Short Communication

Light Response of the Circadian Waves of the APRR1/TOC1 Quintet: When Does the Quintet Start Singing Rhythmically in Arabidopsis?

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We previously identified a novel class of proteins, named Arabidopsis pseudo-response regulators (APRRs), each of which (APRR1/TOC1, APRR3, APRR5, APRR7, APRR9) has an intriguing structural design containing an N-terminal pseudo-receiver domain and a C-terminal CONSTANS motif. Expression of these APRR1/TOC1 family members is under the control of a coordinate transcription, termed ‘circadian waves of APRR1/TOC1 quintet’, may be a basis of a presumed Arabidopsis biological clock (named ‘bar code clock’) [Matsushika et al. (2000) Plant and Cell Physiol. 41: 1002]. Here we further characterized the event of circadian waves, by demonstrating that certain light stimuli are crucial determinants to induce the robust circadian waves, and accordingly, the first-boosted and light-induced APRR9 appears to be primarily responsible for this light response of the circadian waves. Also, as such a light stimulus, a red light pulse that is presumably perceived by phytochromes appears to be sufficient to induce (or synchronize) the APRR1/TOC1 circadian waves.

Key words:

Abbreviations: APRR, Arabidopsis pseudo-response regulator; TOC, timing of CAB.

Circadian rhythms are an old issue of plant physiology (Garner and Allard 1920), and a newly emerging paradigm of plant molecular biology (Anderson and Kay 1996, Thomas and Vinc-Pru 1997, Kreps and Kay 1997, Koornneef et al. 1998, Pichulla 1999, Somers 1999, Staiger and Heinzen 1999, Murtas and Miller 2000, Samach and Coupland 2000, and references therein). According to the current views, many biological rhythms such as those corresponding to photosynthetic activity, leaf movement, stomatal aperture, and flowering time, are regulated by the presumed plant circadian clocks. Such biological clocks presumably consist of input pathways, a central oscillator, and output pathways. The central oscillator is thought to autonomously generate a rhythm through a transcription/translation positive/negative feedback loop. In this respect, results from plant molecular biology have begun to shed light on the molecular bases of circadian rhythms in higher plants (see the reviews listed above). For example, a light-driven entrainment of the Arabidopsis circadian clock has been assumed to be mediated by phytochrome A (PhyA), phytochrome B (PhyB), and cryptochromes (Cry1 and Cry2), thus affirming the roles of these photoreceptors as input regulators to the circadian clock, although an objection was claimed recently (Yanovsky et al. 2000). The Early Flowering 3 (ELF3) gene has also been proposed to act in an input pathway (Hicks et al. 1996, Zagotta et al. 1996). With regard to the central oscillator, several genes were recently proposed to encode potential clock-related components. Both the MYB-related 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 et al. 1997, Wang and Tobin 1998, Schaffer et al. 1998). Recently, two LOV/PAS-containing ZEITLUPE (ZTL) and FKF1 (flavin-binding, kelch repeat, F box) genes were also reported to encode clock-associated proteins (Somers et al. 2000, Nelson et al. 2000). The Timing of CAB 1 (TOC1) gene is also intriguing, because a semi-dominant (toc1-1) mutant has shortened periods of several rhythmic markers including the CAB gene, and also of the leaf movement and stomatal conductance rhythms (Millar et al. 1995, Somers et al. 1998). Since this single toc1-1 mutation interferes with a wide range of clock-controlled output processes, TOC1 most likely functions upstream or within the oscillator itself. These recent results, mainly from the forward-genetics of Arabidopsis thaliana, have provided us with clues for understanding the plant circadian clock. Nevertheless, clarification of the underlying molecular mechanism is at a very early stage.

Our recent results from reverse-genomics provided new insight into the Arabidopsis circadian clock (Matsushika et al.

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We previously identified a novel class of proteins, named pseudo-response regulators (APRRs), which might be implicated in the His-to-Asp phosphorelay signal transduction (Imamura et al. 1999, Makino et al. 2000). Each of *Arabidopsis* pseudo-response regulators, APRRs (APRR1, APRR3, APRR5, APRR7, APRR9), has an intriguing structural design containing not only an N-terminal pseudo-receiver domain, but also a common C-terminal CONSTANS motif that is shared by the flowering-time CO protein (Putterill et al. 1995, Robert et al. 1998). Interestingly, it was recently reported that APRR1 is identical to TOC1 (Strayer et al. 2000). More importantly, all these members of the APRR1/TOC1 family are subjected to a coordinate circadian rhythm at the level of transcription, as mentioned above (see Abstract) (Matsushika et al. 2000). Based on these data, we previously proposed that the sequential and rhythmic events of APRR-transcription, termed ‘circadian waves of APRR1/TOC1 quintet’, may be a basis of a biological clock (named ‘bar code clock’) (Matsushika et al. 2000).

To clarify the issue addressed here, first of all, we would like to consider the principle of the circadian waves (Fig. 1), which implies the following views.

(i) Each mRNA of APRRs starts accumulating after dawn sequentially at approximately 2 h intervals in the order of APRR9→APRR7→APRR5→APRR3→APRR1.

(ii) First of all, the wave of APRR9-mRNA is always boosted immediately after dawn, irrespective of the entrained photo-period conditions.

(iii) The order and intervals of such sequential expression is little affected by the entrained photo-period conditions, if any.

(iv) Except for that of APRR1/TOC1, the shape of each peak is apparently sharp, and does not change significantly, if any, regardless of the photo-period conditions.

(v) In this respect, APRR1/TOC1-mRNA is somewhat unique in that its expression (or accumulation) is prolonged for a while longer, as compared with in the case of others. In other words, the peak of APRR1/TOC1 does not drop sharply, rather it has an evident shoulder. As a result, although both APRR3 mRNA and APRR1/TOC1 mRNA seem to start accumulating concomitantly, APRR1/TOC1 reaches its plateau a few hours later than APRR3.

(vi) Consistent with the fact that the circadian wave of APRR9 is first boosted always after dawn, its expression of mRNA is rapidly and transiently induced, when dark-grown etiolated seedlings were exposed to white light. The question here is when and how does such an event of the circadian waves of the APRR1/TOC1 quintet start robustly.

To this end, extensive northern hybridization analyses were carried out for APRR mRNAs, as follows (Fig. 2). We sowed seeds of *A. thaliana* (Columbia ecotype) on appropriate

Fig. 1 Schematic representation of the circadian waves that is exhibited by the five members of the APRR1/TOC1 family of pseudo-response regulators. To gain insight into the circadian rhythms, exhibited by the five members of the APRR1/TOC1 family, the experimental data of northern hybridization were quantified and schematically illustrated (Matsushika et al. 2000). To clarify the profiles, the maximum level (or amplitude) of each transcript was taken approximately and arbitrarily.

Fig. 5 A schematic view that provides insights into a putative biological clock of Arabidopsis, which is based on the circadian waves of the APRR1/TOC1 quintet. Details are given in the text.
agar-plates (Makino et al. 2000), and they were then kept at 4°C for 2 d under dark (i.e. imbibition and vernalization). After plants were exposed to white light for 3 h in order to enhance germination, they were kept at 22°C under dark conditions to have etiolated seedlings. At 6th day, the etiolated plants were harvested at every 3 h intervals to isolate total RNA. At 7th day, we turned on white light, and then plants were grown under the conditions of 12 h light/12 h dark. During the

Fig. 2 Northern hybridization analyses for the expression of APRRs with the etiolated seedlings and its light response. The etiolated seedlings were grown as schematically indicated at the lower part. (A) At the time indicated, they were exposed to white light (12 h light/12 h dark). At indicated intervals, plants were harvested to prepare total RNA fractions (Makino et al. 2000). (B) Alternatively, they were exposed to continuous white light. To examine the fluctuation of expression of the APRR1/TOC1 family of pseudo-response regulators, northern hybridization analyses were carried out with each denoted probe specific (APRR1/TOC1, APRR3, APRR5, APRR7, and APRR9, respectively) (Matsushika et al. 2000). In order to quantify each transcript, the ubiquitin (UBQ10) transcript was used as an internal and loading reference (in panel A, note that the amounts of RNA loaded were very small, for example, in the lanes denoted by asterisks). To clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, approximately and arbitrarily. In panel B, the quantified data for APRR3 and APRR7 are not shown, for clarity (see the raw data in the upper part).
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following 2 d, total RNA was further prepared at 3 h intervals (Fig. 2). The results of northern hybridization analyses carried out with each specific probe denoted (APTT1/TOC1, APRR9, CCA1, and UBQ10). The specific probe for CCA1 was according to the previous report (Wang et al. 1997), namely, 5'-GGCCTAAGCCTAAACAAACATCC and 5'-GGGATTCTTCTGTTCCAGAAAGG. Both the autoradiogram (A) and quantified data (B) are shown. In panel B, to clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, arbitrarily.

Fig. 3 Northern hybridization analyses for the APRRs in the etiolated plants treated with red light. Plants were grown under dark conditions for 6 d (see Fig. 2), the etiolated seedlings were exposed to red light for 2 min, and then they were further incubated for the indicated times under the dark conditions. Total RNA samples were prepared at intervals, as indicated. With these RNA samples, northern hybridization analyses were carried out with each specific probe denoted (APTT1/TOC1, APRR9, CCA1, and UBQ10). The specific probe for CCA1 was according to the previous report (Wang et al. 1997), namely, 5'-GGCCTAAGCCTAAACAAACATCC and 5'-GGGATTCTTCTGTTCCAGAAAGG. Both the autoradiogram (A) and quantified data (B) are shown. In panel B, to clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, arbitrarily.

observed. This profile is essentially the same as that observed previously for the light/dark entrained plants, grown as long as for 21 d (Matsushika et al. 2000).

The above result prompted us to examine such a light responsive profile of the APRR1/TOC1 quintet under more critical photo-conditions. Namely, the dark-grown etiolated plants were transferred under the growth conditions of constant light, and then extensive northern hybridization analyses were done for all of the APRR1/TOC1 quintet members, including APRR3, APRR5, and APRR9 (Fig. 2B). When plants were exposed to white light, in the following 3 d, free-running and robust circadian waves of the APRR1/TOC1 quintet became markedly evident. It should be also noted that the period (or interval) of the circadian waves was just about 24 h, although the examined plants had never been entrained by the light/dark conditions of 24 h photo-period. It was thus demonstrated that a light exposure appears to be a crucial determinant to induce the free-running and robust APRR1/TOC1 circadian waves.

In above experiments, white light was used with an intensity of approximately 120 μE m\(^{-2}\) s\(^{-1}\). In higher plants, the circadian clock is assumed to be entrained and reset at least partly by red and far-red light, which is perceived by phytochromes. We then tested whether such particular light treatments could also exaggerate the profile of the APRR1/TOC1 circadian waves, particularly, the expression of APRR9 mRNA. To this end, etiolated plants were treated with red light for 2 min, and they were then grown under the dark conditions. The results of northern hybridization analyses show that such a brief red light illumination (2-min pulse, 660 nm, 30.5 μmol of photon m\(^{-2}\) s\(^{-1}\)) was sufficient to induce a transient increase of APRR9 mRNA (note also that the induction occurs very rapidly within 30 min) (Fig. 3). APRR9 mRNA peaked at about 1 h, and then declined by 4 h to a basal level. Consistent with the results with the white light treatment of Fig. 2, an enhanced expression of APRR1/TOC1 mRNA was also observed after a while (after 8 h). Such a profile of the red light response of APRR9 is very similar to that reported previously for CCA1 by Wang and Tobin (1998), as indeed demonstrated here as a reference (Fig. 3). In other experiments, the red pulse (2 min) was followed by a far-red light treatment (5 min, 750 nm, 36.5 μmol of photon m\(^{-2}\) s\(^{-1}\)), or the plants were treated by far-red light alone (5 min). After these treatments, they were grown under the dark conditions for 45 min and 120 min respectively. The results showed that the level of such a transient induction of APRR9 mRNA by the red pulse was reduced (repressed) significantly, when the red pulse was followed by the far-red treatment (Fig. 4). Also, the induction of APRR9 mRNA by a far-red light alone was less evident, as compared with in the case of the red light treatment. These results are best interpreted by assuming that phytochromes play a critical role at least partly for the light induction of APRR9 mRNA, and consequently, the circadian waves.

We previously observed an intriguing event, termed circa-
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Fig. 4 Northern hybridization analyses for the APRRs in the etiolated plants treated under various light conditions. Plants were grown under dark conditions for 6 d (see Fig. 2), and then, the etiolated seedling was exposed to the light conditions, as specified (i.e. red pulse (2 min), red pulse (2 min) were followed by far-red pulse (5 min), far-red pulse alone (5 min)). After these treatments, plants were put back to under the growth conditions of dark. After 45 min and 120 min total RNA samples were prepared, respectively. With these RNA samples, northern hybridization analyses were carried out, with each specific probe denoted (APTT1/TOC1, APRR9, and UBQ10). For APRR9, both the autoradiogram and quantified data are shown (panel B). In panel B, to clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, arbitrarily. Note that the conditions of such light treatments are essentially the same as those described previously (Inaba et al. 1999). In details, for red-pulse irradiation, monochromatic light with a peak emission at 660 nm was supplied at an intensity of 30.5 μmol m−2 s−1 for 2 min. The intensity was measured by a quantum sensor (model LI-190SA, LI-COR, Lincoln, NE). For far-red-pulse irradiation, monochromatic light with a peak emission at 750 nm was supplied at an intensity of 36.5 μmol m−2 s−1 for 5 min. This intensity was measured by thermopiles (model MIR-100Q, Mitsubishi Oil Chemicals, Tokyo, Japan). A pair of modified slide projectors (Cabin II) each equipped with a 300-W halogen lamp (Phillips, Eindhoven, The Netherlands) was used as a source. The light was filtered through a combination of a red-interference filter (maximum wavelength =660 nm, DIF-BPF-2, Vacuum Optics, Tokyo, Japan) and a heat-cut filter (CF-B) to obtain red light and through a far-red interference filter (maximum wavelength =750 nm, DIF-BPF-2) and a long-wavelength heat-cut filter (CF-A) to obtain far-red light.

induce the free-running and robust circadian waves (Fig. 2), and accordingly, the light-induced APRR9 appears to be a crucial determinant for the subsequent event. Also, as such a light stimulus, a red light pulse that is presumably perceived by phytochromes appears to be sufficient to induce (or synchronize) the APRR1/TOC1 circadian waves (Fig. 3, 4). These findings are intriguing in that a presumed circadian clock must adjust itself so as to synchronize to solar and seasonal day/night cycles. We know nothing about the molecular mechanisms by which the APRR1/TOC1 quintet makes a rhythm. However, our findings, together with those of Kay’s group, led us to envisage a naive view, as illustrated in Fig. 5 (see page 335). Based on the general concept as to the biological oscillation, one can assume that the APRR1/TOC1 family members together generate an autonomous rhythm through a transcription/translational positive/negative feedback loop, and serve as an oscillator. In this respect, it was found here that the expression of APRR9 is modulated by certain light stimuli at its transcriptional level. This event appears to be important to link the presumed intrinsic oscillation of the APRR1/TOC1 circadian waves to the entrained external photo-period. In this process, phytochromes play a role, at least partly, as red light was shown to be an effective stimulus for the APRR9 induction. It is thus tempting to speculate that phytochromes (perhaps A and B) exert their roles in concert with PHYTOCHROME INTERACTING FACTOR 3 (PIF3) for the light-dependent induction of APRR9 mRNA. PIF3 is a MYC-related basic helix-loop-helix DNA-binding protein, and it can recognize the G-box-like sequence (CACGTG) (Martinez-Garcia et al. 2000). This transcription factor together with phytochromes at least partly responsible for a light-responsive induction of CCA1 (Wang et al. 1997, Martinez-Garcia et al. 2000). Interestingly, the APRR9 gene also has two CACGTG sequences in its presumed promoter region, as pointed out previously (Martinez-Garcia et al. 2000). Upon the expression of APRR9 is activated (or modulated), a positive cascade of transcription may be exaggerated by an unknown mechanism, which in turn results in a sequential and coordinate expression of the remaining APRR family members, as observed in this study (Fig. 2). Each member of APRRs appears to be accumulated in the nuclei at a certain time (note that the C-terminal amino acid sequence of APRR1, including its C-motif, encompasses a nuclear-localization signal, Makino et al. 2000). Then, each APRR protein may play a distinctive role at a given timing at an interface of rhythmic output pathways. These views are very naive, and none of the aspects assumed above has yet been fully proven. However, they will provide us with at least preliminary bases for understanding the biological meanings of the APRR1/TOC1 circadian waves.

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References


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