A small family of genes, named ARABIDOPSIS PSEUDO RESPONSE REGULATOR (APRR), are intriguing with special reference to circadian rhythms in plants, based on the fact that one of the members (APRR1) is identical to TOC1 (TIMING OF CAB EXPRESSION 1) that is believed to encode a clock component. In Arabidopsis plants, each transcript of the APRR1/TOC1 quintet genes starts accumulating after dawn rhythmically and one after another at intervals in the order of APRR9 → APRR7 → APRR5 → APRR3 → APRR1/TOC1. To characterize such intriguing circadian-associated events, we employed an established Arabidopsis cell line (named T87). When T87 cells were grown in an appropriate light and dark cycle, cell autonomous diurnal oscillations of the APRR1/TOC1 quintet genes were observed at the level of transcription, as seen in intact plants. After transfer to the conditions without any environmental time cues, particularly in constant darkness, we further showed that free-running circadian rhythms persisted in the cultured cells, not only for the APRR1/TOC1 quintet genes, but also other typical circadian-controlled genes including CCA1 (CIRCADIAN CLOCK ASSOCIATED 1), LHY (LATE ELONGATED HYPOCOTYL) and CCR2 (COLD CIRCADIAN RHYTHM RNA BINDING 2). To our knowledge, this is the first indication of cell autonomous circadian rhythms in cultured cells in Arabidopsis thaliana, which will provide us with an alternative and advantageous means to characterize the plant biological clock.

Keywords: Arabidopsis thaliana — Circadian rhythms — Cultured cells.

Abbreviations: APRR, ARABIDOPSIS PSEUDO RESPONSE REGULATOR; TOC1, TIMING OF CAB EXPRESSION 1; CCA1, CIRCADIAN CLOCK ASSOCIATED 1; CCR2, COLD CIRCADIAN RHYTHM RNA BINDING 2; DD, continuous darkness; LD, 12 h light/12 h dark; LL, continuous white light; LHY, LATE ELONGATED HYPOCOTYL.

In general, circadian rhythms are driven by an endogenous biological clock(s) that regulates many biochemical, physiological and behavioral processes in a wide variety of organisms (Dunlap 1999). In higher plants too, there are a wide range of biological processes that are controlled through such a circadian clock. They include movement of organs such as leaves and petals, opening of stomata, daily fluctuations of metabolic activities such as respiration, photosynthesis, and gene expression (Harmer et al. 2000, Schaffer et al. 2001). Thus, clarification of the biological clock (central oscillator) is a paradigm of current plant molecular biology (Barak et al. 2000, Murtas and Millar 2000, McClung 2000, Samach and Coupland 2000, Putterill 2001, Somers 2001, Carre 2001, Mouradov et al. 2002, and references therein). With regard to the Arabidopsis circadian clock, several genes were proposed to encode potential components. Both the CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) genes are implicated in a part of a feedback loop that is closely associated with the circadian clock (Wang and Tobin 1998, Schaffer et al. 1998, Green and Tobin 1999). These homologous transcription factors with a single Myb-domain are the best candidates for such clock components. Another gene, TIMING OF CAB EXPRESSION 1 (TOC1), is also believed to encode a component of the central oscillator (Somers et al. 1998). Furthermore, reciprocal and intimate interactions were observed between the functions of CCA1/LHY and TOC1 (Alabadi et al. 2001, Alabadi et al. 2002, Makino et al. 2002, Matsushika et al. 2002a, Mizoguchi et al. 2002).

We have been extensively characterizing a novel family of proteins, termed Arabidopsis pseudo response regulators (APRRs), with special reference to circadian rhythms (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), based on the fact that one of them (APRR1) is identical to TOC1 (Matsushika et al. 2000, Strayer et al. 2000). More interestingly, each transcript of APRRs starts accumulating after dawn rhythmically and one after another at intervals in the order of APRR9 → APRR7 → APRR5 → APRR3 → APRR1/TOC1. This event was referred to as “circadian waves of the APRR1/TOC1 quintet”. It was thus assumed that not only APRR1/TOC1 itself, but also other APRR1/TOC1 quintet members might be implicated as part of an Arabidopsis circadian clock. Indeed, several lines of solid evidence have already

1 Corresponding author: E-mail, tmizuno@agr.nagoya-u.ac.jp; Fax, +81-52-789-4091.
been provided to support the notion that APRR9 and APRR5 (together with APRR1/TOC1) must also be taken into consideration for a better understanding of the molecular links between circadian rhythms, control of flowering time through the photoperiodic long-day pathway, and also light signaling-controlled plant development (Matsushika et al. 2002b, Sato et al. 2002).

In our previous studies, we mainly adopted Northern blot hybridization analyses in order to examine the expression profiles of certain circadian-regulated genes, including the APRR1/TOC1 quintet genes, for which mRNA was prepared exclusively from intact plants. Alternatively, in this study we employed an Arabidopsis cultured cell line in the hope of gaining further insight into the functions of the APRR1/TOC1 quintet. Axelos et al. (1992) have previously established a cell line (named T87) from the ecotype Columbia plant. With this cell line, we showed that cell autonomous circadian rhythms were observed, not only for the APRR1/TOC1 quintet genes, but also for CCA1 and LHY, under the conditions without any environmental time cues (particularly, in constant dark).

T87 cells were grown in liquid JPL medium (100 ml) with 500 ml flask, under continuous light at 22°C (Fig. 1A, right panel, note that cells are chemomixotrophic, i.e. they derive their carbon source partially from photosynthetic fixation of CO2 and partially from sucrose in the medium). Aliquots were spread on Gamborg’s B5 agar-plates, and then they were incubated (or entrained) under the 12 h light/12 h dark (LD) cycle for 18 d (Fig. 1A, left panel). The well-grown cells were harvested at intervals (3 h), and RNA fractions were prepared. We first examined whether or not each expression of the APRR1/TOC1 quintet genes shows a diurnal oscillation in the cells cultured under the LD cycle (i.e. in entraining LD). Northern blot hybridization analyses were carried out with probes specific for each of APRR9, APRR7, APRR5, APRR3, APRR1/TOC1 (Fig. 1B). The hybridized bands were also quantified (Fig. 1C), based on the UBQ10-value as a loading and internal reference (Fig. 1E). In the cultured cells, the APRR9 transcript was rapidly induced immediately after dawn, whereas that of APRR1 started accumulating upon the onset of evening. It was clearly seen that these APRR1/TOC1 quintet genes were expressed in a sequential and rhythmic manner in the characteristic order of APRR9 → APRR7 → APRR5 → APRR3 → APRR1/TOC1.
The circadian rhythms in *Arabidopsis* are entrained under the 12 h light/12 h dark cycle for 18 d at 22°C. T87 cells were first grown with liquid JPL medium. Aliquots of cells were spread on Gamborg's B5 agar-plates, and then they were incubated (or entrained) under the 12 h light/12 h dark cycle for 18 d at 22°C. Details were the same as those given in the legend to Fig. 1. Plates were transferred to constant white light (LL), as schematically shown (see the horizontal bars). Cells were harvested at intervals (3 h), and RNA fractions were prepared. (A) Northern blot hybridization analyses were carried out with probes each specific for the APRR1/TOC1 quintet genes. (B) The detected bands were also quantified. Other details were essentially the same as those given in the legend to Fig. 1.

These rhythmic profiles with an approximately 24 h period were essentially the same as those previously observed in intact plants (Matsushika et al. 2000). In other words, this is indeed the event that has been referred to as “circadian waves of the APRR1/TOC1 quintet”. It should be also noted that the same circadian events were seen, even when the suspension-cultured cells in the liquid medium (instead of the solid-cultured cells on the agar medium) were directly analyzed in entraining LD conditions. As mentioned above, several genes have been proposed to encode proteins that function within, or close to, the central oscillator. Among them, *CCA1* and *LHY* are particularly intriguing, because these homologous gene products are the best candidates for central oscillator components, as mentioned above. It is thus of interest to examine whether or not the *CCA1* and *LHY* transcripts also show oscillated expression profiles in T87 cells in entraining LD. The results showed that both *CCA1* and *LHY* exhibited a diurnal and robust oscillation with a peak at dawn, respectively (Fig. 1D, upper two panels). We then examined more examples of circadian-regulated genes. In plants, a well-known hallmark of circadian-regulated events is a rhythmic expression of *CAB2* (encoding light-harvesting chlorophyll-a/b-binding protein) (Millar et al. 1995). The *CAB2* gene with its peak at morning is the most downstream target of a circadian output pathway. Another hallmark of clock-regulated transcriptional events is a rhythmic expression of the [*COLD CIRCADIAN RHYTHM RNA BINDING 2*](https://academic.oup.com/pcp/article-abstract/44/3/360/1864726) (*CCR2*) gene (also known as *AtGRP7*), the peak of which is seen at evening in plants (Carpenter et al. 1994, Heintzen et al. 1997). These well-known circadian-controlled genes were also examined in T87 cells (Fig. 1D, lower two panels). They each showed a diurnal oscillation with a peak at the anticipated timing, respectively, as can be seen in intact plants. Taken together, as far as the entraining LD conditions are concerned, T87 cells have the ability to generate diurnal rhythms to oscillate the transcription of certain genes, such as *APRR1*/*TOC1*, *CCA1*, *LHY*, *CAB2* and *CCR2*, all of which are known to be circadian-controlled ones in intact plants.

The most critical view as to the circadian rhythm in plants is that when plants are deprived of environmental time cues (e.g. light/dark cycle and temperature cycle) and placed in constant (“free-running”) environmental conditions, circadian rhythms must persist with a period of around 24 h, often for many days. We next needed to address this critical issue. T87 cells were grown under the entraining LD conditions as described above, and then they were transferred to continuous white light (LL conditions). For the *APRR1*/*TOC1* quintet genes, no free-running rhythm was observed in T87 cells (Fig. 2). In other words, after transfer to LL, their rhythms were dampened very rapidly. The *APRR9* transcript no longer appeared, while others were expressed more or less constitutively. These events in T87 cells were quite different from those seen in intact plants.

We then adopted constant dark (DD) conditions, under which the light signal was completely deprived. Provided that T87 cells were transferred to DD, free-running rhythms were seen, to some extent, with regard to the *APRR1*/*TOC1* quintet genes (Fig. 3). In contrast to LL, the sustained *APRR9* peak appeared at the anticipated phase (i.e. subjected dawn) on the first day in DD, and it persisted even on the second day (Fig. 3A, B). A similar event was also seen for the *APRR7* transcript. Including *APRR5*, *APRR3* and *APRR1* together, the free-running circadian waves were seen in DD, at least for the first day (Fig. 3A, B). To evaluate these events, the circadian waves of the *APRR1*/*TOC1* quintet in intact plants were examined in DD, as critical references. Wild-type plants (Columbia) were grown for 22 d under the 12 h light/12 h dark cycle, and then they were transferred to constant dark. RNA samples were prepared from leaves at appropriate intervals (3 h), and then Northern blot hybridization analyses were carried out with regard to the *APRR1*/*TOC1* quintet genes (Fig. 3C). The profiles observed for the *APRR1*/*TOC1* quintet genes in plants in DD were considerably similar to those observed for T87 cells in DD (compare panels B and C). For instance, the quantified profiles of the free-running *APRR7* and *APRR3* rhythms in T87...
cells in DD were well superimposed with those seen in plants (Fig. 3D, red lines for cells, and blue lines for plants). These results suggested that the circadian-regulated free-running waves of the APRR1/TOC1 quintet genes persist in DD, at least partly, in T87 cultured cells, in a similar manner as those observed in intact plants.

To further verify the above notion, the transcripts of CCA1, LHY and CCR2 were also examined in T87 cells, grown in either LL or DD (Fig. 4). As in the case of the APRR1/TOC1 quintet genes, the free-running rhythms of CCA1, LHY and CCR2 did not persist in LL (see each upper panel in A, B and C). In contrast, their rhythms were clearly sustained at least for 2 d in DD (see each middle panel in A, B and C). These circadian profiles in T87 cells were compared with those in intact plants in DD (see each pair of lower panels in A, B and C). The free-running rhythms of CCA1, observed for both cells and plants, were very similar to each other. The first robust peak of CCA1 appeared at the anticipated timing (subjected morning), which was followed by the less robust second peak with an approximate 24 h period. Indeed, the quantified profile of the CCA1 rhythm in T87 cells in DD was well superimposed to that in plants (Fig. 4D, red lines for cells, and blue lines for plants). Essentially the same event was observed for another circadian-associated gene, LHY. The same was true for another hallmarked gene, CCR2, although the free-running rhythm of CCR2 showed a different phase (i.e. with peak at subjective evening), as compared with CCA1 and LHY (with peak at subjective morning). Note also that these circadian events, observed here for plants, were well consistent with those reported previously for the CCA1, LHY and CCR2 rhythms in DD (Wang and Tobin 1998, Park et al. 1999).

Within the last 5 years, intensive molecular studies have been conducted to understand the mechanisms by which the model Arabidopsis plant generates free-running circadian rhythms (Barak et al. 2000, Murtas and Millar 2000, McClung 2000, Samach and Coupland 2000, Putterill 2001, Somers 2001, Carre 2001, Mouradov et al. 2002, and references therein). These studies were conducted exclusively with use of intact plants or detached organs. In this study, we employed an established Arabidopsis cultured cell line (T87) (Axelos et al. 1992), with which Yuasa et al. (2001) recently succeeded in demonstrating that oxidative stress activates an Arabidopsis homologue of mitogen-activated protein kinase in the cultured cells. To our knowledge, we succeeded for the first time in demonstrating that this established cell line T87 also retains the ability to generate circadian rhythms, at least in part. T87 cells entrained in LD generated robust and diurnal oscillations with regard to certain circadian-regulated genes, such as the APRR1/TOC1 quintet genes, the CCA1 and LHY clock-component genes, and the typical CAB2 and CCR2 output genes (Fig. 1),
as do the intact plants. More importantly, such cell autonomous rhythms persist, even without environmental time cues (i.e. in DD), as can be seen in the intact plants (Fig. 3, 4). Unfortunately, such free-running rhythms were not observed in LL. The reason for this is not clear. In any event, as far as both the LD and DD conditions were concerned, T87 cells will provide us with alternative and advantageous means to characterize the Arabidopsis biological clock at the molecular level. For instance, light-pulse phase response curves in circadian rhythms could be easily examined, and pharmacological approaches to circadian physiology could also easily be taken.

As has been extensively debated, central circadian pacemakers that control animal behaviors are located in the brains of insects and rodents. Nevertheless, the location of such a central pacemaker has not been determined in plants. Rather, isolated plant organs and tissues support circadian rhythms, indicating that these explants each contain a circadian clock (Sai and Johnson 1999, Thain et al. 2000, Thain et al. 2002). It has been also proposed that the circadian systems of organs and localized areas of tissues in plants appear to be functionally independent on each other (Thain et al. 2002). In this context, our findings of cell autonomous circadian rhythms in cultured cells are consistent with the above view, and also will shed more light on such longstanding debates.

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