Note

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Dual Luciferase Assay for Monitoring Transient Gene Expression in Higher Plants

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Abstract

To investigate the use of the DLRA system in plants, expression of firefly luciferase (F-luc) and *Renilla* luciferase (R-luc) reporter genes in plant cells introduced by microprojectile bombardment was examined. Although the relative activity of F-luc and R-luc varied among types of plant cells, the assay system is highly sensitive and can be applicable to various plant cells.

Abbreviations

GUS, β-glucuronidase; CAT, chloramphenicol acetyltransferase; CaMV, cauliflower mosaic virus; CAB, chlorophyll a/b-binding protein; DLRA, dual-luciferase reporter assay.

By fusing the reporter gene to a genomic DNA fragment, and examining the expression of the reporter gene under the control of the promoter, we can monitor the promoter activity indirectly as the activity of reporter gene product. Several reporter gene assay systems have been developed for monitoring gene expression. In higher plants, the GUS gene has been the most popular for studying tissue and organ-specific expression in transgenic plants (Jefferson, 1987). Firefly luciferase (F-luc) also is one of the most used widely reporter genes because its enzyme activity is closely coupled to transcription, and the assay system is rapid and sensitive (Ow *et al.*, 1986; Koncz *et al.*, 1990; Millar *et al.*, 1992). Recently, *Renilla* luciferase (R-luc) is also being widely used as a genetic reporter for higher plants (Mayerhofer *et al.*, 1995). Although the assay of R-luc is rapid, the sensitivity is somewhat limited by autoluminescence. Because of this problem, R-luc is used mainly as a co-reporter to F-luc.

An assay format called the dual-luciferase reporter assay (DLRA) has been designed to sequentially quantify enzymatic activities of F-luc and R-luc from a single sample (Sherf *et al.*, 1996). The integration of the two luciferase assays provides an efficient means for incorporating an internal standard into measurements of reporter gene expression levels. Because of its versatility and higher sensitivity compared with conventional procedures, we applied DLRA for monitoring gene expression by the microprojectile bombardment-mediated transient expression of reporter genes in higher plants (Seki *et al.*, 1991; Millar *et al.*, 1992). In this report we show that the DLRA is highly sensitive and can be applicable to various higher plant cells. In addition, we also discuss possible problems associated with the assay system.

In order to express luciferase genes in plant cells, we constructed expression vectors for firefly and *Renilla* luciferases based on the pBI221 plasmid harboring CaMV35S promoter. To evaluate the efficiency of DLRA protocol in plant cells, we examined the transient expression of reporter genes encoding firefly and *Renilla* luciferases by microprojectile bombardment. The 35S::F-luc construct was made by replacing the GUS coding region of pBI221 (Clontech) with a LUC+ fragment from pSPluc+ (Promega). The LUC+ coding region had been excised by digestion with *Bgl*II and *Eco*RV and then was cloned into *Sac*I blunt-*Bam*HI sites of pBI221. To make the *CAB1*:: F-luc plasmid, we isolated a 1.4 kb genomic DNA fragment of the Arabidopsis *CAB1* gene by PCR using the following primers;
5'-CGCAAGCTTACTCCTCAATCACACTCC-TATA-3' and
5'-CCGCCATGGTTGAGTGCAGCACAAAG-TAA-3'.

The PCR product was digested with *Hin*dIII and *Nco*I and then cloned into *Hin*dIII-*Nco*I sites of 35S::F-luc. The 35S::R-luc construct was made by excising the *Renilla* luciferase coding region from pRL-null (Promega) by digestion with *Xba*I blunt-

*Bgl*II and then cloned into *Sac*I blunt-*Bgl*II sites of p35SGFP (Clontech). The intron sequence derived from pRL-null was removed by digestion of the plasmid with *Nhe*I and *Sma*I then self-ligated.

The microprojectile bombardment of plant cells was carried out using the model PDS-1000/He particle delivery system (BioRad) according to the manufacturer's instructions. For three independent assays, 25 μl aliquots of gold microcarrier (1.6 μm) suspensions were coated with 0.5 μg of each plasmid DNA and spread onto the macrocarriers. Samples were placed 6-9 cm from the stopping screen and bombarded at 1100 psi under a vacuum of 28 inches of mercury. Five-day-old tobacco BY-2 cells (2 m*l*) maintained in a liquid medium (Nagata *et al.*, 1981) were spread on 3 % agar plates and briefly allowed to dry to remove excess liquid. After bombardment, cells were harvested with 2 m*l* of liquid medium, transferred to a flask, and then incubated at 26 ℃ with gentle agitation.

For luciferase activity measurements, samples of 200 μl of tobacco BY-2 cells were transferred to a 1.5 m*l* microfuge tube and centrifuged at 10000g for 1 min to remove the liquid medium. Cells were dissolved in 250 μl of ice cooled passive lysis buffer (Promega), homogenized using a disposable plastic pestle (Kontes) and cleared by a 1-min centrifugation at 10000g, at 4 ℃. Other plant tissues were homogenized in 2 m*l* of passive lysis buffer using an ice-cooled mortar and pestle.

For luciferase activity measurements, 10 μl of cell lysate was transferred into the luminometer tube containing 50 μl LARII solution (Promega), mixed by tapping, and then placed in a luminometer (model LB9501; Berthold). Then, 50 μl of Stop & Glo solution (Promega) was added to the reaction mixture for detection of R-luc activity. Each reading of luciferase activity was 10 seconds in the luminometer.

For β-glucuronidase activity measurement, we used a GUS-Light chemiluminescent detection kit (Tropix). The substrate solution (70 μl) was added to 10 μl of cell lysate, diluted with 90 μl of passive lysis buffer containing 10 mM 2- mercaptoethanol and incubated for 1 h at 30 ℃. After addition of 100 μl of accelerator solution, GUS activity was measured for 10 seconds in the luminometer.

Fig. 1 shows the results of time-course measurements of activities for F-luc, R-luc and GUS reporter genes expressed under the control of the CaMV 35S promoter in tobacco BY-2 cells. Although we used dual-luciferase assay reagents optimized primarily for animal cells, the detection levels of F-luc and R-luc were sensitive enough for plant cells. Expression levels of R-luc were

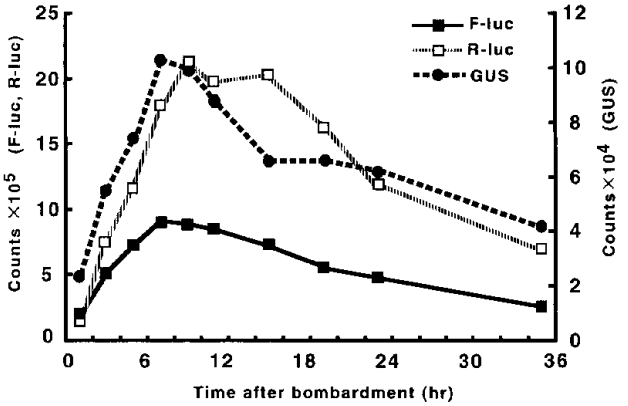

Fig. 1. Time-course measurement of firefly and *Renilla* luciferases and GUS activities in tobacco cells.
Tobacco tissue culture cells (BY-2) were bombarded with plasmid DNAs expressing *F-luc*, *R-luc* and *GUS* genes under control of the cauliflower mosaic virus 35S promoter. Enzymatic activity was measured by DLRA and the chemiluminescence GUS detection kit (GUS-Light). Each measurement is shown as a 10-second reading by a luminometer.

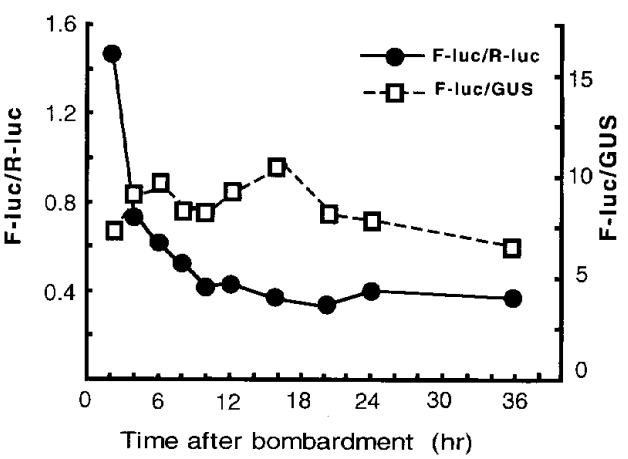

Fig. 2. Time-course of F-luc/R-luc activity and F-luc/GUS activity ratios in tobacco cells.
Each activity ratio was calculated from the time-course measurements of enzymatic activities shown in Fig. 1.

distinctly higher than those of F-luc, but the background level of R-luc was approximately 10 times higher than that of F-luc under the assay conditions used in this study (data not shown).

As shown in **Fig. 2**, a rapid decrease in F-luc/R-luc ratio was evident up to 4 h after bombardment. On the other hand, a relatively constant F-luc/GUS ratio was observed. In this experimental condition, however, the F-luc/R-luc ratio appeared to be constant up to 10 h after bombardment.

To confirm the versatility of this assay system, we conducted transient expression experiments using

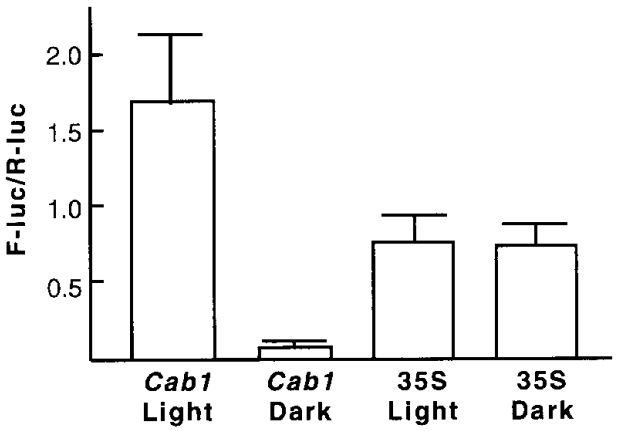

Fig. 3. Activities of Arabidopsis *Cab1* and CaMV35S promoters in spinach leaf tissue under different light conditions.
Spinach leaves were bombarded with plasmid DNAs harboring *Cab1*:: F-luc or 35S::F-luc and incubated under continuous white light (50 μmol m^{-2} sec^{-1}) or dark conditions for 16 h at 24℃. 35S::R-luc was used as a control and the enzymatic activities were measured by DLRA. Values are mean ±SE for three independent assays.

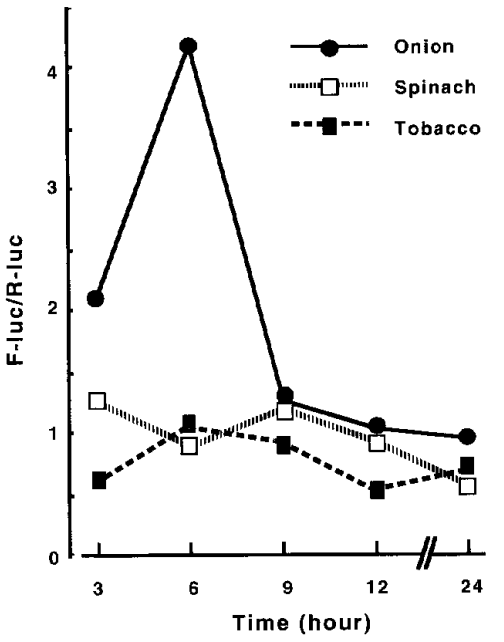

Fig. 4. Time-course measurement of F-luc/R-luc activity ratios in various plant tissues.
Onion epidermal cells, spinach and tobacco leaves were bombarded with equal amount of 35S::F-luc and 35S::R-luc plasmids. Samples were harvested and assayed at each time point.

an inducible promoter. Because of its high expression level and light responsiveness, we used the *CAB1* gene promoter from *Arabidopsis thaliana* (Ha and An, 1988) for this purpose. A plasmid construct containing the *CAB1*::F-luc gene was introduced into spinach leaves together with 35S::R-luc by microprojectile bombardment. After 16 h of incubation in a moisture chamber under different light conditions, a portion of the sample was ground in the lysis buffer with a mortar and pestle and assayed for luciferase activity. **Fig. 3** shows the results of the transient expression experiment of the light-responsive promoter in spinach leaves under continuous light and dark conditions. Compared with the CaMV35S promoter, the expression level of the Arabidopsis *CAB1* promoter is strongly induced under the continuous light condition. These results suggest that the data obtained by the dual-luciferase system faithfully reflects the mRNA levels of inducible gene expression by transient assay in plant cells.

Next we compared the activities of F-luc and R-luc in various plant tissues by expressing both genes under the control of an identical promoter-terminator cassette. We introduced 35S::F-luc and 35::R-luc plasmids into plant cells by microprojectile bombardment and compared their activities by the dual-luciferase assay. In our assay conditions, no significant difference in F-luc/R-luc ratio was observed between tobacco and spinach leaves, but a relatively high F-luc/R-luc ratio was detected in onion epidermal cells (**Fig. 4**). Moreover, the tissue specificity was evident when luciferase genes were transiently expressed in different tissues of lily. As shown in **Fig. 5**, the result clearly demonstrates that the relative activity of 35S::F-luc in leaf is about 4 times higher than that in pollen mother cells.

Unlike transgenic plant tissues where all the cells

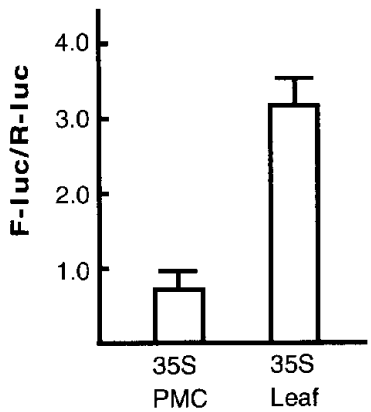

Fig. 5. Activities of F-luc and R-luc in lily pollen mother cell (PMC) and leaf tissue.
Lily pollen mother cells and leaves were bombarded with equal amount of 35S::F-luc and 35S::R-luc plasmids. After 24 h of incubation in the dark, enzymatic activities were measured by DLRA. Values are mean ±SE for six independent assays.

are transformed by the reporter gene, only a portion of the cells are transformed and express reporter genes in tissues transiently transformed by microprojectile bombardment. Therefore, compared with the reporter gene expression analysis for transgenic plants, the assay for transiently expressed reporter genes requires higher sensitivity. Because of its low background levels and wide linear range of detection, it is clear that F-luc is more suitable as a reporter for transient assay compared with other reporter genes such as GUS and CAT (Topfer *et al.*, 1988).

In this study, we used R-luc as a co-reporter to F-luc and found that DLRA can be a powerful tool for the transient reporter gene assay of plant cells. Because the assay procedure is rapid and simple, DLRA is particularly useful when a large number of samples are to be analyzed.

The time course measurement of reporter gene activities carried out in the present study indicated that the stability of enzymatic activity of F-luc is lower than that of R-luc. Because an identical promoter-terminator cassette was used, we speculated that the relatively low stability of F-luc is mainly due to the instability of the F-luc mRNA or protein *in planta* as described before (Thompson *et al.*, 1991). A previous study suggested that the F-luc activity in transgenic plants is unstable especially in the presence of its substrate, luciferin. Therefore, the instability of F-luc has been regarded as an advantage when considering the characteristics of the reporter gene required for the monitoring of mRNA levels *in planta* (Millar *et al.*, 1992).

While the DLRA is more rapid and convenient our results suggest that, although the assay protocol is rather tedious and time-consuming, the GUS gene is also reliable and useful as a co-reporter to F-luc. On the other hand, the use of R-luc as a reporter gene is rather limited because of higher background levels generated by autoluminescence.

As shown in **Fig. 4** and **Fig. 5**, tissue- or cell type-specific differences in relative luciferase activities were evident. It is possible that the differences in stability of luciferases is one of the major determinants, but additional regulatory steps such as transcriptional regulation, mRNA stability and translation efficiency may also be involved in the mechanisms by which tissue- or cell type-dependent relative luciferase activity is affected.

Previously, GUS-gene-dependent artifacts including ectopic expression in pollen of transgenic tobacco have been described (Uknes *et al.*, 1993). Although not yet reported, it is possible that the reporter-gene-dependent artifacts may be associated with the assay system using F-luc and R-luc. Clearly, further studies are required to elucidate the factors involved in reporter gene activities.

Acknowledgments

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