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Protocols

Introduction of a Plant Intron into the Luciferase Gene of *Photinus pyralis*

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Abstract: We describe a new luciferase reporter gene, *luc*^{INT}, for early detection of luciferase activity in *Agrobacterium* transformation studies, and present improved techniques for the extraction of luciferase that decrease the time needed to quantitate luciferase activity. The *luc*^{INT} reporter gene combines the PIV2 intron from *GUS*^{INT} with *luc**, the modified luciferase gene. *luc*^{INT} is expressed in plant cells but not in *Agrobacterium*, allowing earlier detection of gene expression in the presence of *Agrobacterium* during transformations in tobacco leaf discs. Stable expression levels of *luc*^{INT} and *luc** in tobacco suspension cultures are compared for two different promoters.

The luciferase gene (*luc*) from the North American firefly, *Photinus pyralis*, has been used as a reporter gene in a variety of organisms, including bacteria (Wood and DeLuca, 1987) and plants (Millar et al., 1993; Ow et al., 1986). Luciferase activity can be visualized in transgenic plants without sacrificing valuable plants or tissue. Luciferase

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The nucleotide sequence data for the gene will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U84006.

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Abbreviations and gene names: 35S, 35S promoter of cauliflower mosaic virus; CaMV, cauliflower mosaic virus; CLR, cell-culture lysis reagent; GFP, green fluorescent protein; GUS, β-glucuronidase; *GUSINT*, *uidA* with the PIV2 intron; *luc*, cDNA encoding luciferase; *luc**, a specially modified *luc*; *lucINT*, *luc* cDNA with the PIV2 intron; LAR, luciferin assay reagent; *nosT*, gene encoding nopaline; *ocs*, gene encoding octopine synthase promoter region; PCC, photon-counting camera; RT-PCR, reverse transcription-PCR; *uidA*, gene encoding β-glucuronidase.

assays are very sensitive, especially when used in combination with a photon-counting camera (PCC) or a luminometer, and as few as 2000 molecules of luciferase have been detected (Wood, 1991). Extraction of luciferase from plant tissues is simple and quick. In addition, quantitation is facilitated by the broad linear range of the luciferase assay, which can extend over eight orders of magnitude (Wood, 1991). Because the half-life of luciferase protein is quite short (Millar et al., 1993; Nguyen et al., 1989; Thompson et al., 1991), reductions in mRNA levels are quickly converted into lower levels of protein expression. Thus, luciferase provides a more dynamic indication of *in-vivo* mRNA levels than the longer-lived β-glucuronidase (GUS; Narasimhulu et al., 1996) or green fluorescent protein (GFP) reporters (Narasimhulu et al., 1996; Ward and Bokman; 1982).

Many eukaryotic promoters are actively expressed in bacterial cells (Vancanneyt et al., 1990), and it often takes four to six weeks to eliminate *Agrobacterium* from plant cells or tissues after transformation (Barghchi, 1995; Suter-Crazzolara et al., 1995). Therefore, it is difficult to examine early gene expression from the reporter genes presently available for plants. Vancanneyt et al. (1990) have shown that GUS activity can be successfully used as a reporter of early gene expression during *Agrobacterium*-mediated transformation if an intron is inserted into *uidA*. Because the bacteria cannot process transcripts containing introns, any reporter activity must be due to expression of a plant gene. Unfortunately, GUS is very stable and the histochemical assay for GUS cannot be applied to living tissue (Suter-Crazzolara et al., 1995). Therefore, a luciferase gene containing an intron would offer many advantages over conventional reporter genes. For example, it would allow early detection of transgene expression *in planta* after *Agrobacterium* transformations without sacrificing the transformant for RT-PCR (Narasimhulu et al., 1996) or histochemical staining for GUS (Vancanneyt et al., 1990). An intron-containing luciferase gene may also facilitate transformant screening without antibiotics, by assaying regenerating plantlets or calli for luciferase activity.

Ideally, the intron used should contain several stop codons to prevent translational read through, and it should also be efficiently removed by plant splicing systems. Plant introns have splice junctions similar to animal introns and are generally AT rich, with an average length of 250 bp (Goodall et al., 1991; McCullough et al., 1993; Shapiro and Senapathy, 1987). The second intron of the potato *ST-LS1* gene (Eckes et al., 1986), is a typical plant intron with an AT content of 80 percent, a length of 189 bp, typical splice junctions, and multiple stop codons in all translational

reading frames. Vancanneyt et al. (1990) created the intron PIV2 from the second intron of *ST-LS1* by altering the internal splice borders to match the consensus sequence of plant introns, and inserting it into *uidA*. Transcripts of the resulting gene, named GUS^{INT}, are spliced effectively in *Arabidopsis* (Vancanneyt et al., 1990), tobacco (Narasimhulu et al., 1996; Rempel and Nelson, 1995), and maize (Narasimhulu et al., 1996).

Here we describe a new luciferase reporter gene that combines the PIV2 intron from GUS^{INT} with a modified firefly luciferase gene. We used *luc**, described by Bonin et al. (1994), in which commonly used restriction sites have been removed, and the translational initiation context has been optimized by Kozak's rules (Kozak, 1983). The new gene, luc^{INT}, is expressed in plant cells but not in *Agrobacterium*.

Materials and Methods

Cloning luc^{INT}

A *Sna* BI site was inserted into *luc** via silent mutagensis overlapping the 51st codon using a PCR primer set, designed to span from the area to be altered to a nearby *Bsi* WI site. The DNA product amplified was the 5′ region of *luc** with the new *Sna* BI site. The new *luc* gene was designated *luc′*. A similar approach was used to create, from a GUS^{INT} plasmid, a *Pvu* II site overlapping the 3′-splice junction of PIV2. A PCR product containing PIV2 was amplified from a GUS^{INT} plasmid, and then cloned into the *Sna* BI site of the *luc′* gene to create the luc^{INT} gene. All areas of the new luc^{INT} gene that were amplified during PCR were sequenced twice in each direction to ensure that there were no PCR-induced mutations. All sequences were as predicted. Standard cloning techniques (Sambrook et al., 1989) were used to create binary vector plasmids (Fig. 1) from pGPTV-*kan* (Becker et al., 1992) in which *luc** and luc^{INT} are driven by either the 35S promoter (Jefferson et al., 1987) or the *ocs* promoter (Koncz et al., 1983). The 3′ region of *nosT* (Jefferson et al., 1987) was used to terminate all the luciferase genes. The resulting constructs, pLkB05, -06, -07, and -08 (Fig. 1), were used to assess the relative activity of *luc** and luc^{INT} at two different promoter strengths. The pLkB05-08 plasmids were mobilized into *Agrobacterium tumefaciens* C58C1 (pGV3850) via triparental mating, using *E. coli* HB101 (pRK2013; Ditta et al., 1980) to facilitate transfer from the DH5 strains.

E. coli luciferase detection

Luminescence of *E. coli* DH5 (Gibco) colonies that contained a *luc′* or luc^{INT} gene on a pBI221-derived plasmid was measured with a

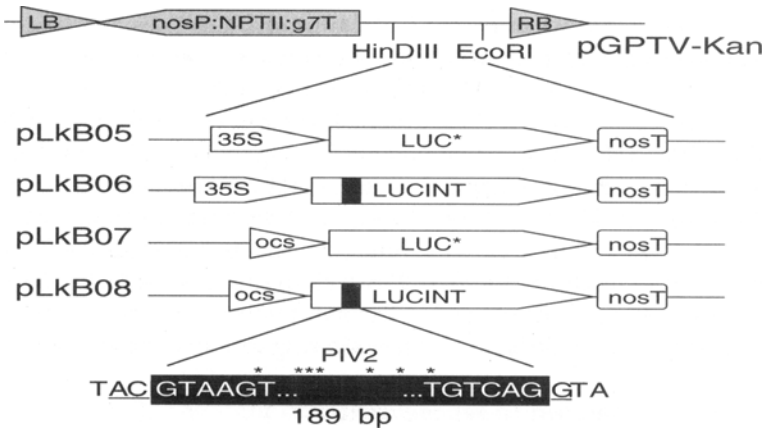

Fig. 1. Schematic diagrams of luciferase constructs. Each construct contains either the *luc** or *lucINT* gene driven by either the CaMV 35S promoter (35S) or the *ocs* promoter. The reporter constructs are arranged in a head-to-head configuration with the NPTII selectable marker driven by the *nos* promoter. A schematic of the PIV2 intron is shown below the *ocs:lucINT* construct (black box). The inframe stop codons of PIV2 are indicated by asterisks, and the interrupted codon is underlined. LB, left T-DNA border; RB, right T-DNA border; *nosT*, nopaline synthase terminator; *nosP:NPTII:g7T*, the gene encoding neomycin phosphotransferase from pGPTV-kan (Becker et al., 1992). Not drawn to scale.

Hamamatasu Argus-50 PCC in the presence of 1 mM D-luciferin (Biosynth). The Hamamatasu Argus-50 PCC settings were 10 V for 10 minutes.

Tobacco leaf discs: infecting with *Agrobacterium* and luciferase detection

Tobacco leaves were surface sterilized with 95% v/v EtOH and 50% v/v Clorox bleach (equivalent to 2.6% w/v sodium hypochlorite), then washed four times in sterile distilled water. Leaf discs were cut aseptically, and plated onto 0.8% w/v Phytagar (Sigma) containing OSMTob medium (Horsch et al., 1985) without any antibiotics. Then, 100 µL of *A. tumefaciens* C58C1 (pGV3850) cultures were added to appropriate culture wells. The 24-well plates (Nunc) were sealed with Parafilm™ and incubated for 3 days at 27° C with constant light. Luminescence was then measured with a Hamamatasu Argus-50 PCC imediately after the addition of 75 µL of 1 mM D-luciferin (Biosynth). The Hamamatasu Argus-50 settings were 5 to 10 V for 3 to 75 minutes.

Stable transformation of NT1 cells

To examine the effect of the PIV2 intron on luciferase expression in stable transformants, we transformed NT1 cells from tobacco suspension cultures as described by An (1985). Transformants were selected on NT1 medium (An, 1985) supplemented with 200 mg/L Timintin (SmithKline Beecham) and 100 mg/L kanamycin (Sigma) for three weeks. Microcalli were clearly visible after three weeks, and 36 putative transformants were isolated by transferring the microcalli to fresh medium. After one week on fresh medium, the first 24 surviving calli were each split into three pieces on separate plates for use in different assay procedures. The stock plates were maintained by transferring calli every two weeks.

Luciferase detection in whole calli

In an attempt to get an early look at the relative activities of the luciferase genes, calli were transferred into wells of a black 96-well plate (Nunc). Each well contained 100 μL NT1 medium supplemented with 200 mg/L Timintin and 100 mg/L kanamycin. The calli were cultured overnight at 27° C to allow them to recover from the transfer. Then, 25 μL of 1 mM D-luciferin was added to each well immediately prior to imaging in the Hamamatasu Argus-50 PCC at 10 V for 5 minutes. Results are shown in Fig. 3. This procedure does not provide a rigorously quantitative measure of expression, but it is a quick and effective method for making initial observations on a large number of transformants.

Extractive luciferase assay

Luminescence results when luciferase, D-luciferin, ATP and O_2 are mixed. This reaction is normally characterized by a sharp peak of luminescence followed by a very rapid decline (Promega, 1993; Wood, 1991), but it can be extended to produce a stable luminescence plateau of 30 to 60 seconds duration when coenzyme A is included in the reaction mix (Ford et al., 1992; Promega, 1993; Wood, 1991). The Promega stable luciferase assay buffer, which contains coenzyme A, was used in our extractive luciferase assays. Since luciferase has a very short half-life in plant cell extracts, extreme care was taken to insure that all samples were treated similarly. All steps were timed, and the extracts were always kept on ice except during the 4° C microcentrifugation. After three weeks of selection, all calli are presumed to be free of *Agrobacterium* because we were unable to recover bacteria by plating on rich non-selective media. First, ca. 0.5 g fresh weight of week-old NT1 cells were placed in a microfuge tube in a wet ice bath, and 200 μL of ice-cold cell-culture lysis reagent (CLR) was added. The tissue was ground with a motor-driven

Kontes pestle for 1.5 to 2.0 minutes at ca. 1000 rpm, and then snap frozen in liquid nitrogen. The samples were then thawed for 10 minutes in a wet ice bath, and cleared by centrifugation for 2.5 minutes and 10,000 x *g* at 4° C, and then returned to the ice bath. Luminescence was measured within ca. six minutes of centrifugation using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). For each measurement, 10 µL of cleared extract was combined with 50 µL of luciferin assay reagent (LAR; Promega). The luminometer automatically injects the LAR into a tube containing the extract to insure an accurate interval between mixing and measurement. Luciferase activity is optimal near room temperature, so it is important for the LAR to be equilibrated to room temperature (22° C) prior to assaying the samples or standards (Promega, 1993). The light emission was measured over a 10-second interval, starting 1.5 seconds after LAR injection. The total amount of protein in the extract was determined using DC protein assay kit (BioRad). A standard curve spanning seven orders of magnitude was generated using recombinant luciferase (Boehringer-Mannhein) in CLR supplemented with 1 mg/mL bovine serum albumin (Millar et al., 1992). The luciferase standard curve and DC protein assays were used to calculate the luciferase specific activity of each callus line in ng equivalents of luciferase per mg of extracted protein.

Results and Discussion

Cloning *luc*^{INT}

The intron PIV2 was designed by Vancanneyt et al. (1990) to be easily inserted into a *Sna* BI site. *luc** lacks a *Sna* BI site, but it was possible to create one via silent mutagensis. The new *Sna* BI site in *luc´* overlaps the 51st codon of the luciferase protein. The sequences at the splice junctions (AC | GTAAG ... TGTCAG | GT) of *luc*^{INT} are identical to those in *GUS*^{INT} (Vancanneyt et al., 1990). *E. coli* DH5 colonies that contained *luc´* on a pBI221-derived plasmid exhibited significant luciferase activity when imaged with a Hamamatasu Argus-50 PCC in the presence of 1 mM D-luciferin (Biosynth). However, DH5 containing the same plasmid with *luc*^{INT} in place of *luc´* gave no detectable luciferase activity (data not shown).

Luciferase expression in *Agrobacterium* and *Agrobacterium*-infected tobacco leaf discs

Agrobacterium harboring the 35S:*luc** plasmid pLkB05 showed significant expression of luciferase (Fig. 2, row B, column 1), but no expression

was detected from *Agrobacterium* containing the 35S:*luc*^{INT} plasmid pLkB06 with either the Hamamatasu Argus-50 PCC (Fig. 2, row D, column 1) or with extractive assays using a luminometer (data not shown). We conclude that, as expected, *Agrobacterium* failed to process the *luc*^{INT} intron correctly. The absence of detectable expression in *luc*^{INT} strains contrasts sharply with the high luminescence from *luc** strains. Bacterial *luc** activity was so high after three days of growth that no plant luciferase expression is detectable (Fig. 2B). In contrast, luminescence is readily detectable after three days from leaf discs exposed to bacteria harboring

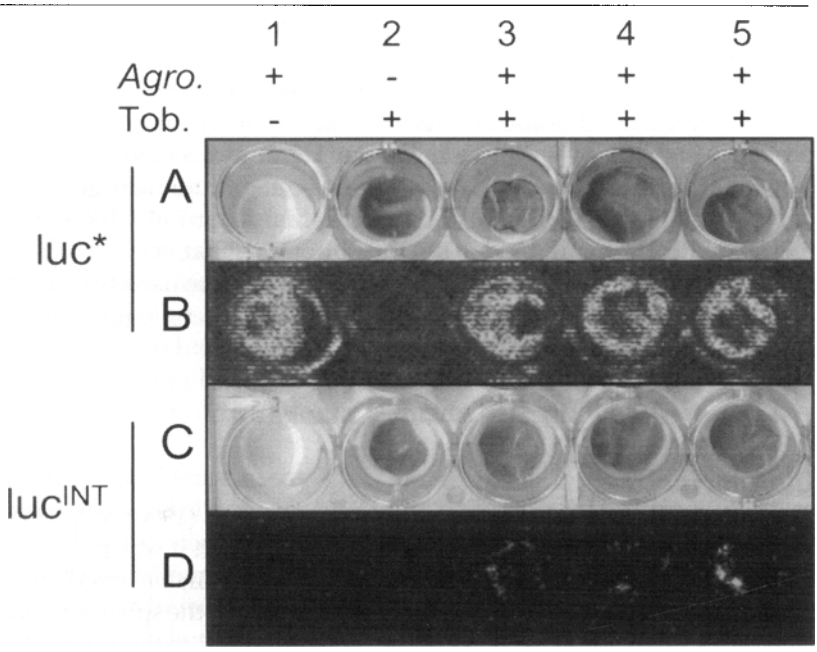

Fig. 2. Expression of luciferase in tobacco leaf discs three days after co-incubation with *Agrobacterium*. *Agrobacterium* and/or tobacco leaf discs were incubated in wells of a 24-well culture plate for three days at 22° C under constant light. Rows *A* and *C* show reflected light images at the time of the assay. Rows *B* and *D* show luminescence images obtained after addition of 75 µL of 1 mM D-luciferin. Luminescence was imaged with a Hamamatasu Argus-50 PCC. In *A* through *D*, wells in columns 2 to 5 contain tobacco leaf discs, while wells in columns 1 and 3 to 5 contain *Agrobacterium*. The *Agrobacterium* is C58C1 (pGV3850, pLkB05) in *A* and *B* and C58C1 (GV3850, pLkB06) in *C* and *D*; these strains contain the 35S:*luc** and 35S:*lucINT* constructs respectively. In (B) the Hamamatasu Argus-50 PCC sensitivity setting was 5 V and photon counting was carried out for three minutes, while in *D* the setting was increase to 10 V and counting was for 10 minutes.

the 35S:*luc*^{INT} construct (compare row D, columns 1 and 3–5 in Fig. 2). The sensitivity setting needed to detect the 35S:*luc*^{INT} expression in tobacco leaf discs is much higher (ca. 103-fold) than that needed to detect the *Agrobacterium* 35S:*luc** expression. Thus, the level of luminescence from the *luc*^{INT} plant cells in Fig. 2D (columns 3–5) is much lower than that seen for *luc** in *Agrobacterium* cells surrounding the leaf discs in Fig. 2B (columns 3–5), emphasizing the importance of preventing bacterial expression when attempting to detect expression in plant cells. It is unlikely that the luminescence observed in *luc*^{INT} plant cells is from some unknown mechanism related to *Agrobacterium* infection because leaf discs incubated with *Agrobacterium* harboring a 35S:GFP construct, pBin-m-gfp5-ER (J. Haseloff, personal communication), do not produce detectable luminescence. Luminescence was detected from 35S:*luc*^{INT} but not 35S:GFP-infected leaf discs, even when photon counting was performed for 75 minutes rather than 10 minutes, as in Fig. 2D (data not shown).

Luciferase expression in stably transformed NT1 cells

Extractive assays were performed to provide more accurate estimates of the luciferase activity levels in the transformed calli. The results of these

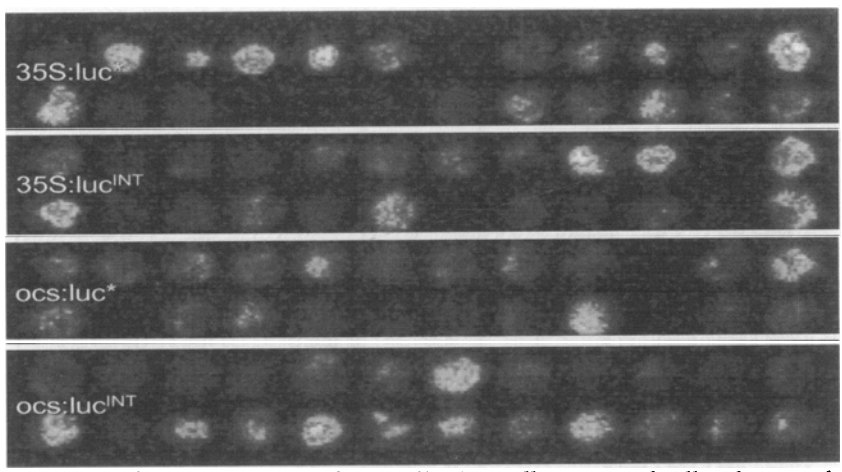

Fig. 3. Luciferase expression from calli. A small portion of callus from each stably transformed line was transferred from a stock plate into a well of a black 96-well culture plate. Each well contained 100 µL of NT1 medium supplemented with 200 mg/L Timintin and 100 mg/L kanamycin. Calli were allowed to recover overnight at 27° C. 25 µL of 1 mM D-Luciferin was then added to each well, and the whole plate was imaged in the Hamamatasu Argus-50 PCC for 5 min at 10 V. There are 24 calli for each construct depicted in Fig. 1. *ocs*, promoter of *ocs*; *35S*, 35S promoter of CaMV.

assays are summarized in Fig. 4. There is no significant difference between the mean values for *luc** and *luc*^{INT} when driven by either the 35S promoter or the *ocs* promoter (Fig. 4A), indicating that *luc*^{INT} performs at least as well as *luc**.

A comparison between extractable luciferase activities and activities estimated from the 96 calli in Fig. 3 demonstrates a positive correlation, but the correlation is weak (r = 0.520). Thus, the callus assays, while useful, should be regarded as semiquantitative. The extractive procedure is, however, simple enough to permit many samples to be assayed rapidly. Furthermore, since *luc*^{INT} is not expressed effectively in *Agrobacterium*, this gene will allow us to assay plant expression earlier after transformation when the bacteria still contaminate the regenerating calli. Therefore, the most limiting time factor involved in transformation studies using these techniques will be the growth rate of the transformed plant material.

In summary, we have generated a new luciferase reporter gene, *luc*^{INT}, that should prove useful for early detection of transgenic luciferase, and we present improved luciferase extraction techniques that decrease the time needed to quantitate luciferase activity. *luc*^{INT} should find many uses

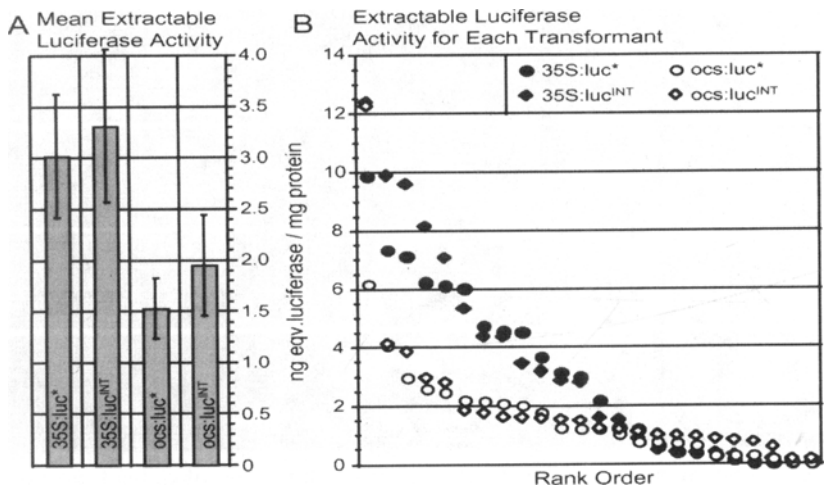

Fig. 4. Extractive luciferase assay means and summary. (A) Mean extractable luciferase activity for the four constructs, each of which is represented by 24 transformants. Error bars indicate the SEM. (B) Extractable luciferase activity in individual transformants. For each construct, data from individual transformants are presented in rank order to facilitate comparison of the activity distributions. *ocs*, promoter of *ocs*; 35S, 35S promoter of CaMV.

other than the early quantitation of transformant luciferase activity demonstrated here. For example, it should be possible with *luc*^{INT} to develop a new *Agrobacterium*-based transient assay protocol.

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