expression in transgenic eggplant. During 50 week measurement, temperature averaged every week in the green house (A) and LUC activity (B) are shown. LUC activity was measured for 50 weeks after infection by *A. tumefaciens* ESR712. The LUC activity of transgenic eggplants No. 4 (■), 9 (●), 25 (□) and 29 (○) were measured.

plant during a one year period, although its expression was variable.

### 3.5 Effect of temperature on LUC activity and growth of transgenic calli

Variation of the LUC activity during the long-term assay was suspected that temperature might have a significant influence. To determine the effect of temperature on LUC activity, transgenic calli were cultured on the incubator varying temperature from  $20$  to  $37^\circ\text{C}$ . The specific activity is shown in Fig. 4A. Over 3 weeks, the LUC activity fluctuation was little at  $20$ – $30^\circ\text{C}$ . However, in the case of  $33$  and  $37^\circ\text{C}$ , the LUC activity decreased drastically within 2 d. At a culture temperature of  $37^\circ\text{C}$ , there was a 97% decrease in LUC activity in less than 24 h. At a culture temperature of  $33^\circ\text{C}$ , the drop occurred over 2 d, and then the activity restored slowly.

The effect of temperature on calli growth is shown in Fig. 4B. An increase in fresh weight at  $30^\circ\text{C}$  was found to be 8.5-fold during 28 d culture. At temperatures above or below this optimum, growth was attenuated. In addition, at  $37^\circ\text{C}$  the transgenic calli were brown in color and found to be non-active.

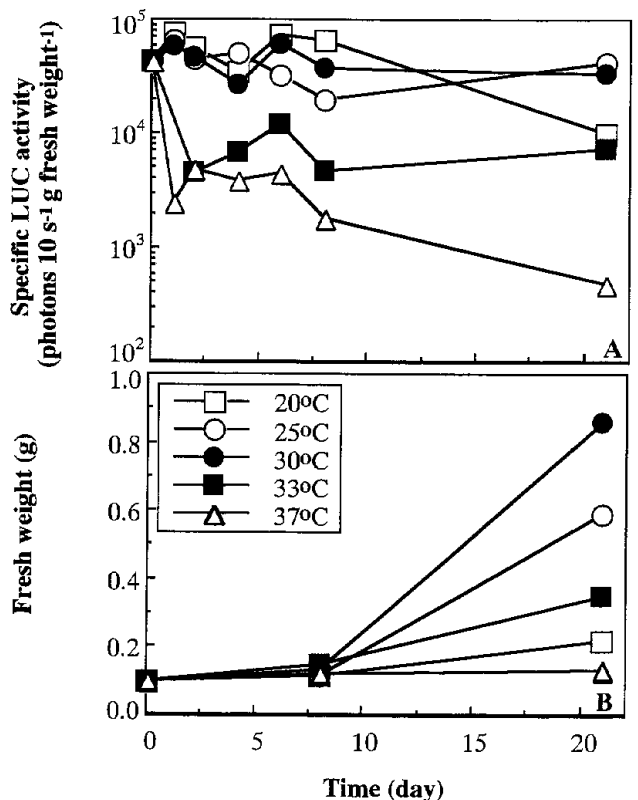


Fig. 4 Effect of culture temperature on LUC activity (A) and growth (B) of transgenic calli. Transgenic calli were incubated in various temperatures for 3 weeks and measured fresh weight and the specific LUC activity.

## 4. Discussion

This work focused on the expression of the *luc* gene in eggplant over an extended period of time. The transformation process was analyzed with respect to both enzyme and gene levels. Among 52 primary transgenic eggplants, 4 (8%) showed expression levels below 100 photons  $10^{-1}$  g fresh weight<sup>-1</sup>, even when the *luc* gene was integrated as judged by the analysis of PCR and its southern blotting. This indicates that the LUC activity in transgenic eggplant can have a spectrum of values. The variation in gene expression might depend on how many copies were integrated or into which site the *luc* gene was inserted. Such factors were not examined in this work. However, they could be very important from the point of view of production of useful gene products.

It is also important to express the inserted gene in a plant tissue at high level over the long term. This is especially true for plant cells. The expression of the *luc* gene in transgenic eggplant was found to be stable for at least 1 year, though its specific activity fluctuated. In our case, the reason behind the specific LUC activity decrease during the time period from 20 to 33 weeks might be due to environmental conditions. Specifically, the 20–33 weeks corresponded to June–September (summer) when it was difficult to control the temperature in the greenhouse to 25°C. At that time, the temperature difference between day and night was in the range of 20–25°C, which might cause plants to fluctuate in the activity of the expressed protein.

This was shown in experiments with transgenic calli. In the cases of 25 and 30°C, calli grew and LUC activity did not decrease during 3 weeks. However, at the temperatures higher than 33°C calli did not grow, which caused a decrease in the LUC activity. Furthermore, the LUC activity decreased drastically within 1 or 2 d following a temperature shift. These results reveal that the decrease in LUC activity is due to an increase in temperature as shown in transgenic calli and eggplants. Therefore,

temperature changes cause fluctuations in the gene expression in transgenic plants.

The luminescence of the transgenic eggplant showed that the *luc* gene was integrated and expressed throughout the whole plant. This is a consequence of the CaMV35S promoter that does not express the gene in a tissue-specific manner. If a promoter that expresses tissue-specifically is used, the *luc* gene could be a good marker for the detection of gene expression at a specific tissue.

In conclusion, our data demonstrate that eggplant can be transformed successfully by an *Agrobacterium*-mediated technique and the expression of *luc* gene is affected by environmental conditions.

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