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Technical Note

Multi-color luciferases as reporters for monitoring transient gene expression in higher plants

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Abstract To investigate the usefulness of multi-color luciferase technology as reporter genes in higher plants, we assayed the transient expression of click beetle luciferase genes introduced into plant cells by microprojectile bombardment. Although their expression levels were relatively low, luminescence from green and red luciferases were separable under the CCD camera equipped with interference filters. Results of time-course experiments and the inducible promoter assay suggest that the multi-color luciferase system optimized primarily for mammalian cells is also applicable to monitor reporter activities in plant cells.

Key words: Click beetle, *in vivo* monitoring, multi-color luciferase, transient assay.

Several reporter gene assay systems have been developed for monitoring gene expression and function in various organisms. Reporter gene systems using fluorescent proteins including Green Fluorescent Protein (GFP) and its derivatives are widely used as reporters for protein tagging. However, the fluorescent protein is not suitable for monitoring gene expression levels because the quantification method for fluorescent reporter proteins is not readily available. On the other hand, a bio-luminescence reporter system using luciferase reporter genes has been developed and widely used for monitoring of gene expression levels in various systems including higher plants (Millar et al. 1992). Sherf et al. developed an assay format called the dual-luciferase reporter assay for quantification of gene expression levels (Sherf et al. 1996s). This assay system was designed to sequentially quantify enzymatic activities of firefly luciferase (F-luc) and *Renilla* luciferase (R-luc) from a single sample. The luminescence of these two luciferases is differentiated by their enzymatic reaction properties (Wood 1998). The integration of the two luciferase assays provides an efficient means for incorporating an internal standard into measurements of reporter gene expression levels and successfully applied for monitoring of plant gene expression (Matsuo et al. 2001; Ono et al. 2004).

A series of cDNA clones encoding luciferases with four different colors was obtained from the bio-luminescent click beetle, *Pyrophorus plagiophthalamus* (Wood et al. 1989; Viviani et al. 2001). These click beetle genes have been developed as a reporter vector system and have become available commercially (Almond et al. 2003). The click beetle luciferase (CB-luc) genes encode CBRluc, which emits red light, and CBG99luc or CBG68luc, which emits green light in the Chroma-Luc™ reporter system (Promega). The CBG99luc gene sequence is 99% identical to CBRluc while the CBG68luc gene is designed to be 68.9% identical to CBRluc to avoid problems associated with the high sequence homology between these genes. All CB-luc genes in the Chroma-Luc™ system have 99% identical amino acid sequences and a few amino acids are responsible for different colors of light. These luciferases catalyze D-luciferin that is also catalyzed by F-luc, and the red and green lights can be separated with interference filters. Therefore, this technology is suitable for monitoring of multiple gene expression levels simultaneously (Almond et al. 2003).

In an attempt to utilize this reporter system for studies of regulated expression of plant genes, we investigated the usefulness of this novel technology for assay of transient gene expression in higher plant cells. Here, we show that the multi-color luciferase technology, optimized primarily for mammalian cells, is also applicable for higher plants. In addition, we also discuss possible problems associated with this technology.

Abbreviations: CaMV, cauliflower mosaic virus; *CAB1*, chlorophyll a/b-binding protein; cDNA, complementary DNA; F-luc, firefly luciferase; GFP, green fluorescent protein; GUS, β-glucuronidase; Nos, nopaline synthase; R-luc, *Renilla* luciferase.

In order to express the multi-color luciferase genes in plant cells, we constructed expression vectors based on the pBI221 plasmid harboring the CaMV35S promoter and *nos*-terminator cassette (Matsuo et al. 2001). The luciferase reporter genes were transiently expressed by microprojectile bombardment. The 35S::luciferase constructs were made by replacing the GUS coding region of pBI221 with a CB-luc gene from the Chroma-Luc™ basic vectors (Promega). The multiple restriction enzyme sites of the basic vectors were removed by digesting *Sma*I and *Nco*I and the CB-luc-coding sequences were excised by digestion with *Nhe*I and *Xba*I-blunt, and then cloned into *Xba*I and *Sac*I-blunt sites of pBI221. The *CAB*1::CB-luc plasmid was made by excising the CBG99luc-coding sequence from the basic vector with *Nco*I and *Xba*I and then cloned into *Nco*I–*Xba*I sites of *CAB*1::Fluc (Matsuo et al. 2001).

The activity of CB-luc genes was assayed after microprojectile bombardment of plant cells as follows. Twenty-five microliters of the gold microcarrier (1.6 μm) coated with 2 μg plasmid DNA was spread onto the macrocarrier. Samples were placed 9 cm from the stopping screen and bombarded using a model PDS-1000/He particle delivery system (BioRad) at 1100 psi under a vacuum of 28 inches of mercury. Six hours after bombardment, an aqueous solution of 0.1 mM D-luciferin potassium salt (Wako) was sprayed and then bioluminescence was observed under a charge-coupled device (CCD) camera system (LAS-3000: FUJIFILM). For the color-specific detection, we used interference filters for wavelengths greater than 610 nm (high pass filter) and wavelengths between 510 nm and 560 nm (band pass filter). The luminescence levels were quantified using Multi Gauge software (FUJIFILM).

As shown in Figure 1, transient expression of red-emitting and green-emitting luciferases in onion and spinach were distinguishable by using high pass (Figure 1C and H) and band pass (Figure 1D and I) filters. Although spinach leaf tissue contains a green pigment, similar images were obtained, suggesting that the presence of pigment does not influence the assay sensitivity. Because the wavelength of green-emitting luciferases overlaps with the wavelength of 610 nm long pass filter transmission, a low level of light emission derived from CBG99luc and CBG68luc was also observed (Figure 1C and H).

In order to evaluate luciferase activity in cell extracts, we conducted a dual luciferase assay using cultured tobacco (BY-2) and onion epidermal cells, with 35S::R-luc as the internal control (Matsuo et al. 2001). BY-2 cells (1 ml) were spread on 6 cm plastic Petriplate and the excess liquid was removed by pipetting. After the bombardment, 1 ml liquid medium was added to the BY-2 cells. Six hours after bombardment, 800 μl of BY-2 cells in Petriplate were transferred into a 1.5 ml-

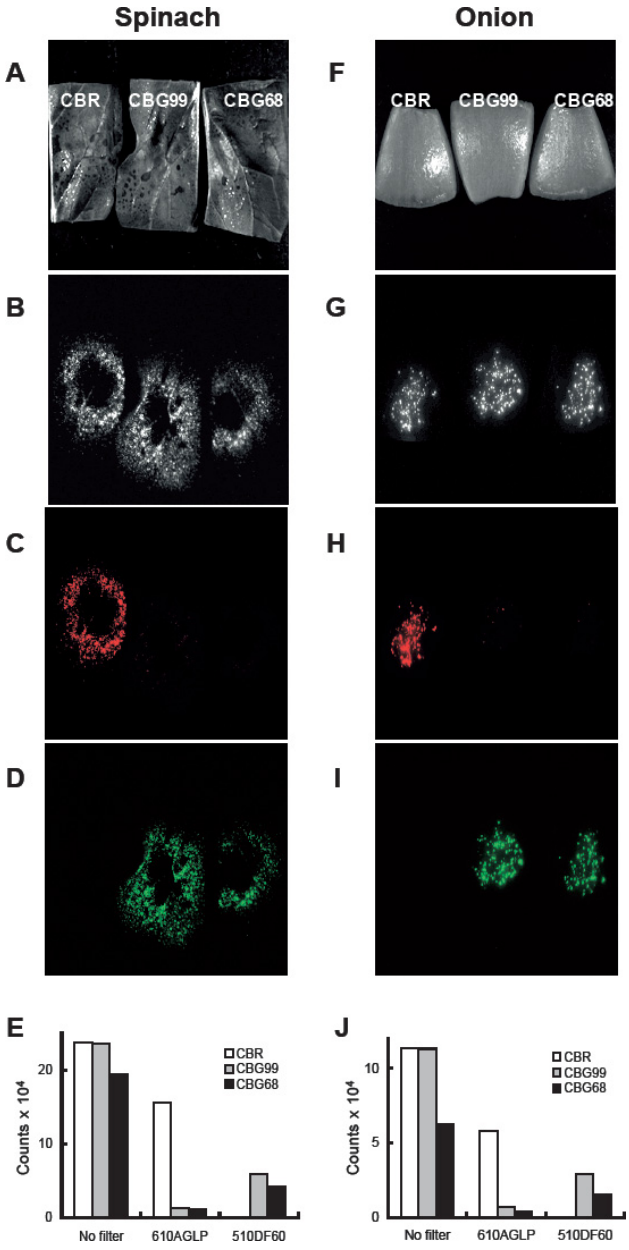

Figure 1. Bioluminescence detection of CB-luc reporters in onion and spinach. Spinach leaf cells (A–E) and onion epidermal cells (F–J) were bombarded with plasmid DNAs expressing CB-luc genes (CBR, CBG99 and CBG68) under control of the CaMV 35S promoter. D-luciferin was sprayed and then the *in vivo* bioluminescence was monitored for 10 min at 26°C using a CCD camera. (A,F) Bright field image. (B,G) Bioluminescence detection (no filter). (C,H) Bioluminescence detection with 610 nm long pass filter (610AGLP). (D,I) Bioluminescence detection with 510–560 nm band pass filter (510DF60). (E,J) Quantification of CB-luc reporter activities by image analysis.

microfuge tube and centrifuged at 2800 *g* for 1 min at 4°C. Then 700 μl of supernatant was removed and cells were dissolved in 100 μl of ice cooled 2× passive lysis buffer (Promega). Cells were homogenized using a hand-held homogenizer (Iuchi) and cleared by centrifugation at 11000 *g*, for 1 min at 4°C. Onion epidermal cells were homogenized in ice-cooled 1× passive lysis buffer using

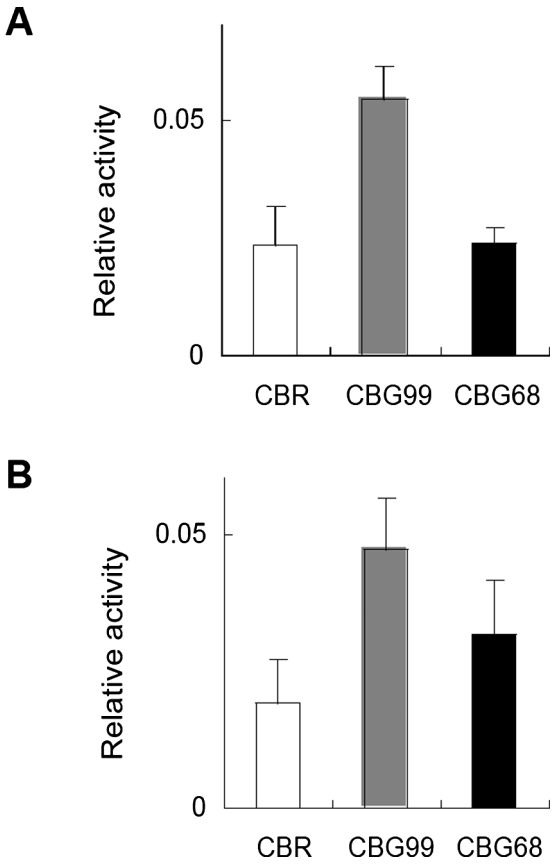

Figure 2. Activities of green and red emitting luciferases in BY-2 and onion epidermal cells. BY-2 (A) and onion epidermal cells (B) were bombarded with equal amount of 35S::CB-luc and 35S::R-luc plasmids. After 6 h of incubation, enzymatic activities were measured by a dual luciferase reporter assay. Each value is the mean±SE for three independent assays.

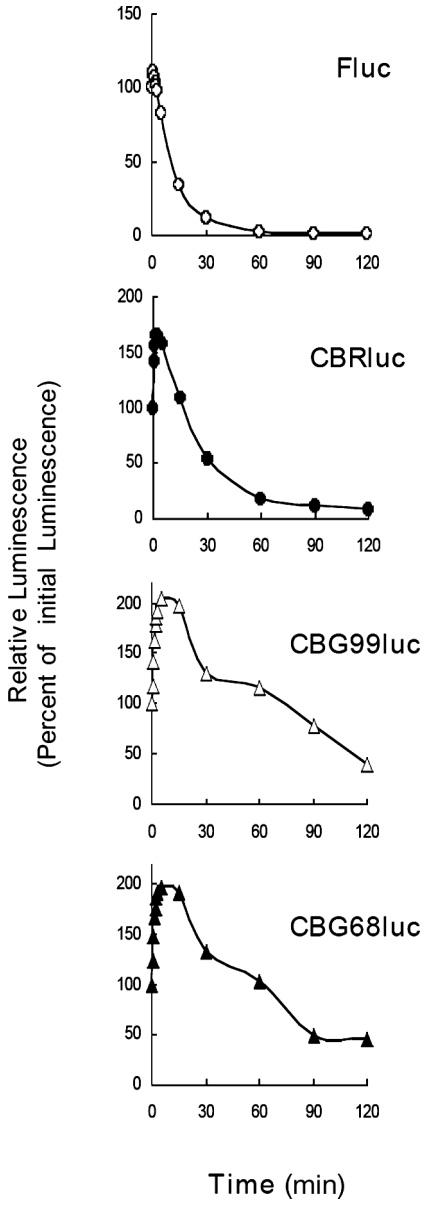

Figure 3. Measurement of luminescent signal kinetics for the red- and green-emitting CB-luc reporters in BY-2 cell extracts. BY-2 cells were bombarded with plasmid DNAs expressing F-luc and CB-luc genes under control of the CaMV 35 S promoter. Samples were lysed and the luminescence initiated with LARII reagent. After addition of LARII reagent, luminescence measured for 10 sec periodically with a luminometer was integrated for 120 min. Relative activities are shown as percent of initial luminescence.

a hand-held homogenizer and cleared by centrifugation at 11000 *g*, for 1 min at 4°C. For detection of luminescence activity, 8 μl of cell lysate was transferred into the luminometer tube (75×12 tube: Nunc) containing 40 μl LARII solution (Promega), mixed by tapping, and then placed in a luminometer (Luminescencer-MCA: Atto). Then, 40 μl of Stop and Glo solution (Promega) was added to the reaction mixture for the detection of R-luc activity.

As shown in Figure 2, among the CB-luc genes the activity of CBG99luc was relatively high in these samples. The activity of CBRluc was significantly lower than that of CBG99luc in both samples, but this observation may be due to the relatively low sensitivity in the red region of the luminometer used in this study.

In order to examine the stability of luciferases in assay solution, we compared the luminescence activities in BY-2 cell lysate. Cell lysate (8 μl) was transferred into the luminometer tube (75×12 tube: Nunc) containing 40 μl LARII solution, and then luminescence levels were measured periodically for 120 min in the luminometer. As shown in Figure 3, the F-luc activity decreased monotonically, but the luminescence levels of CB-luc increased within a few minutes followed by a gradual signal decay. Moreover, CBG99luc and CBG68luc had more stable activity than F-luc and CBRluc under this assay condition. These results suggest that care must be taken when quantifying CB-luc activities in cell lysate.

Next, we investigated the luciferase activity in BY-2 cells. We introduced 35S::CBRluc, 35S::CBG99luc, 35S::CBG68luc and 35S::F-luc plasmids into cells by microprojectile bombardment and monitored their

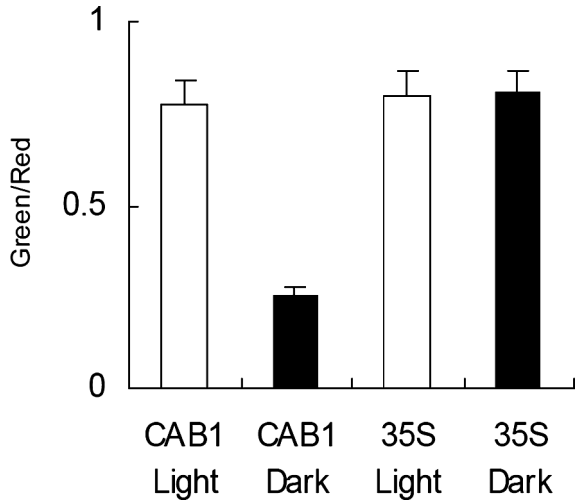

Figure 5. Activities of Arabidopsis *Cab1* and CaMV35S promoters in spinach leaf tissue under different light conditions. Spinach leaves were bombarded with plasmid DNAs harboring *Cab1*::CBG99luc or 35 S::CBG99luc and incubated under continuous white light (50 μmol m^{-2} sec^{-1}) or dark conditions for 21 h at 26°C. 35S::CBRluc was used as a control and the luminescence activities were measured with a CCD camera using interference filters. Quantification of each promoter activity was conducted by image analysis. Each value is the mean±SE for three independent assays.

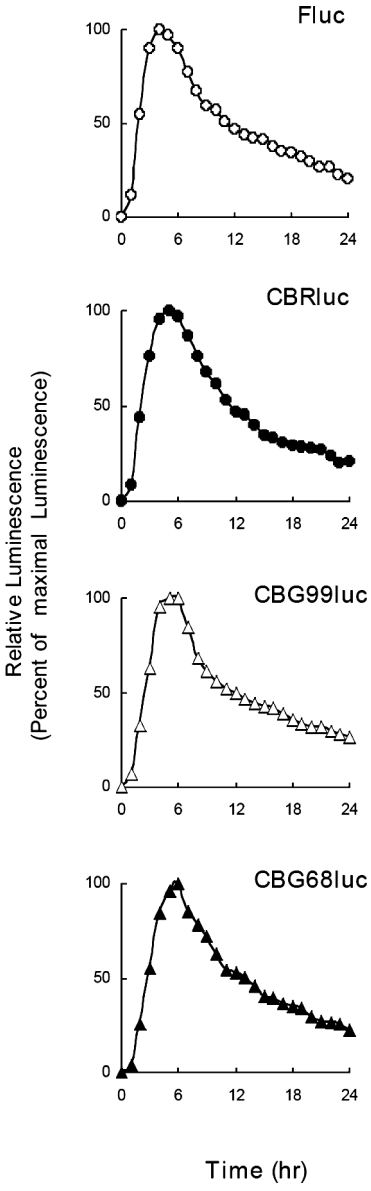

Figure 4. F-luc and CB-luc activities in BY-2 cells with passage of time. BY-2 cells were bombarded with plasmid DNAs expressing F-luc, and CB-luc genes under control of the CaMV 35 S promoter. After D-luciferin application, luminescence for 10 min measured periodically for 24 h with a CCD camera was integrated and the images were processed by Multi Gauge software. Relative activities are shown as percent of maximal luminescence.

activities by *in vivo* luminescence detection using CCD camera. As shown in Figure 4, the four luciferases exhibited similar expression profiles. Maximal luminescence levels were observed between 4 and 6 hour after bombardment, and then decreased gradually over 24 hours. These results suggest that these luciferases are suitable for comparative studies of promoter activity in dual-reporter format *in vivo*.

To confirm the versatility of this assay system, we conducted transient expression experiments using an inducible promoter. Because of its high expression level and light responsiveness, we exploited the *CAB1* gene promoter from *Arabidopsis thaliana* for this purpose (Ha and An 1988; Matsuo et al. 2001). A plasmid construct containing the *Cab1*::CBG99luc gene was introduced into spinach leaves together with 35S::CBRluc by microprojectile bombardment. After 21 h of incubation under different light conditions, bioluminescence was observed using interference filter sets as described above. As shown in Figure 5, the expression level of the Arabidopsis *Cab1* promoter is strongly induced under the continuous light condition. These results suggest that the assay system is able to monitor the regulated expression of an inducible promoter by transient assay in plant cells.

The results of this study indicated that the CB-luc genes encoding green and red luciferases can be used as reporters in the assay of transient expression in higher plant cells. The color separation by the interference filters enables us to monitor dual-luciferase expression levels simultaneously. However, green and red luciferases show relatively low luminescence compared to F-luc and R-luc in plant cells (Figure 2). Because of the differences in stability in assay solution using cell extracts, the assay conditions need to be modified before this CB-luc-based multi-color luciferase reporter system can be applied for detection of luminescence *in vitro* (Figure 3).

On the other hand, this technology is particularly useful for *in vivo* monitoring of the expression of multiple genes by bioluminescence detection. Unlike the

dual-luciferase assay based on the chemiluminescence reaction, which requires unique substrates for differentiation of F-luc and R-luc, the CB-luc assay system requires only beetle luciferin as the substrate for luminescence detection. This advantage provides versatility of the luciferase gene reporter system and enables us to conduct an *in vivo* assay with transiently transformed cells using the dual-reporter format. This multi-color luciferase system is expected to be useful for monitoring gene expression in transgenic plants: Precise gene expression levels can be monitored as activities relative to the internal co-reporter that monitor cell viability as well as basal gene expression levels *in planta*.

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