Short Communication

An In vivo Dual-Reporter System of Cyanobacteria Using Two Railroad-Worm Luciferases with Different Color Emissions

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In vivo genetic reporter systems using luciferase enzymes enable the real-time monitoring of gene expression in living cells. We have challenged concurrent monitoring of two independent promoter activities within the same cells to precisely compare their characteristics in vivo. In this report, we describe a simple dual-reporter system capable of simultaneously monitoring two promoter activities in living cyanobacterial cells. Two railroad-worm luciferases catalyzing the bioluminescent emissions of different colors served as the dual reporters; each emission was successfully separated by interference filters to estimate the individual bioluminescence signals using photomultiplier tubes. Using this system, we clearly demonstrated the difference in the expression profiles between promoters in the same cells.

Keywords: Bioluminescence — Circadian rhythm — Cyanobacterium — Luciferases — Railroad worms — Real-time monitoring.

The real-time monitoring technique is preferable for studies on gene expression requiring successive data collection. Studies of circadian rhythms would benefit greatly from such methods. A reporter system is indispensable for the monitoring of circadian rhythms in cyanobacterial cells because this organism does not exhibit any visible circadian phenomena. A large number of promoters have been monitored using a bacterial luciferase; the promoter activity of many examined genes in Synechococcus elongatus PCC 7942 displayed circadian rhythmicity (Liu et al. 1995). Although detailed expression profiles of each gene can be demonstrated using in vivo single-reporter systems, any comparison of between the promoter characteristics of individual genes is very difficult. Reporter activities are highly dependent upon cellular conditions including concentration of substrates of reporter enzyme reactions, potential of hydrogen, and metabolic activities affecting overall transcription/translation levels. Thus, it would be necessary to equalize global cellular conditions between different reporter lines; however, information about them in living cells is inaccessible. A simple solution is to concurrently compare two reporter-promoter constructs of interest in the same living cells. Since quantification of bioluminescence of luciferases is much better than that of fluorescent proteins in continuous monitoring of reporters (Hakkila et al. 2002), we attempted to develop a bioluminescence dual-reporter system in cyanobacteria. We selected two luciferases derived from railroad worms: PxvGR of Phrixothrix vivianii that catalyzes the emission of green bioluminescence (λmax = 549 nm) and PxhRE of P. hirtus that catalyzes the emission of red bioluminescence (λmax = 622 nm) (Viviani et al. 1999). These two luciferases provide a valuable advantage in that the difference between the wavelengths of their respective emissions is large enough to separate the signals with interference filters. Both enzymes share similar biochemical reaction mechanisms allowing use of the firefly luciferin substrate. Bacterial luciferase from Vibrio harveyi has been used for cyanobacteria; this luciferase (λmax = 490 nm) may serve as the shorter-wavelength reporter (Hastings 1978). The chemistry of light emission, however, differs from that of insect luciferases. It was reported that bacterial and firefly luciferases could report the same promoter activity in a different manner (Min and Golden 2000). Firefly luciferase might be another choice. The emission wavelength, however, is strongly dependent upon pH conditions; the λmax varies from 562 nm to 616 nm according to the pH changes in the assay (Seliger and McElroy 1964). In contrast, the emission wavelengths of both railroad-worm luciferases are stable against pH changes (Viviani et al. 1999).

We have therefore constructed two kinds of promoter-reporter genes using the two distinct railroad-worm luciferases. A construct, NS2trcPxvGR, containing the coding region of the PxvGR gene under the control of the trc promoter, was introduced into Synechococcus elongatus PCC 7942.
An in vivo dual-reporter system of cyanobacteria cells (Fig. 1). We chose the E. coli trc promoter since it was known to perform in cyanobacteria as a strong promoter (Ishiura et al. 1998). The construct integrated into Neutral Site 2 (NS2) in the genome through homologous recombination to create the PtrcGR transformant strain. The “Neutral Site” is a genetic locus demonstrating a tolerance against introduced transgenes without any observable changes in phenotype (Andersson et al. 2000). We then constructed a plasmid vector, NS1kaiPpxhRE, carrying the coding region of the PxhRE gene under control of a kaiBC promoter segment. The kaiB and kaiC genes function centrally in circadian clock oscillations in Synechococcus; both are polycistronically transcribed (Ishiura et al. 1998). This plasmid construct carried sequences targeting another Neutral Site (NS1) in the cyanobacterial genome for integration by homologous recombination. This transformant strain was named PkaiRE. We also established a double transformant strain, PtrcGRPkaiRE, which had been transformed with both NS2trcPxvGR and NS1kaiPpxhRE (Fig. 1A). It should be noted that the difference between NS1 and NS2 had little influence on the expression profiles of transgene in it (data not shown).

To separate the emissions of the PxvGR and PxhRE luciferases, we chose two interference filters based on their emission spectra (Viviani et al. 1999). The apparatus used for the real-time dual-reporter counting system is detailed in Fig. 1B. Every 30 min, a sample dish was successively monitored using two photomultiplier tubes performing photon counting for 30-s periods. The bioluminescence of the living cyanobacterial strains, PtrcGR and PkaiRE, was successively quantified by the two photomultipliers: one using a 540 nm filter (PM540) and one using a 630 nm filter (PM630). Photon numbers are plotted against time to represent the bioluminescence of the PtrcGR strain counted by PM540 (green triangles) or PM630 (red squares) (Fig. 2A). Fluctuation of reporter gene expression was effectively monitored by PM540. PM630 detected much lower bioluminescence levels in the same sample. The correlation of
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photon emissions between PM540 and PM630 is represented in Fig. 2B. The count of PM630 at each time point was about 6% of that of PM540 for PtrcGR. Fig. 2C represents the bioluminescence of the PkaiRE strain counted by PM540 and PM630. In this case, the count of PM540 at each time point was only 1.5% of that of PM630 (Fig. 2D). These results indicated that bioluminescence of the PtrcGR and PkaiRE strains was effectively eliminated by the 630 nm filter and by the 540 nm filter, respectively. A cyanobacterial strain carrying a construct in which PxvGR was driven by the kaiBC promoter showed a lower level of bioluminescence than the PkaiRE strain did, but patterns of bioluminescence kinetics between these strains were similar to each other, suggesting that PxvGR and PkhRE luciferases were likely to show parallel molecular behavior in cyanobacterial cells (data not shown).

We then monitored the bioluminescence of the PtrcGR-PkaiRE strain expressing both PxvGR and PkhRE luciferases. The photon counts emitted by this strain measured using PM540 and PM630 are plotted against time (Fig. 2E). The patterns of PM540 and PM630 readings in this strain look similar to those of the single reporter strains, PtrcGR and PkaiRE, respectively, supporting the idea that the two promoter activities are retained in the dual reporter strain and can be detected without significant crosstalk. As the bioluminescence from PxvGR was picked up by PM630 at a higher rate than that of PkhRE by PM540, we present corrected data (Fig. 2F) calculated from the linear regression in Fig. 2B. Since the raw and corrected data show few differences, correction of bioluminescence data can be omitted for thePtrcGR-PkaiRE strain. The correction of data would be necessary in such cases where a sample shows considerable difference in expression levels between PxvGR and PkhRE luciferases.

Based on these preliminary assays, we tried to display the difference in circadian rhythmicity between the trc and kaiBC promoters. The circadian rhythmicity of the trc promoter activity appears to be unsteady compared to that of the kaiBC promoter in single luciferase reporter assays. Fig. 3 exhibits profiles of bioluminescence from PtrcGR and PkaiRE cells continuously monitored for 5 d under continuous light conditions. While the kaiBC promoter (strain PkaiRE) sustained robust rhythmicity during the experimentation period, the trc promoter (strain PtrcGR) damped the circadian rhythm, exhibiting unclear rhythmicity in 5 d. This phenomenon in PtrcGR may be explained as the result of the difference of promoter activity between trc and kaiBC. It is impossible, however, to rule out the possibility that the introduction of Ptrc-reporter transgene into the genome might make the cellular conditions unstable. Namely, cellular concentrations of components for luciferase enzyme reaction might fluctuate unstably, or the circadian clock system itself might be altered in the PtrcGR.
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The dual reporter system has resolved this issue. Bioluminescence of the PtrcGRPkaiRE strain, monitored using this system (Fig. 3C), demonstrated that the profiles of bioluminescence detected by PM540 (the trc promoter activity) and PM630 (the kaiBC promoter activity) were similar to the patterns observed for the PtrcGR and PkaiRE strains, respectively. Thus it can be concluded that the activity of the kaiBC promoter, but not that of the trc promoter, sustained a robust circadian rhythm for long periods.

This example provides a simple application of the in vivo dual-reporter system to biological studies. The simplicity of both the molecular basis and the measurement device should enable extensive application of this system. Any organisms into which exogenous DNA can be introduced will be possible systems for utilization of this method. This method provides a powerful tool in the analysis of plural promoters. Using a railroad-worm luciferase as an internal control reporter, multiple promoter activities can be evaluated with high reliability in vivo. In vivo estimation of reporter gene activities should be more efficient than in vitro systems as the former does not require the extraction procedures required for the latter. These additional experimental procedures become an obstacle to massive data collection required for time-lapse analyses and the screening of new genes/promoters. The in vivo dual-reporter system we have developed is a simple and promising solution to the problems associated with conventional assays of promoter activity.

For the experiments, S. elongatus PCC 7942 was used as the cyanobacterial strain. Culture and transformation methods have been described previously (Kutsuna et al. 1998). Cells (30–50 cells) were spread on 40 mm polystyrene dishes (Asahi Techno Glass, Tokyo, Japan) of BG-11 agar medium containing 0.5 mM α-luciferin (Firefly, sodium salt; BIOSYNTH AG, SG, Switzerland). Sample dishes were first incubated under constant white light (34 μmol m⁻² s⁻¹) at 30°C for 5 d, then treated with 12-h darkness for entrainment of circadian clock prior to bioluminescence measurements.

Bioluminescence of cyanobacteria was monitored using the modified luminescence dish monitor (LDM) system (Kutsuna et al. 1998). To capture bioluminescence efficiently, two photomultiplier tubes (R329P, Hamamatsu Photonics K.K., Toyooka, Shizuoka, Japan) were used. Interference filters (MY0540 and MY0630, Asahi Spectra, Co. Ltd, Nishinasuno, Tochigi, Japan) were used for isolation of bioluminescence derived from PxvGR and PxhRE luciferases, respectively. Maximum transmittances of these filters were 60% at 540 or 630 nm with a bandwidth (at 50% of the maxima) of 10 nm. In the dual luminescence dish monitor (dLDM) system, each sample dish was subjected to a repeated sequence of the following measurement steps: 30–40 s darkness, 30-s measurement of green bioluminescence, 30–40 s darkness, 30-s measurement of red bioluminescence, and 28-min white light (34 μmol m⁻² s⁻¹). Signals from the photomultiplier tubes were processed by a photon counting unit (C6465, Hamamatsu Photonics K.K., Toyooka, Shizuoka, Japan), then introduced to an electronic pulse counter. Microsoft Excel 2001 was used for statistical analyses.

To make the plasmid construct, NS2trcPxvGR, the coding region of the PxvGR gene was subcloned under the control of the trc promoter into the NcoI site of the pTrc99A vector (Amersham Biosciences, Piscataway, NJ, U.S.A.). A region including the trc promoter (Ptrc), PxvGR, and the rRN B terminator (TrrnB) was amplified by PCR using the following primer set:

trcUBglII: AATGAGATCTGCGCCGACATCATAACGGTTC
trcLBglII: GGCCAGATCTCAGGATGGCCTTCTGC.

The amplicon was digested by a restriction enzyme, BgIII, to make cohesive ends.

pNS2KmT was a pBR322-based vector including the 3-kbp BamHI-digested NS2 region. As a selective marker for cyanobacterial transformation, the kanamycin-resistance gene

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Fig. 3  Circadian behavior distinguishable between the trc promoter and the kaiBC promoter in the same living cells. Bioluminescence of cyanobacterial strains, PtrcGR (A), PkaiRE (B), and PtcGRPkaiRE (C), were monitored for 5 d under constant light conditions. Values of PM540 (green triangles) and PM630 (red squares) emissions are plotted.
was inserted at the BstEII site within the NS2 region. A BamHI site adjacent to the kanamycin resistance gene, inside the NS2 region, was used to introduce the Ptri-PxvGR-TrrnB amplicon.

To make the NS1kaiPxhRE plasmid construct, the coding region of the PxhRE gene under the control of the trc promoter was subcloned into the NcoI site of the Ptrc99A vector. The promoter region of the kaiBC operon from the stop codon of kaiA to the start codon of kaiB was used in this construction. kaiA and kaiB are located in tandem (Ishiura et al. 1998). The trc promoter in Ptrc99A was replaced by the kaiBC promoter (PkaiBC) 41-bp upstream of the NcoI site in Ptrc99A. This 41-bp region includes a Shine-Dalgarno sequence to enhance translation. The pNS1Cm was a pBR322-based vector including the 2.9-kbp BamHI-digested NS1 region. As a selective marker for cyanobacterial transformation, the chloramphenicol resistance gene was inserted at the XhoI site within the NS1 region. An XhoI site adjacent to the chloramphenicol resistance gene, inside the NS1 region, was used to introduce the region including PkaiBC-PxhRE-TrrnB from the above construction.

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