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Firefly Luciferase as a Reporter for Plant Gene Expression Studies

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Reporter enzymes often are used to monitor gene expression. Several reporter gene systems have been developed which differ in ease of use, cost, sensitivity, versatility and safety. We have used luciferase from the North American firefly Photinus pyralis *extensively as a reporter in studies of plant gene expression. We found that luciferase has several features that make it a particularly attractive reporter when compared to the frequently used CAT, GUS and neo genes. In this article, we review some of these characteristics and present expression plasmids and assay procedures optimized for use with plant suspension culture cells.*

Introduction

The function of *cis*-acting regulatory sequences in gene expression is the focus of much research today. Promoter sequences, 5´ and 3´ untranslated regions (UTRs), and introns qualitatively and quantitatively affect gene expression. We are interested in determining how these sequences modulate gene expression in plant cells. To facilitate our studies, we attached these regulatory regions to reporter genes such as chloramphenicol transacetylase (CAT), neomycin phosphotransferase (neo), beta-glucuronidase (GUS) and firefly luciferase (1,2). In this article, we discuss the advantages of firefly luciferase compared to other reporter genes. We also present a series of chimeric plasmids that express luciferase in plant cells and the protocols used to assay luciferase activity.

The first luciferase gene cloned was from the North American firefly *Photinus pyralis* (3,4). The native gene contains several introns, but a full-length cDNA also has been isolated. The active enzyme is a single polypeptide with a mass of 62kDa. The luciferase reaction (Figure 1) emits yellow-green light (560nm) and requires only the enzyme, ATP, Mg2+, O2 and the substrate luciferin. In fireflies, the luciferase reaction occurs in the peroxisomes of a specialized light organ, but the reaction can also occur in bacteria and cell-free extracts. The only equipment required to detect luciferase activity is a photon measuring device such as a luminometer or a scintillation counter.

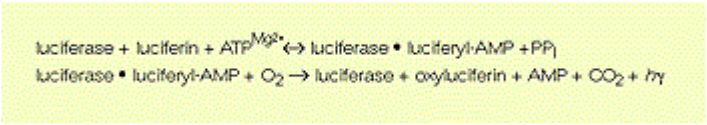
1. 

Figure 1. The luciferase reaction.

Firefly luciferase is a nearly ideal reporter gene for plant cells. We list the advantages of luciferase over other frequently used reporters below and in Table 1.

1. The luciferase assay is quick and easy. The assay simply involves mixing the cell extract with assay solution and placing the mixture in a photon measuring device. Assay times are usually less than a minute. In our experience, 50 luciferase assays can be performed in about 1 hour. In contrast, the standard CAT and neo assays require laborious extractions, chromatography, or gel separations (5,6,7).
2. The luciferase assay is very sensitive. Luciferase assays are 10- to 1000-fold more sensitive than the standard CAT assay (4,8); as little as 10^-20 moles of luciferase protein can be detected. The ability to quantify very low expression of reporter enzyme is an advantage when testing sub-optimal regulatory sequences. Since plants have little or no endogenous light-producing activity, even small quantities of luciferase enzyme can be measured without interference from background noise. Plant tissues do contain endogenous enzyme activities which mimic beta-galactosidase, CAT, neo and GUS, and which can result in excessive background readings.
3. Luciferase can be detected in several ways. Generally, luciferase is assayed from cell-free extracts, although we and others (9,10) have detected expression *in vivo* using film or video imaging technology (VIM, Figure 3). Luciferase also can be detected *in situ* using immunohistochemistry (11).
4. The reagents used for the luciferase assay are not hazardous. By contrast, standard CAT and neo assays use radioisotopes and/or solvents which are hazardous and have high disposal costs. CAT and neo ELISA assays (12) are commercially available, but are expensive.
5. The luciferase assay is economical. Luciferase assays cost slightly less than neo assays and 10 to 50 times less than CAT assays (reference 5 describes a more economical CAT assay for plants). Since typical experiments use multiple constructions, and repetitions are performed to ensure accuracy, a low-cost assay conserves financial resources.

In addition to luciferase, we find that the GUS gene is an exceptional reporter for plants. As shown in Table 1, it also is safe, sensitive, and versatile. In transfection experiments comparing several luciferase constructs, expressing a second reporter gene activity to correct for variation in transfection efficiency and protoplast recovery is essential to accuracy. As a result, we often use both luciferase and GUS expression constructs in each transfection. We find that the chief advantage of GUS over luciferase is the ease with which histochemical staining can be performed to determine tissue localization of expression.

Table 1. A Comparison of Several Plant Reporter Genes.

	firefly luciferase	**GUS (footnote 1)**	**CAT (footnote 2)**	**neo (footnote 3)**
Time (time per 50 assays; approximate)	1 hour	~3 hours	>3 hours	>3 hours
Specialized equipment required	luminometer (footnote 4) or scintill-ation counter	fluorometer (footnote 5)	none	none
Hazards	none	none	radioactive substrates; hazardous chemicals	radioactive substrates; hazardous chemicals
Additional	VIM, immuno-	histo-	ELISA	ELISA

assay methods	chemistry, photographic film	chemical stain, activity stain in denaturing SDS gel, immuno-chemistry		
Endogenous background activity (footnote 6)	none	yes in standard GUS buffer (17); none when using CCLR	yes; can be heat inactivated	yes; must be gel separated
Translational fusions (footnote 7)	yes; N- and C-terminal	yes; N- and C-terminal	no	no

Footnotes:

1. *We assay GUS activity by flourimetry using the substrate MUG (4-methylumbelliferyl-beta-D-glucuronide).*
2. *Comments are for the standard CAT assay described in reference 6.*
3. *Comments are for the neo assay described in reference 7.*
4. *We have used luminometers from Analytical Luminescence Laboratory (model #2010) and Analytical Scientific Instruments (model #3010).*
5. *We have used a Hoefer Scientific Instruments Fluorometer (model TKO-100) for GUS assays.*
6. *Background activity can vary between plant species and tissues.*
7. *The activity of fusion proteins will vary.*

Luciferase expression vectors

Our laboratory studies the effects of promoters, 5´ and 3´ UTRs, and introns on gene expression in plant cells. We constructed a series of luciferase expression vectors in which the various regulatory regions can be interchanged easily. In Figure 2, we present the restriction maps of several of these constructs, showing the unique restriction sites that separate the promoter, the luciferase cDNA and the 3´ polyadenylation region. A detailed description of each plasmid has been published elsewhere (2,13). The luciferase gene in pJD300, pJD301 and pJD312 is transcribed from the cauliflower mosaic virus (CaMV) 35S promoter, which is active in both monocots and dicots. pAL61, which contains the maize *Adh1* promoter and first intron, expresses well in monocots but poorly in dicots. Each construction features an *Nco I* (CCATGG) restriction site engineered at the luciferase start codon. This modification serves two purposes: 1) the ATG is within the Kozak consensus (14) for efficient translation initiation in eukaryotes, and 2) the *Nco I* site may be used to generate N-terminal fusion proteins. We have constructed several active N-terminal fusion proteins in pAL61.

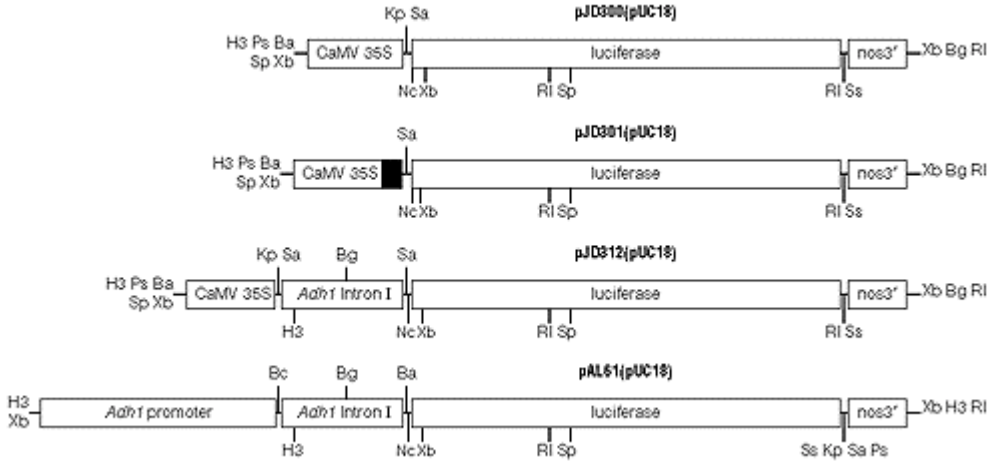
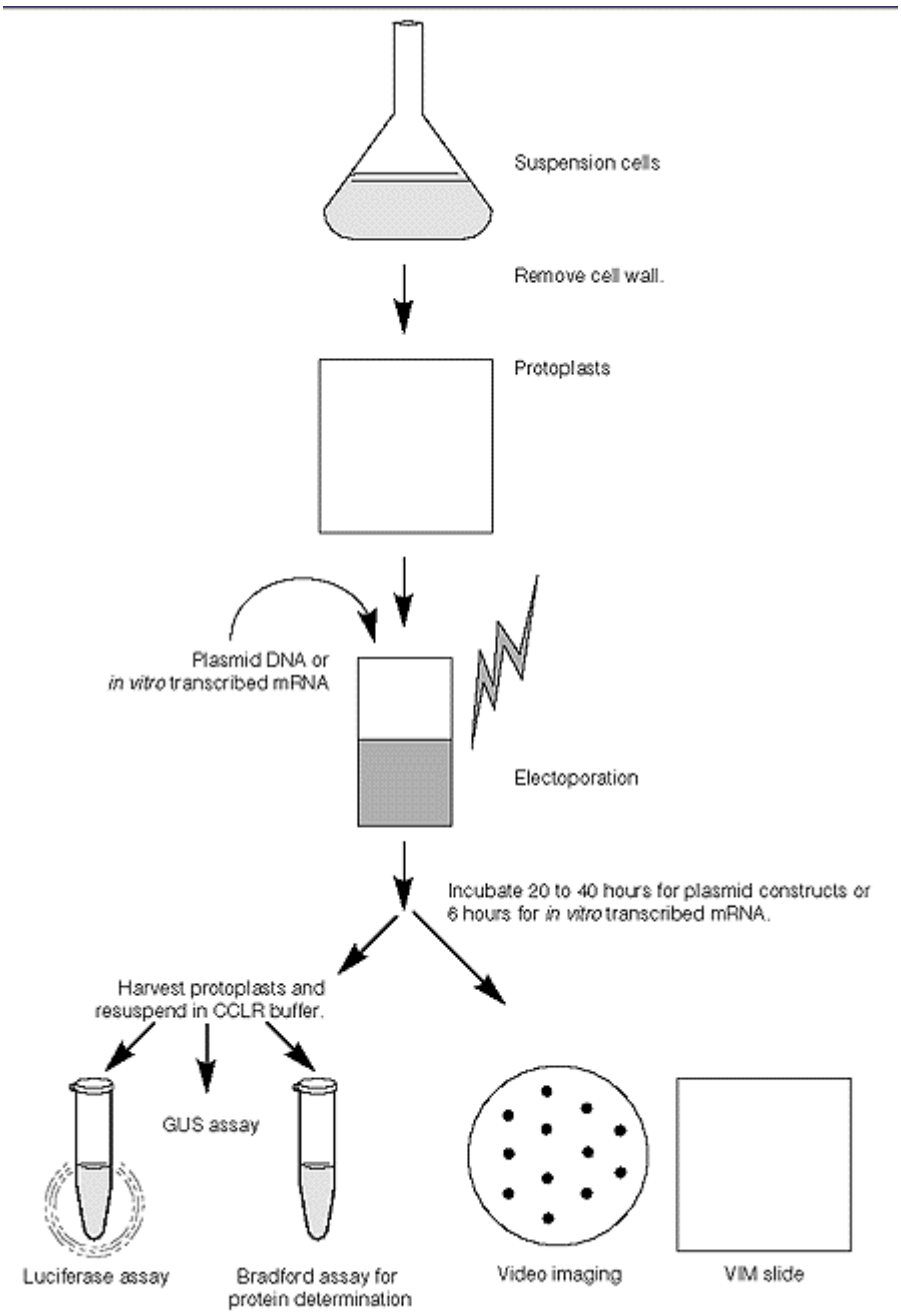
1. 

Figure 2. Restriction maps of several luciferase expression vectors. The name of each expression vector is followed in parentheses by the name of the plasmid vector carrying the diagrammed transcription units. The shaded box in pJD301 represents the tobacco mosaic virus (TMV) omega 5´ UTR translational enhancer sequence. The restriction sites are abbreviated as: *Bam*H I, Ba; *Bcl* I, Bc; *Bgl* II, Bg; *Eco*R I, RI; *Hin*d III, H3; *Kpn* I, Kp; *Nco* I, Nc; *Pst* I, Ps; *Sal* I, Sa; *Ssp* I, Sp; *Sst* I, Ss; *Sma* I, Sm; *Xba* I, Xb.

Plasmids for synthesizing capped, polyadenylated luciferase mRNA *in vitro* using T7 phage RNA polymerase have also been constructed (10). After transfecting the mRNA into plant protoplasts, luciferase activity can be detected in cell extracts or visualized using VIM (Figure 3). *In vitro*-synthesized mRNAs have been used to study the effects of RNA regulatory elements such as 5´ and 3´ UTRs (15,16) and to determine mRNA stability in the cytoplasm (10).



1.

Figure 3. An outline of the transient assay protocol for plant cells. In the lower right, tobacco protoplasts were transfected with luciferase mRNA. Luciferin was added and the light output was detected by video imaging; the red and yellow colors represent areas of high light output.

Transient expression assay using luciferase expression vectors in plants

We generally use a transient expression assay to test chimeric luciferase plasmids. Luciferase constructs are introduced into plant suspension cells by electroporation or particle bombardment. Most assays are performed in maize BMS (Black Mexican Sweet) suspension cells. Excellent results also have been obtained with tobacco, carrot and rice suspension lines.

An outline of the electroporation procedure is shown in Figure 3 and described in detail elsewhere (13). First, the cell wall is removed by digestion with a mixture of cellulase, hemicellulase and pectolyase. The protoplasting enzymes are washed away, and approximately 5 x 10^6 protoplasts are electroporated using 10-50µg of the luciferase expression plasmid or 2-5µg of capped, polyadenylated mRNA. A GUS-expressing reporter construct is usually included in each transfection to correct for protoplast viability and recovery. To insure reproducibility, a master mix of each luciferase plasmid plus the GUS internal control is prepared. Aliquots of the mix are distributed to individual cuvettes for electroporation.

After incubation for 1-2 days, the transfected protoplasts are harvested by centrifugation and passively lysed by resuspending in 0.4ml Cell Culture Lysis Reagent (CCLR, Promega) or a modification of this formulation, CCLR (see section, Modified Extraction and Assay Buffer Formulations for Plant Cells, below). The disrupted protoplasts are spun in a microcentrifuge for greater than 1 minute to pellet cellular debris and then placed on ice. The extract can be stored at -80°C for at least a month without loss of luciferase or GUS activities.

The extracts are assayed for luciferase activity in the following manner. Extract (20µl) is placed in a luminometer cuvette or a scintillation vial and allowed to reach ambient temperature. Immediately before counting, 200µl of Luciferase Assay Reagent (LAR, Promega) or a modification of this reagent, LAR (see section, Modified Extraction and Assay Buffer Formulations for Plant Cells, below) is added to the extract, and the mixture is placed in a photon quantifying device. If a luminometer is utilized, detection times range from 10-30 seconds.

Since both luciferase and GUS activities are stable in CCLR, aliquots of the same extract may be used for GUS assays; GUS assay procedures for plants have been described in detail (13,17). The raw luciferase data can be corrected using either GUS activity or protein determination. Table 2 reports typical results for BMS cells transfected with two of the plasmids shown in Figure 2.

Table 2. Transient Expression Assay Using the Luciferase Reporter Gene.

			Luciferase activity	
Plasmid	Extraction buffer	GUS activity (pmol/min/20µl)	LAR assay (lu/30 sec)	GUS corrected (lu/30sec/ pM MU/min)
control	std	0	156	0
pJD300	std	23.7	320,629	13,628 +/- 2,261
control	CCLR	0	167	0
pAL61	CCLR	19.2	602,496	31,585 +/- 3,668

Electroporations using BMS protoplasts and the plasmids indicated were performed as described in the text. The composition of the standard extraction buffers has been published in reference 13. GUS assays were done as described in reference 17. The 30 second luciferase readings for 20µl of extract using the LAR were corrected with the GUS activity in 20µl of extract; the results from 2 or 4 replicates were averaged and a standard deviation is given as a ± value. lu, light unit; MU, methylumbelliferone; std, standard.

Tricks, Tips and troubleshooting

Photon counting devices

Many commercially available luminometers have an automatic injection device to deliver the substrate luciferin to the reaction mixture. Using a luminometer and standard reaction conditions (4), the cuvette is placed in the counting chamber and the luciferase reaction is initiated by injecting luciferin into the cuvette. After an initial burst, light production falls off rapidly because the end-product, oxyluciferin, inhibits the enzyme, luciferase. Since the cuvette is already in the counting chamber, the initial burst of light is detected. While a luminometer with an autoinjector is preferable for the standard luciferase assay, the high price of these instruments might preclude purchase.

Less expensive luminometers (without automatic injectors) and scintillation counters can also be used to measure photons (18), but because light production falls off rapidly under standard reaction conditions, the handling time between luciferin addition and placement in the counting chamber makes it impossible to detect the initial burst of light.

Promega's Luciferase Assay System is an improvement over previous assays due to increased enzymatic turnover which results in enhanced light production that is essentially constant (t1/2 = 5 minutes) for several minutes. Using the LAR, light production is essentially constant over several minutes, allowing time to mix enzyme and substrate manually and insert the reaction in the luminometer or scintillation counter. If a scintillation counter is used, the coincidence counter can be turned off or the raw data can be mathematically corrected (19).

Detecting luciferase in *E. coli*

Luciferase activity can be detected *in vivo* in *E. coli* (20). This feature can be used to screen for bacterial colonies that contain the luciferase reporter gene in a chimeric plasmid. When the luciferase gene is present in *E. coli*, it is usually expressed at detectable levels, even in the absence of a bacterial promoter. This 'leaky' expression can be used to pre-screen colonies for luciferase activity prior to preparing and analyzing plasmid DNA. In addition, luciferase activity detected in the bacterial assay provides an assurance that the gene has not been altered by mutation.

To perform the bacterial assay, a fresh colony is collected with a toothpick, and the cells are smeared onto the bottom of a luminometer cuvette. The cells are resuspended in 50µl of LB broth containing 100mM sodium citrate, pH 5.5 and 1mM luciferin and light output is measured. Although the results are not quantitative, light production is diagnostic for presence of the luciferase gene.

Modified extraction and assay buffer formulations for plant cells

The reagents used for luciferase assays can be purchased or prepared in the laboratory. The Luciferase Assay System from Promega was developed for use with mammalian cells, but the lysis reagent (CCLR) and assay reagent (LAR) also work well with plant cells. We have simplified the CCLR and LAR for use with plant protoplasts without any loss of luciferase activity. The modified formulations for the Cell Culture Lysis Reagent (CCLR) and Luciferase Assay Reagent (LAR) are: CCLR is 100mM potassium phosphate, pH 7.8, 1mM EDTA, 7mM 2-mercaptoethanol, 1% Triton® X-100, 10% glycerol and LAR is 20mM Tricine, pH 7.8, 5mM MgCl2, 0.1mM EDTA, 3.3mM DTT, 270µM Coenzyme A, 500µM luciferin, 500µM ATP.

DNA versus mRNA transfections

The protocols for introducing DNA plasmids and *in vitro* transcribed mRNA into plant cells are essentially identical, with one exception. DNA constructs must localize to the nucleus and undergo transcription before luciferase translation can occur, thus the optimal assay time is 20-40 hours after transfer. For mRNA transfections, the protoplasts are electroporated **immediately** after RNA addition to prevent message degradation by RNases. Because the mRNA is rapidly translated in the cytoplasm, luciferase expression peaks approximately 6 hours after transfer.

Expressing both luciferase and GUS in each transfection

Luciferase has low activity in the standard GUS extraction buffer (17) containing both Triton X-100 and SDS. We find that GUS enzyme activity is stable in CCLR, obviating preparation of dual extracts using the luciferase and GUS extraction buffers. Moreover, an endogenous glucuronidase activity can be detected in BMS extracts prepared with the standard GUS extraction buffers that is not present in extracts prepared with CCLR. We suggest that CCLR be used even when working only with the GUS reporter gene.

Standardizing the raw luciferase data

Most of our studies of gene regulation utilize the luciferase reporter gene. Because of the variation inherent in sample handling, we include a GUS expression construct in each luciferase reporter transfection for correction of differences in transfection efficiency, protoplast recovery and sample variability. Using this regime, the expression data are expressed as light units (lu)/10 seconds per pmol methylumbelliferone (MU)/minute. While we strongly recommend the use of a second reporter gene for standardization, the luciferase specific activity also can be determined by measuring the protein concentration of the extract and then expressing the result as lu/10 seconds per mg protein. CCLR interferes with the Bradford assay, resulting in artifactually high protein concentration measurement. If a protein determination is called for, we recommend preparing cell extracts in the absence of Triton® X-100 and disrupting protoplasts by sonication.

Conclusions

Firefly luciferase is a superior reporter gene for plant cells. The enzyme assay is easy and inexpensive and requires no radioactive or hazardous materials. Since plants contain little to no endogenous luminescence, background is minimal. Luciferase assays are adaptable to applications that require sensitive quantitation or simply a 'yes' or 'no' determination of gene expression. The GUS reporter gene also has many favorable qualities, and we find it advantageous to use both reporters in the same transfected cells. Continued improvement of existing luciferase assays and development of new detection technologies such as video imaging will extend the applications of luciferase as a reporter system.

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Ordering Information

Product	Cat.#
Luciferase Assay System	E1500

This system contains sufficient reagents for 100 assays.

Product	Cat.#
Luciferase Assay Reagent	E1483

This system contains sufficient reagent for 1,000 assays.

Product	Size	Cat.#
Luciferase Cell Culture Lysis Reagent, 5X	30ml	E1531

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