# Promoter tagging in plantain using the luciferase reporter gene

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## ABSTRACT

For the isolation of promoters in plantain an *Agrobacterium*-mediated tagging approach was chosen. A promoterless luciferase reporter gene next to the left border of the T-DNA (pluc19) was introduced into embryogenic cells of the plantain 'Three Hand Planty'. Five months after transformation, individual cell colonies in 24-well plates were screened *in vitro* for baseline luciferase activity. Positive lines showing activated luciferase expression were further screened at plantlet stage. The frequency of cultures with luciferase activity comparable to or higher than the CaMV35S promoter control was 0.15%. Cell lines were also screened at 8°C four months after transformation with a new T-DNA tagging construct containing an improved luciferase reporter gene next to the right border (pETKUL2). Screening of approximately 15,887 cell colonies revealed 155 (0.98%) with luciferase activation at 26°C. Twenty-two independent cell colonies (0.14%) showed responsive luciferase expression at 8°C. The isolation of flanking plant DNA sequences was accomplished by TAIL PCR, an average of four flanking sequences per line was obtained. Sequences were analyzed for conserved regions and promoters elements. Six out of 11 flanking T-DNA sequences of three promoter tagged lines showed vector backbone integration or T-DNA rearrangements. Five putative promoter sequences were isolated and analyzed, one of which turned out to be a putative GDP-mannose pyrophosphorylase gene.

## MATERIALS AND METHODS

Embryogenic cell suspensions from the plantain cultivar 'Three Hand Planty' (THP; AAB group) were cocultivated with *Agrobacterium* strain EHA105 harbouring one of two plasmids (Fig. 1) wich carry a promoterless luciferase reporter gene next to a T-DNA border. Two tagging vectors were used: pluc19 (Mudge *et al*. 1998) carrying the wild type luciferase gene (*luc*) from the North American firefly *Photinus pyralis* next to the left border, and pETKUL2 (Remy *et al*. unpublished) with a codon-optimized *luc*$^{+}$ gene (Sherf and Wood 1994) next to the right border.

After a 2-3 month selection for putative transgenic pluc19 lines on cell culture medium (ZZ), individual cell colonies were subcultured on regeneration medium (RD1) for 1-3 months and screened in 24-well plates. For lines transformed with pETKUL2, screening for baseline luciferase activation (BLA) was done 2-3 months after transformation on ZZ medium with over 700 cell colonies per petri dish. Subsequently, the transgenic lines were subjected to cold stress. Luciferase (LUC) activation pattern of cell colonies was monitored on a temperature-controlled plate. Luciferase assays were performed by the application of 0.1 mM luciferin to the colonies. Bisected meristems and leaf discs from greenhouse plants were placed horizontally on filter paper saturated with half strength MS medium and sprayed twice with 0.1 mM luciferin. Light emission was captured in a light-tight box using a liquid nitrogen-cooled CCD camera (Versarray™ 512 B LN, Roper Scientific) coupled to a light sensitive lens (50 mm/f 1.2, Nikon). Live and luciferase images were analyzed with Metamorph software 5.0r3 (Universal Imaging).

Isolation of T-DNA flanking sequences was accomplished with Thermal Assymetric Interlaced (TAIL) PCR (Liu *et al*. 1995) with some modifications.

Sequences were analyzed with BLASTn and Fasta3 programs for homology search. PlantCARE database (http://oberon.fvms.ugent.be:8080/PlantCARE/index.html) was used to detect putative promoter elements and TATA boxes.

## RESULTS


**Figure 1.** T-DNA from plasmids pluc19 (a) and pETKUL2 (b). (a) pluc19 = *luc*-T*nos*: luciferase - nopaline synthase terminator, P*nos*-*neo*-T*nos*: nopaline synthase promoter - neomycin phosphotransferase - nopaline synthase terminator.
(b) pETKUL2 = *luc*+-T*nos*: codon optimized luciferase – nopaline synthase terminator, P35S-*neo*-35SpolyA: CaMV35S promoter – neomycin phosphotransferase – CaMV35S poly A signal.
LB: left border, RB: right border.

Luciferase screening of 2,014 putative transgenic pluc19 lines showed a BLA frequency of 0.15% on RD1 medium in 24-well plates. The pETKUL2 lines with an average of 700 colonies on ZZ medium per petri dish showed a frequency of 0.98% (Table 1). Twenty-two independent cell colonies out of 15,887 showed responsive luciferase activity at 8°C (Table 1).

**Table 1.** Baseline luciferase activity (BLA, no stress) and responsive luciferase activity (RLA) at cold stress (8°C) in cell colonies of the plantain cultivar THP 2-5 months after *Agrobacterium*-mediated transformation carrying the promoterless luciferase T-DNA constructs pluc19 and pETKUL2

| Screening Stage | Tagging construct | Total Nr. cell colonies screened | BLA frequency (%) * | RLA frequency (%) at 8°C * |
|---|---|---|---|---|
| RD1 | pluc19 | 2,014 | 0.15 | NT |
| ZZ | pETKUL2 | 15,887 | 0.98 | 0.14 |

* Percentage of independent transgenic lines showing BLA or RLA at 8°C during cell culture stage (RD1, ZZ) over the total number of independent lines screened.

NT = Not tested.

The main advantages of pETKUL2 over pluc19 are: the distance between the T-DNA borders and the start codon of the luciferase gene (32 vs. 662 bp), the codon optimized luciferase gene (*luc*$^{+}$ vs. *luc*), and the location of the reporter gene in the T-DNA (RB vs. LB) (Fig. 1). Higher luciferase expression and tagging frequency (Table 1) were obtained with pETKUL2.

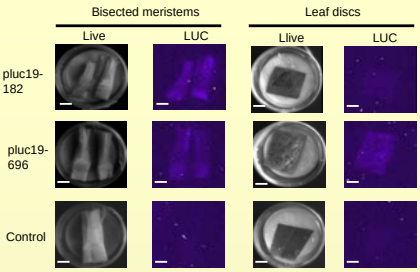


**Figure 2.** Live and LUC images of two promoter tagged lines and an untransformed control plant. Bisected meristems and leaf discs from greenhouse plants were screened for luciferase activity after application of 0.1 mM luciferin. Images were taken after 6 hours in complete darkness to reduce background. Blue, green = weak luciferase expression; red, white = strong luciferase expression. Bar = 1 cm.

Transgenic cell colonies were screened for luciferase activation at 8°C cold stress. Three different expression patterns of cell colonies can be seen in Figure 4: (1) enhanced, (2) repressed, and (3) unchanged luciferase expression compared to that at 26°C. Around 70% of the cold-responsive lines showed a repressed expression at 8°C, and only 12% showed an enhanced response (Table 2)

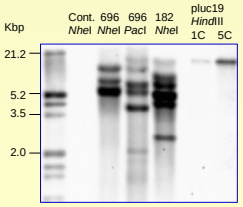


**Figure 3.** Southern blot analysis of two independent pluc19 tagged lines (696, 182). Cont. = untransformed plant control. Ten µg of total digested DNA were hybridized to a DIG-labelled *luc* probe. 1C, 5C = 1 and 5 copy number reconstructions.

Southern analysis of two "constitutive" promoter tagged pluc19 lines (696, 182) showed three to six integrations of the T-DNA (Fig. 3). Isolation of 11 flanking sequences of T-DNA was accomplished with TAIL-PCR (Liu *et al*. 1995). Different T-DNA rearrangements and backbone integration of the plasmid was obtained. One sequence from line pluc19-182 proved to be similar to part of the first exon of the GDP-mannose pyrophosphorylase gene from *Arabidopsis thaliana* and *Oryza sativa*, with a TATA box included. This line showed luciferase expression in the meristem region but not in the leaf of greenhouse plants (Fig. 2). As multiple flanking sequences were isolated, RT-PCR will be performed to confirm the activating sequence.

**Table 2.** Different luciferase activation patterns of pETKUL2 cell colonies screened at cold stress. Colonies were subjected to 26°C for 4 hours and then to 8°C for 18 hours

| Luciferase activation | Cell colonies | |
|---|---|---|
| | Number | % |
| Enhanced | 3 | 12 |
| Repressed | 19 | 73 |
| Unchanged | 4 | 15 |
| **TOTAL** | **26** | **100** |

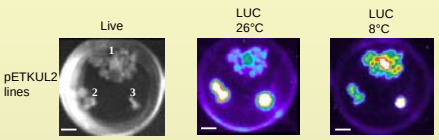


**Figure 4.** Live and LUC images of three different cold expression patterns in cell colonies of the plantain cultivar THP carrying independent T-DNA integrations of pETKUL2. Blue, green = weak luciferase expression; red, white = strong luciferase expression. Bar = 1 cm.

## CONCLUSIONS AND PERSPECTIVES

1. Baseline luciferase activation frequency reached 0.15% at regeneration stage (RD1) using the tagging vector pluc19. Activation frequency using the tagging vector pETKUL2 was 0.98% at cell colony stage (ZZ).
2. Promoter tagged lines showed three to six T-DNA integrations, RT-PCR will be performed to confirm the flanking plant sequence(s) responsible for the luciferase activation.
3. Four flanking sequences per promoter tagged line were obtained with TAIL PCR.
4. The frequency of responsive luciferase activation under cold stress was 0.14% using pETKUL2, cold-activated screening will be continued.
5. Cold- and heat-responsive promoters will be tagged in banana.
6. Further characterization of the pluc19 line with homology to the GDP-mannose pyrophosphorylase gene from *Arabidopsis thaliana* and *Oryza sativa* will be performed.

**References**

Mudge *et al.*, 1996. Austr. J. Plant Physiol., 23: 75-83.

Liu *et al.*, 1995. Genomics, 25: 674-681.

Sherf and Wood, 1994. Promega Notes, 49: 14-21.

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