Characterization of Plant Circadian Rhythms by Employing Arabidopsis Cultured Cells with Bioluminescence Reporters

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Recent intensive studies have begun to shed light on the molecular mechanisms underlying the plant circadian clock in Arabidopsis thaliana. During the course of these previous studies, the most powerful technique, elegantly adopted, was a real-time bioluminescence monitoring system of circadian rhythms in intact plants carrying a luciferase (LUC) fusion transgene. We previously demonstrated that Arabidopsis cultured cells also retain an ability to generate circadian rhythms, at least partly. To further improve the cultured cell system for studies on circadian rhythms, here we adopted a bioluminescence monitoring system by establishing the cell lines carrying appropriate reporter genes, namely, CCA1::LUC and APRR1::LUC, with which CCA1 (CIRCADIAN CLOCK-ASSOCIATED1) and APRR1 (or TOC1) (ARABIDOPSIS PSEUDO-RESPONSE REGULATOR1 or TIMING OF CAB EXPRESSION1) are believed to be the components of the central oscillator. We report the results that consistently supported the view that the established cell lines, equipped with such bioluminescence reporters, might provide us with an advantageous means to characterize the plant circadian clock.

Keywords: Arabidopsis — Circadian rhythm — Cultured cells — Luciferase-reporter — Plant clock.

Abbreviations: APRR, ARABIDOPSIS PSEUDO-RESPONSE REGULATORS; CCA1, CIRCADIAN CLOCK-ASSOCIATED1; CCR2, COLD CIRCADIAN RHYTHM RNA BINDING2; CT, circadian time; DD, continuous darkness; D/L, dark/light; LD, 12 light/12 dark; L/D, light/dark; LL, continuous light; LHY, LATE ELONGATED HYPOCOTYL; TOC1, TIMING OF CAB EXPRESSION1; LUC, luciferase gene; PRC, phase response curve.

Introduction

Circadian rhythms are driven by an endogenous biological clock(s) that regulates many biochemical, physiological, and behavioral processes in a wide variety of organisms (for reviews, see Dunlap 1999, Young and Kay 2001). In higher plants too, there are a wide range of biological processes that are controlled through the circadian clock (for a review, see McClung 2000). Indeed, intensive molecular studies have been conducted to understand the mechanisms, by which the model higher plant Arabidopsis thaliana generates clock-controlled circadian rhythms, and also by which the circadian clock controls the fundamental biological processes, such as the photoperiodicity-dependent control of flowering time (for reviews, see Barak et al. 2000, Mouradov et al. 2002, Eriksson and Millar 2003, Yanovsky and Kay 2003). As the result, a number of clock-associated components have been identified (for a recent review, see Eriksson and Millar 2003). They include the best candidates of clock components, CCA1 (CIRCADIAN CLOCK-ASSOCIATED1) (Wang and Tobin 1998, Green and Tobin 1999) and LHY (LATE ELONGATED HYPOCOTYL) (Schaffer et al. 1998). They are homologous Myb-related transcription factors. TOC1 (TIMING OF CAB EXPRESSION1) is also believed to be another component of the central oscillator (Somers et al. 1998). TOC1 is a member of a small family of proteins, designated as ARABIDOPSIS PSEUDO-RESPONSE REGULATORS (APRR) (Matsushika et al. 2000, Strayer et al. 2000). Other members of the APRR1/TOC1 quintet (APRR3, APRR5, APRR7, and APRR9) were also demonstrated to play circadian-associated roles (Makino et al. 2000, Makino et al. 2001, Makino et al. 2002, Matsushika et al. 2000, Matsushika et al. 2002a, Matsushika et al. 2002b, Murakami-Kojima et al. 2002, Sato et al. 2002, Nakamichi et al. 2003, Yamashino et al. 2003, Ito et al. 2003, Yamamoto et al. 2003). A current consistent model as to the Arabidopsis circadian oscillator illustrates that it is comprised of three main factors, CCA1, LHY, and TOC1 (APRR1) (Alabadi et al. 2001, Alabadi et al. 2002, Mizoguchi et al. 2002, Mas et al. 2003, and for a review, see Eriksson and Millar 2003). According to the model, the CCA1/LHY Myb-related transcription factors act redundantly in a manner such that they directly repress the transcription of TOC1 through binding to the promoter of TOC1, and conversely, the transcription of CCA1/LHY is activated by TOC1, directly or indirectly. Consequently, such mutual interactions between CCA1/LHY and TOC1 result in a formation of a transcriptional negative/positive feedback loop (Alabadi et al. 2001), which is reminiscent of the fundamental oscillators proposed for many other organisms (Young and Kay 2001).

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During the course of such plant studies, the most powerful technique, elegantly adopted, was a bioluminescence monitoring system of circadian rhythms. This system employed a transgenic plant carrying a transgene, in which a hallmark circadian-controlled gene was fused to the firefly luciferase gene \((LUC)\), so as to easily monitor a given circadian profile in plants in real-time. Such reporter genes, mainly used, are \(CAB2\) (encoding a light-harvesting chlorophyll-\(a/b\)-binding protein) (Millar et al. 1995) and \(CCR2\) (\(COLD\ \text{CIRCADIAN\ RHYTHM\ RNA\ BINDING}2\)) (Mas et al. 2003). Recently, transgenic plants carrying \(CCA1::LUC\) or \(TOC1::LUC\) have also been employed (Doyle et al. 2002, Alabadi et al. 2001). These studies were conducted exclusively with use of intact transgenic plants.

Although central circadian pacemakers that control animal behaviors are located in the brains of insects and rodents, isolated plant organs and tissues support circadian rhythms, indicating that these explants contain each circadian clock (Sai and Johnson 1999, Thain et al. 2000, Thain et al. 2002). Also, the circadian systems of plant organs and localized areas of tissues appear to be functionally independent on each other (Thain et al. 2002). Considering these, we previously attempted to employ an established \(Arabidopsis\) cultured cell line (named T87) (Axelos et al. 1992), in order to monitor such a cell autonomous circadian rhythm, with the assumption that such cultured cells would provide us with an alternative and advantageous means to characterize the plant biological clock at the molecular level. Indeed, another group has also successfully employed another \(Arabidopsis\) cell line to characterize the properties of the circadian-associated factor named \(ZTL/ADO1\) (\(ZEITLUPE/ADAGIO1\)) (Kim et al. 2003). These studies suggested that certain \(Arabidopsis\) cultured cells might retain an ability to generate circadian rhythms, at least partly. In this study, we further attempted to adopt a bioluminescence monitoring system by establishing transgenic cell lines carrying appropriate reporter genes, namely, \(CCA1::LUC\) and \(APPR1::LUC\). Here we report the results that consistently suggested that these cell lines, equipped with bioluminescence reporters, might provide us with an advantageous means to characterize the plant biological clock.

## Results

### Transgenic cell lines carrying bioluminescence reporter genes

A set of 5'-upstream DNA sequences with varied lengths of the \(CCA1\) gene were fused to a modified firefly luciferase gene (named \(LUC^+\), hereafter simply referred to as \(LUC\) for clarity of this text) on a versatile \(Agrobacterium\) vector. Three types of \(CCA1::LUC\) fusions were constructed here (they were designated a, b, and c, respectively) (Fig. 1A). One construct
(labeled a) is a \textit{CCA1}-promoter::\textit{LUC} fusion, whereas others (labeled b and c, respectively) contain portions of the \textit{CCA1}-coding-region fused in frame to \textit{LUC} (i.e., protein fusion). According to an \textit{Agrobacterium}-mediated DNA delivery method, we isolated several independent transgenic cell lines, carrying each corresponding \textit{CCA1}::\textit{LUC} transgene, by scoring the intensities of bioluminescence for hygromycin-resistant transformants (see Materials and Methods). These transformants were purified and established by successively cultivating on CIM agar-plates containing hygromycin (Fig. 1B, upper panel on the right-hand side). It should be noted that, for each construct, we established three independent lines (a-L1, a-L2, and a-L3, so forth), each of which was assumed to carry a given \textit{CCA1}::\textit{LUC} transgene at a chromosomal region different from each other. In the following experiments, these independent \textit{CCA1}::\textit{LUC} transgenic cell lines were characterized simultaneously in order to obtain reliable and reproducible results.

With these cell lines, a few blocks of callus-like cells were put into a glass vial containing fresh CIM agar-medium, followed by incubation for further 3 d (Fig. 1B, upper panel on the left hand side). Luciferin was added by spraying it into the vials, and then they were placed in a bioluminescence detection system (Kondo et al. 1993), which was conditioned in the 12 light/12 dark (LD) cycle at the constant temperature of 22°C (see Fig. 1B).

When the intensities of bioluminescence were monitored in LD for the cells carrying \textit{CCA1}::\textit{LUC}, they showed a robust rhythm with a period of approximately 24 h (Fig. 1B, lower panel). Although the maximum intensities were varied among these constructs, in every case the bioluminescence profiles were well synchronized with peaks at dawn every day. This is consistent with the fact that \textit{CCA1} is a typical example of circadian-controlled ‘morning gene’ in plants. The presented results were obtained using the transgenic lines, a-L1, b-L1, and c-L1, respectively. However, it should be noted that the consistent and reproducible results were also obtained for other independent transgenic lines (a-L2 and -L3, b-L2 and -L3, and c-L2 and -L-3) (data not shown). It should also be emphasized that both the promoter (construct-a) and protein (construct-b) fusions showed essentially the same diurnally oscillated profile. This suggested that the promoter region of \textit{CCA1} is sufficient to generate the oscillation, and also that the 5’-promixal half of the \textit{CCA1} coding region in construct-c is not implicated in the oscillation.

\textit{Cultured cells show diurnal rhythms with reference to both \textit{CCA1}::\textit{LUC} and \textit{APRR1}::\textit{LUC}}

With regard to the observed diurnal rhythm of \textit{CCA1}::\textit{LUC} (c-L1) in T87 cells (Fig. 1B), we reproducibly confirmed the result with another independent cell line (c-L2), as mentioned above (Fig. 2A, red line). In this confirmatory experiment, we further prepared RNA from the cells (they were separately cultured under essentially the same conditions), in order to directly detect the transcript that was derived from the endogenous \textit{CCA1} gene, by Northern blot hybridization with a specific probe (Fig. 2A, blue columns). The diurnally oscillated profile of bioluminescence was well coincident with that of the intrinsic transcript of \textit{CCA1}. This result promoted us to construct an alternative cell line, by employing another hall-marked clock gene, \textit{APRR1} (or \textit{TOC1}). In this case also, three independent \textit{APRR1}::\textit{LUC} transgenic lines (labeled L1, L2, and L3) were established (see Fig. 1A). The established cell lines

\begin{figure}
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\caption{Cultured cells show diurnal rhythms with reference to both \textit{CCA1}::\textit{LUC} and \textit{APRR1}::\textit{LUC}. (A) Transgenic cells, carrying \textit{CCA1}::\textit{LUC} (construct-c-L2, see Fig. 1A) were subjected to the bioluminescence assays, as exactly described in Fig. 1. The cells were entrained in the light/dark (L/D) cycle, as schematically illustrated. RNA samples were also isolated from the cells (separately cultured under essentially the same conditions), in order to directly detect the transcripts that was derived form the endogenous \textit{CCA1} gene, by Northern blot hybridization with a specific probe (see blue columns). In these experiments, \textit{UBQ10} was also analyzed as an internal and loading reference. The hybridized bands were detected with a phosphoimage analyzer (BAS-2500, Fujifilm, Japan). Then, the measured intensities of each band were normalized (by dividing with the \textit{UBQ10}-value). Based on these values, the relative amounts of mRNA of \textit{CCA1} (or transcript) were calculated and expressed as arbitrarily units, in which the maximum level was taken arbitrarily as 10. (B) Transgenic cells, carrying \textit{APRR1}::\textit{LUC} (construct-L1), were also subjected to both the bioluminescence and Northern blot hybridization assays, exactly as described above for \textit{CCA1}::\textit{LUC}. In these experiments, data were collected for three independent transgenic lines (L1, L2, and L3). Essentially the same bioluminescence profiles were obtained, thus representative results are shown (i.e., construct-L1).}
\end{figure}
carrying APRR1::LUC were also analyzed under the same conditions as those described above (Fig. 2B, for L1). The bioluminescence intensities of APRR1::LUC also showed a diurnally oscillated profile with a period of about 24 h. Notably, it peaked at evening, the phase of which was quite different from that of CCA1::LUC. The consistent and reproducible results were obtained from other transgenic lines (for L2 and L3) (data not shown). This is well consistent with the fact that APRR1 is a typical example of circadian-controlled ‘evening gene’ in plants. Such a rhythm of APRR1::LUC was also coincident with that of the transcript from the endogenous APRR1 gene (Fig. 2B, red columns). From these results, we concluded that the established transgenic cell lines show robust and diurnally oscillated rhythms with reference to both CCA1::LUC and APRR1::LUC.

Rhythmic waves of the APRR1/TOC1 quintet genes

To further confirm the significance of the diurnal rhythms observed for CCA1::LUC and APRR1::LUC in T87 cells, we further constructed transgenic lines carrying APRR9::LUC, APRR7::LUC, APRR5::LUC, and APRR3::LUC, respectively (two independent lines, L1 and L2, for each). As demonstrated previously (Matsushika et al. 2000), these APRR1/TOC1 quintet genes in plants are all subjected to circadian rhythms, in such a manner that each APRR transcript starts accumulating after dawn sequentially in LD in the order of APRR9→APRR7→APRR5→APRR3→APRR1/TOC1. To see if such circadian-related events were also seen in cultured cells, the expressions of APRR9::LUC, APRR7::LUC, APRR5::LUC, and APRR3::LUC were monitored in LD (Fig. 3), as exactly described for APRR1::LUC (see Fig. 2). In every case including repeatedly monitored APRR1::LUC (Fig. 3, lowermost panel), all the APRR::LUC constructs showed diurnal rhythms in each characteristic manner (Fig. 3). When compared the overall profiles with each other, it was clear that the bioluminescence of each APRR::LUC fluctuated after dawn rhythmically and sequentially in the order of APRR9→APRR7→APRR5→APRR3→APRR1/TOC1 (Fig. 3). For these examinations, we used given APRR::LUC transgenic lines (namely, L1 for each). When we repeated the experiments with other independent transgenic lines (L2 for each), essentially the same events were observed (data not shown). The rhythmic profiles observed for the set of APRR::LUC fusions in cultured cells were reminiscent of the circadian waves of the APRR1/TOC1 quintet observed previously in plants (Matsushika et al. 2000). These results further supported the significance of the diurnally oscillated rhythms that could be observed in T87 cells.

Fig. 3 Cultured cells show diurnal rhythms with reference to the APRR::LUC quintet members. As indicated, transgenic cells carrying either APRR9::LUC, APRR7::LUC, APRR5::LUC, APRR3::LUC, or APRR1::LUC were subjected to the bioluminescence assays, exactly as described in Fig. 1. In these experiments, data were collected for two independent transgenic lines (L1 and L2) of APRR9::LUC, APRR7::LUC, APRR5::LUC, APRR3::LUC. Essentially the same bioluminescence profiles were obtained, thus representative results are shown (i.e., construct-L1 for each). Note also that the reproducibility was evident, when one compared the results between APRR1::LUC(L1) in Fig. 2B and APRR1::LUC (L1) in Fig. 3.
Both CCA1::LUC and APRR1::LUC show free-running rhythms in continuous light and dark. (A) Growth curves of T87 cells, grown in JPL liquid medium in continuous light (LL) and darkness (DD), are shown. The growth conditions are given in detail in Materials and Methods. (B and C) The transgenic cell lines, carrying either CCA1::LUC or APRR1::LUC, were subjected to essentially the same procedures as those described in Fig. 1 and 2. After being entrained for 3 d in LD, the cells were released into either continuous light (panel B, LL) or darkness (panel C, DD), as schematically indicated. The bioluminescence assays were carried out. In these experiments, data were independently collected for three transgenic lines (L1, L2, and L3) for each. Essentially the same bioluminescence profiles were obtained, thus the representative results are shown (i.e., construct-L1 for each).

Both CCA1::LUC and APRR1::LUC show free-running rhythms in continuous light and dark.

Next, a critical viewpoint as to the circadian rhythm is that even when plants were deprived of environmental time cues (e.g., light/dark cycle and/or temperature cycle), the circadian rhythm must persist with a period of near 24 h, often for many days. We next addressed this critical issue (i.e., free-running rhythm), with reference to the transgenic cell lines each carrying CCA1::LUC, APRR1::LUC. The cells were treated with essentially the same procedures as those described in Fig. 1. And then, after entraining in LD for 3 d, the cells were released into either continuous light (LL, 50 μmol s⁻¹ m⁻²) or darkness.
(DD) (Fig. 4). In these experiments, it should be first noted that T87 cells are chemomixotrophic. In other words, they derive their carbon source mainly from sucrose in the medium, and perhaps partly from photosynthetic fixation of CO₂. Thus, the important fact was that the suspension-cultured cells in the liquid medium could grow well under both the LL and DD conditions, as indeed demonstrated in Fig. 3A. This made it possible to monitor the cells in both LL and DD for several days, because we did not need to fear about severe defect of growth in DD. Such free-running rhythms were monitored for CCA1::LUC (c-L1) and APRRI::LUC (L1), and the representative results are shown in Fig. 4B (for LL) and Fig. 4C (for DD).

The oscillated profiles of CCA1::LUC and APRRI::LUC in LD were fully sustained at least for several days, even after released into either LL or DD. Under both the conditions, CCA1::LUC showed a free-running rhythm with a peak at subjective morning, whereas APRRI::LUC display a free-running rhythm with a peak at subjective evening. These results were reproducibly confirmed using other transgenic cell lines (c-L2 and L3 for CCA1::LUC, L2 and L3 for APRRI::LUC) (data not shown). The period of the free-running CCA1::LUC rhythm was estimated to be 24.9±0.5 h (n = 8) in LL, and 27.1±1.1 h (n = 8) in DD, the values of which were considerably longer than that (ca. 24 h) of the entrained CCA1::LUC rhythm (see Fig. 2). The same was essentially true for APRRI::LUC. In any case, it was possible to monitor a free-running rhythm for both CCA1::LUC and APRRI::LUC in T87 cells, under the conditions without environmental time cues.

For the above experiment with CCA1::LUC, we employed construct-c (i.e., a protein fusion). To verify the critical event of free-running rhythm, we further employed other construct-a and -b (see Fig. 1A). Their free-running rhythms were monitored in both LL and DD, and we observed free-running rhythms for both the CCA1::LUC constructs (construct-a-L1 and construct-b-L1), and both in LL and DD (Fig. 5). The same results were obtained when other independent transgenic lines were examined (i.e., construct-a-L2 and L3, and construct-b-L2 and L3) (data not shown). It should thus be emphasized that both the promoter (construct-a) and protein (construct-b) fusions showed essentially the same free-running circadian profiles (compared between Fig. 5A and 5C in LL, and also between Fig. 5B and D in DD), which were also very similar to that observed for construct-c (see Fig. 4). As emphasized earlier, these results suggested again that the promoter region of CCA1 is sufficient to generate the free-running rhythm, and also that the 5'-prominal half of the CCA1 coding region is not implicated in this respect.

A single light/dark or dark/light cue is sufficient to generate a robust free-running rhythm

With regard to the experiments above, we first entrained the cells in the LD cycle for several days, in order to generate the synchronized and robust rhythm of CCA1::LUC. However, we also observed the following intriguing events (Fig. 6).

When the cells were kept in either LL or DD for a long time (>6 d), the rhythms of CCA1::LUC dampened. The monitored intensities of bioluminescence were reduced to their trough-levels (e.g., see the profile during –48 h to 0 h in DD of Fig. 6A), essentially the same event was seen (period = 25.0±0.6 h, n = 8), and it persisted for several days without any further light/dark (L/D) signal (Fig. 6A). Essentially the same event was seen (period = 26.5±0.7 h, n = 8), when the cells grown in LL was released to DD (i.e., LL→DD

Fig. 6 A single light/dark or dark/light cue is sufficient to generate a robust free-running rhythm. (A, upper panel) Transgenic cells carrying CCA1::LUC in vials (see Fig. 1B) were kept in continuous darkness (DD) for 7 d, and then they were released to continuous light (LL). (A, lower panel) Conversely, the transgenic cells, kept in continuous light (LL), were transferred in the dark (DD). During the time course, they were subjected to bioluminescence assays, as schematically illustrated. (B) The results of both the above experiments were superimposed to each other. It should be noted that the time was set as 0 (or subjective dawn), when light was turned on, whereas the time was set as 12 (or subjective dusk), when light was turned off.
events were observed, when the cells with

stratified in Fig. 6B. It should be noted that essentially the same
circadian phase, as schematically demon-

strated in Fig. 6B. It should be noted that essentially the same events were observed, when the cells with APRR7::LUC and APRR5::LUC were examined (data not shown). It was thus suggested that a single external L/D cue is sufficient to generate a robust free-running rhythm in cultured cells, and that a single dark/light (D/L) cue is also equally sufficient to result in so. We were little surprised to observe these events, because essentially the same circadian-associated events have already been well documented for intact plants (for a review, see Bunning 1967, and references therein). Indeed, we also confirmed such events for intact plants by characterizing the circadian rhythms of the APRR1/TOC1 quintet genes with Northern blot analyses (data not shown). Rather, we would like to emphasize that a sustainable and robust free-running rhythm could be generated in cultured cells in DD by applying only a single L/D cue. This is intriguing, because under such conditions in DD in intact plants, we often experienced that the rhythms of CAB2::LUC and CCA1::LUC were very rapidly dampened (except for the CCR2::LUC rhythm).

Temperature compensation

It is claimed that the period of a circadian rhythm remains relatively constant over the range of physiologically relevant temperatures. This event is referred to as ‘temperature compensation’, which means that the circadian clock maintains its pace over a range of temperatures (Kondo et al. 1993, Johnson 1999). The CCA1::LUC cells were entrained in the condition of 12 h darkness at varied temperatures (18°C, 22°C, and 26°C). They were then released into LL, in order to monitor the free-running rhythms at each different temperature. The periods of such free-running rhythms were statistically estimated (Fig. 7). The measured period of the rhythm at 26°C was considerably shorter than that observed at 18°C. However, a large difference of temperatures (26°C vs. 18°C) resulted in a relatively narrow difference of periods, suggesting that the properties of rhythms generated in cultured cells appear to fulfill the diagnostic criteria of a circadian rhythm, namely, ‘temperature compensation’ (Fig. 7).

Phase response curve

As emphasized above, since the putative clock(s) in cul-
tured cells continued to run in DD for several days, it was pos-
sible to examine the so-called phase response curve (PRC) (for a review, see Johnson 1999). A pulse of light administered prior to subjective dawn advances the phase of the clock, whereas the identical pulse applied after dusk delays the phase of the clock. In Arabidopsis plants, PRCs for pulses of red and blue light in DD were recently examined (Covington et al. 2001). Finally, such classical PRC analyses should provide us with an insight into the nature of putative clock in cultured cells, in terms of its light responsiveness (or resetting by light).

The free-running rhythms in DD were first generated in cultured cells by the LL→DD cue, as exactly described above. Beginning after one full day in DD, each vial containing cells was exposed to white light pulses (5 min, 5 μmol s⁻¹ m⁻²) at appropriate timing. The rhythms of CCA1::LUC were further monitored for both the light-exposed and reference cells. Such representative raw data are shown in Fig. 8A. In one case (upper panel), the light pulse resulted in an advanced phase of rhythm in the light-treated cells, whereas in another case (middle panel), the light pulse showed little effect on the phase of rhythm. As the last case (lower panel), the light pulse resulted in a delayed phase of rhythm. The periods and phases of these observed rhythms after treated with the light pulses were estimated, and then they were converted to circadian time (CT) (see Materials and Methods). Degrees of such observed phase shifts were plotted against CT, when the cells were exposed to the light pulses. This gave us a figure of the PRC (Fig. 8B), from which the following views emerged. The cells exhibited little resetting in the subjective morning, when the light pulses resulted in small phase advances/delays. Later in the subjective evening, the light stimuli elicited phase delays. A phase shift was observed in the subjective night, when phase delays became phase advances, but this phase shift was not so large (±4.5 h) in the context of CT. This figure of PRC in the sub-
saturating light pulse (5 min, 5 μmol s⁻¹ m⁻²) was similar to that of the so-called type-1 PRC in general (Johnson 1999). When we examined PRC with a saturating light pulse (30 min 25 μmol s⁻¹ m⁻²), a much larger phase shift (±9 h) was ob-
served during the subjective night. The obtained figure of PRC in the saturating light pulse was somewhat similar to that of the so-called type-0 PRC (Johnson 1999), but it was less characteristic as compared with a typical type-0 PRC (data was not shown). Therefore, PRC analyses with cultured cells remain more extensively performed with various light intensities. However, the results of our preliminary PRC analyses are compatible with the idea that the free-running robust rhythms in cultured cells were somehow generated by a light-controlled clock-like function.

**Discussion**

In this study, we attempted to show that the bioluminescence assay system could be adopted to characterize the circadian rhythms in Arabidopsis cultured cells (named T87) (Fig. 1), based on the facts that: (i) both CCA1::LUC and APRR1::LUC showed diurnal oscillations in cells entrained in LD, and such observed rhythms peaked at each characteristic phase (subjected dawn and dusk) (Fig. 2), as in intact plants; (ii) the significance of the above observation was further confirmed by analyzing other distinct types of APRR::LUC in T87 cells (i.e., APRR9::LUC, APRR7::LUC, APRR5::LUC, APRR3::LUC), with which we observed the circadian waves of the APRR1/TOC1 quintet (Fig. 3); (iii) they also showed robust free-running rhythms in both LL and DD (Fig. 4, 5); (iv) a single D/L (alternatively, D/L) cue was sufficient to generate sustainable and robust free-running rhythms (Fig. 6); (v) in these respects, it should be noted that both the CCA1-promoter and CCA1-protein fusions showed the consistent properties, as compared to each other (Fig. 5). Thus, the rhythm monitored in cultured cells appears to reflect a transcriptional event; (vi) the periods of resulting rhythms remained relatively constant over the range of temperatures (Fig. 7); (vii) classical PRC analyses could be carried out, at least partly (Fig. 8). Therefore, the properties of rhythms in cultured cells, monitored with several LUC reporters, consistently fulfilled the diagnostic criteria of the circadian rhythm, namely, ‘free-running’, ‘entrainment’, and ‘temperature compensation’. Nevertheless, we do not know whether the circadian rhythms observed through monitoring bioluminescence reporter genes in cultured cells precisely (or completely) reflect the clock function(s) in intact plants.

**Fig. 8** Phase response curve (PRC). (A) The free-running rhythms in DD were first generated in cultured CCA1::LUC cells by the LL→DD cue, as exactly described in Fig. 4. Beginning after one full day in DD, each vial containing cells was exposed to white light pulses (5 min, 5 μmol s⁻¹ m⁻²) at appropriate timings (as indicated). The rhythms of CCA1::LUC were further monitored for both the light-exposed (red lines) and reference (blue lines) cells. Such three representative data are shown (upper panel, middle panel, and lower panel, respectively). The free-running rhythm of CCA1::LUC in DD showed a period of 26.4±0.3 h (independently examined samples = 14). The light pulses might reset the subjective day/night cycle, the event of which might result in either phase advances or phase delays, as also illustrated. (B) Thus, the periods and phases of these observed rhythms after treated with the light pulses were estimated, and then they were converted to circadian time (CT). Degrees of such observed phase shifts were plotted against CT, when the cells were exposed to the light pulses. In detail, the abscissa is the CT of the light pulse served, from CT0 to CT24. CT0 is defined as the beginning of the subject day, and CT12 is defined as the beginning of subject night. The ordinate is the magnitude of phase shift expressed in CT. Advances are plotted as positive value, delays are plotted as negative. To convert ‘real’ hour to CT hour, the number of real time hours is multiplied by 24, and divided by the free running period (see above). If the value of CT were over 24, 24 should be subtracted from it, because the CT between CT0 and CT24 is the duration of the endogenous free running period. To create PRC, at least two peaks (i.e., window of 60 h) were detected after being exposed to the light pulse, as indeed seen in this figure. This protocol gave us the figure of the PRC, as illustrated in panel B.
Recently, transgenic plants carrying a CCA1::LUC transgene have been characterized with a bioluminescence monitoring system (Doyle et al. 2002). The observed free-running rhythms for CCA1::LUC in plants in LL and DD are considerably similar to those observed for CCA1::LUC in cultured cells in this study (e.g., both in phase and amplitude). However, the rhythms in DD in cultured cells were more robustly sustained, as compared with those observed in plants. Transgenic plants carrying a TOC1::LUC transgene were also characterized to some extent (Alabadi et al. 2001). The observed free-running rhythms for TOC1::LUC in LL in plants are also similar to those observed for TOC1::LUC in cultured cells in this study (e.g., both in phase and amplitude). Through Northern blot hybridization analyses, it was previously reported that the transcript of TOC1 showed a rhythm for more than two cycles in DD (Strayer et al. 2000). In any event, it should be more extensively examined whether or not T87 cells have a circadian clock comparable to that of intact plants. Clarification of this issue must await further examination. However, our results of this study are compatible with the idea that T87 cells retain a clock-like function, at least in part.

In our previous study on rhythms in T87 cells (Nakamichi et al. 2003), we failed to observe free-running rhythms of APRR1::TOC1 and CCA1 in LL, which we analyzed by Northern blot hybridization. In this study, we observed rhythms in LL with reference to the same circadian-controlled genes. The reason for this discrepancy is not clear. In the previous study, however, we cultured cells on agar-plates for more than 2 weeks in order to harvest an amount of cells large enough for RNA preparation. In this study, we could characterize rhythms of fresh cells cultured for just several days on agar-plates, by means of a very sensitive bioluminescence assay system (see Fig. 1A). Therefore, the growth-phase might be a critical parameter to observe a reliable and reproducible rhythm in T87 cells, of which we were indeed more cautious in this study. In any case, we must be very cautious of culture conditions (e.g., growth-phase) to monitor circadian rhythms in T87 cells.

As emphasized above, it is not certain whether the circadian rhythms in T87 cells perfectly reflect the clock function(s) in intact plants. However, no matter what molecular mechanisms underlie in the cell autonomous clock-like events, the results of this study suggested that the cultured cell system might provide us with an alternative and advantageous means to characterize the plant biological clock at the molecular level, as has been suggested previously by us (Nakamichi et al. 2003), and also by others (Kim et al. 2003). Keeping the limitations in mind, and taking the advantages of cultured cells with the bioluminescence monitoring system, one can take various biochemical and pharmacological approaches to circadian physiology; some of which might otherwise be difficult to perform with use of intact plants. The results from such approaches should shed light on the mechanisms underlying the clock function in higher plants, as has been successfully demonstrated for the circadian clock of the unicellular cyanobacterium (Kondo et al. 1993). It may also be noted that rat cultured cells with a promoter-LUC construct were recently employed to characterize the expression of a given mammalian circadian-controlled gene (Ueda et al. 2002).

Materials and Methods

Growth conditions of cells

Axelos et al. (1992) have previously established a cell line (named T87) from the A. thaliana ecotype Columbia. T87 cells were provided from The RIKEN Plant Cell Bank (http://www.ptc.riken.go.jp/pcb/indexPCB.html) (RIKEN, Yokohama, Japan). They were usually grown in a liquid medium (named JPL) or a solid medium (named CIM) under continuous white light (50 µmol m⁻² s⁻¹) at 22°C. When cells were grown in the liquid medium, they were incubated in 100 ml of JPL medium containing 1.5% sucrose and 1-naphthalenacetic acid (1 µM NAA) in 500 ml flask, by shaking on a rotary shaker (120 rpm). The contents of JPL medium are exactly the same as those described by Axelos et al. (1992). Occasionally (e.g., every 10 d), an aliquot (10 ml) of well-grown liquid culture was filtered through an autoclaved nylon mesh (0.5 mm in diameter), and then the harvested cells were inoculated again into fresh JPL medium. Under these standard growth conditions, cells in JPL medium grew well in both continuous light and darkness (see Fig. 4A). By this procedure, suspension-cultured cells are stably maintained in our laboratory. When cells were grown on the solid medium, an aliquot of vigorously growing cells in JPL medium was spread on CIM agar (0.6%) medium containing 3.0% sucrose and 1 µM NAA. The contents of CIM medium are: 3.3 g liter⁻¹ of Gamborg’s B5 medium salt mixture (Wako Co. Ltd., Osaka, Japan) and 0.5 g liter⁻¹ of 2-morpholinoethanesulfonic acid monohydrate (MES). Cells grew well on this solid medium (see Fig. 1B). Alternatively and occasionally (e.g., every 10 d), several blocks of well-grown calli on the solid medium were directly transferred onto fresh CIM agar-medium. By this procedure, solid-cultured cells are stably maintained in our laboratory.

Construction of CCA1::LUC and APRR1::LUC

A set of 5'-upstream DNA sequences encompassing the CCA1 gene was amplified by polymerase chain reaction (PCR) with the following sets of primers (see Fig. 1A): construct-a (5'-CTGAAGCTTCTAAGCaTGGTTATAGCTTAGAC-3') and 5'-GGTTCTAGTGGTTATAGCTTAGAC-3' of CCA1::LUC. A 5'-upstream sequence encompassing the APRR1 promoter was also amplified by PCR with the following primers (see Fig. 1A): 5'-CTGAAAGCTTCTGACATGTTATAGCTTAGAC-3' and 5'-GGTTCTAGTGGTTATAGCTTAGAC-3' of APRR1::LUC. The template used was the total genomic DNA from plants. These isolated DNA fragments were fused to the modified fire fly luc+ gene on pSP-luc+ (Promega, Madison, WI, U.S.A.) at the HindIII and NcoI sites. Note that we newly created a SacI site at the 5'-proxial end of luc+ on these recombinant plasmids. The resulting CCA1::LUC and APRR1::LUC constructs were cloned onto the HindIII and SacI sites of an Agrobacterium binary vector (named pA-BH). For other APRR1::LUC constructs, details will be described elsewhere. T87 cells were transformed according to an Agrobacterium-mediated DNA delivery method. We isolated several independent transgenic cell lines carrying each corresponding promoter::LUC transgene, as follows. First, T87 cells were grown in JPL medium, and then an aliquot (5 ml, 30 mg ml⁻¹ of wet cells) was mixed with the Agrobacterium cells (1 mg of wet cells) harboring the binary vectors
containing each corresponding promoter::LUC constructs. After incubation for 48 h, T87 cells were extensively washed with JPL medium containing cefotaxime (200 µg ml⁻¹), and then they were further incubated for 48 h in JPL medium with cefotaxime. The vigorously growing cells were spread on non-charged Hybond-NX filters (Amersham Pharmacia, Buckinghamshire, U.K.), which were placed on CIM agar-plates with cefotaxime. They were grown in the absence of hygromycin B for 48 h. The resulting filters were lifted and transferred onto fresh CIM agar-plates with cefotaxime and hygromycin B (10 µg ml⁻¹). After incubation for about 2 weeks, hygromycin-resistant cells appeared on the plates. To establish transgenic cell lines, the resulting hygromycin-resistant callus-like cells were further selected by successively transferring onto fresh CIM agar-plates with cefotaxime and hygromycin B at 10-day intervals. Accordingly, we established several independent transgenic cell lines each carrying an appropriate transgene that was characterized in this study (i.e., CCA1::LUC and APRR1::LUC, see Fig. 1A).

RNA preparation and Northern blot hybridization

From T87 cells, RNA samples were prepared by essentially the same procedures according to the conventional ATA method, as described previously (Nakamichi et al. 2003). They were subjected to Northern blot hybridization, as also described previously. Briefly, RNA was separated in agarose gels (1%) containing 0.67 M formaldehyde, then transferred to Hybond-N+ membranes (Amersham Pharmacia). The fixed membranes were hybridized with 32P-labeled DNA fragments in 6× standard saline phosphate and EDTA (1× SSPE = 0.18 M NaCl, 10 mM phosphate buffer, 1 mM EDTA, pH 7.4), 5× Denhardt’s solution, and 0.5% SDS containing 10% dextran sulfate and 100 µg ml⁻¹ salmon sperm DNA, at 65°C for 18 h. The membranes were washed once with 2× SSPE and 0.5% SDS for 15 min at room temperature, once with 2× SSPE and 0.5% SDS for 30 min at 65°C, and then with 0.2× SSPE and 0.5% SDS for 15 min at 65°C. The washed membranes were exposed and analyzed on a phosphoimager analyzer (BAS-2500II) (FujiXerox, Tokyo, Japan).

Bioluminescence assays

T87 transgenic cells were grown on CIM agar-plates in continuous light (LL), (50 µmol m⁻² s⁻¹), as described above. A few blocks of vigorously growing callus-like cells were put into a glass vial containing CIM agar medium (see Fig. 1B). They were usually incubated for 3 d in LL at 22°C. If necessary, however, they were incubated for 7 d in LL, or 7 d in the dark (DD) (e.g., see Fig. 8). The cells in the vials were sprayed with a fine mist containing luciferin (5 mM) dissolved in water containing 0.01% Triton X-100, and then they were further incubated for 24 h. Bioluminescence assays were carried out with the monitoring system that has been developed previously by Kondo et al. (1993). By this system, the intensities of bioluminescence were detected for each vial by a photomultiplier detector-tube (Hamamatsu 931B) (Hamamatsu Photonics, Hamamatsu, Japan). The photomultiplier detector was enclosed in a light-tight box. A microcomputer controlled the box containing photomultiplier detector to move along a guide rail to measure bioluminescence sequentially from in-line-placed vials every 30 min. So, the cells in a vial was exposed to dark period for 5 min every 30 min (2 min of darkness to allow chlorophyll fluorescence, then two 1.5 min measurements of bioluminescence). However, this brief dark-exposure could not have major effect of the circadian rhythms in intact plants (Millar et al. 1995), and in cultured cells (our unpublished data). The methods of the output from photomultiplier detector to microcomputer, and analysis software were also as described by Kondo et al. (1993). This software contains an equation to compute a period of peaks in a given profile (Kondo, unpublished method). To estimate the period of rhythms, the data for three cycles from at least three independent experiments were adopted. To create PRC, at least two peaks (i.e., window of 60 h) were detected after being exposed to the light pulse (as indeed seen in Fig. 8). The bioluminescence monitoring system was in a light-controlled and temperature-controlled chamber to maintain constant condition of light and temperature. It may be also noted that another luminometer (ARGUS-50, Hamamatsu Photonics, Hamamatsu, Japan) was occasionally used to conveniently detect the intensities of bioluminescence of T87 cells, grown on CIM agar-plates.

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References


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