The Arabidopsis Pseudo-response Regulators, PRR5 and PRR7, Coordinate
Play Essential Roles for Circadian Clock Function

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In Arabidopsis thaliana, a number of clock-associated protein factors have been identified. Among them, TOC1 (TIMING OF CAB EXPRESSION 1) is believed to be a component of the central oscillator. TOC1 is a member of a small family of proteins, designated as ARABIDOPSIS PSEUDO-RESPONSE REGULATOR, including PRR1/TOC1, PRR3, PRR5, PRR7 and PRR9. It has not been certain whether or not other PRR family members are also implicated in clock function per se. To clarify this problem, here we constructed a double mutant line, which is assumed to have severe lesions in both the PRR5 and PRR7 genes. Resulting homozygous prr5-11 prr7-11 young seedlings showed a marked phenotype of hyposensitivity to red light during de- etiolation. In addition, they displayed a phenotype of extremely late flowering under long-day photo-period conditions, but not short-day conditions. The rhythms at the level of transcription of certain clock-controlled genes were severely perturbed in the double mutant plants when they were released into continuous light (LL) and darkness (DD). The observed phenotype was best interpreted as ‘arrhythmic in both LL and DD’ and/or ‘very short period with markedly reduced amplitude’. Even under the light entrainment (LD) conditions, the mutant plants showed anomalous diurnal oscillation profiles with altered amplitude and/or phase with regard to certain clock-controlled genes, including the clock component CCA1 (CIRCADIAN CLOCK-ASSOCIATED 1) gene. Such events were observed even under temperature entrainment conditions, suggesting that the prr5-11 prr7-11 lesions cannot simply be attributed to a defect in the light signal input pathway. These pleiotropic circadian-associated phenotypes of the double mutant were very remarkable, as compared with those observed previously for each single mutant. Taking these results together, we propose for the first time that PRR5 and PRR7 coordinately (or synergistically) play essential clock-associated roles close to the central oscillator.

Keywords: Arabidopsis — Circadian rhythms — Control of flowering — Light signaling — Pseudo-response regulator.

Abbreviations: DD, darkness; FFT-NLLS, fast Fourier transform non-linear least squares; LD, 12 h light/12 h dark; LL, continuous light.

Introduction

Recent intensive studies on the model plant Arabidopsis thaliana have begun to shed light on the molecular mechanisms underlying a variety of circadian-controlled biological events, including control of flowering time and photomorphogenic responses (for reviews, see McClung 2000, Eriksson and Millar 2003, Yanovsky and Kay 2003, and references therein). The clock (or oscillator) is central to such circadian rhythms (Somers 2001, Young and Kay 2001), and the current candidates for Arabidopsis clock components are CCA1 and LHY (LATE ELONGATED HYPOCOTYL), which are homologous and redundant Myb-related transcription factors (Schaffer et al. 1998, Wang and Tobin 1998, Green and Tobin 1999, Mizoguchi et al. 2002). TOC1 is also believed to be another component of the central oscillator (Somers et al. 1998a, Strayer et al. 2000, Alabadi et al. 2001). These two types of clock components are proposed to form an autoregulatory feedback loop (Alabadi et al. 2001, Alabadi et al. 2002), whereby TOC1 is accumulated in late day and early night, and promotes the transcription of CCA1 (and LHY). The subsequent rise of CCA1 (and LHY) protein levels during the early and mid day acts to repress the transcription of TOC1 through direct binding to cis-elements of the TOC1 promoter.

In this connection, we have been characterizing a small family of proteins consisting of five members each designated as ARABIDOPSIS PSEUDO-RESPONSE REGULATOR (PRR1, PRR3, PRR5, PRR7 and PRR9) (Makino et al. 2000, Matsushika et al. 2000, Makino et al. 2001, Makino et al. 2002, Matsushika et al. 2002a, Murakami-Kojima et al. 2002, Murakami et al. 2004, Nakamichi et al. 2003, Yamashino et al. 2003, Nakamichi et al. 2004). During the course of these studies, we have been adopting the somewhat unusual four-letter nomenclature ‘APRR’, whereas many other researchers currently favor using ‘PRR’ (Eriksson et al. 2003, Kaczorowski and Quail 2003, Somers et al. 2004). Furthermore, rice (Oryza sativa) has a homologous set of five pseudo-response regulators, which were also circadian controlled, and thus we referred to these as OsPRRs (Murakami et al. 2003). Considering these facts, we would like to adopt hereafter the three-letter nomenclature ‘Arabidopsis thaliana PRR’ in order to avoid any further confusion. In any case, it should be emphasized that PRR1 is identical to the TOC1 clock component (Matsushika et al. 2000, Strayer et al. 2000).

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The PRR family proteins are unique to plants (Mizuno 2004), and these homologous nuclear proteins commonly contain an N-terminal domain similar to the phospho-accepting receiver domain of two-component response regulators (Mizuno 1998, Matsushika et al. 2000) followed by the additional C-terminal (CCT) motif also found in the CO (CONSTANS) transcription factor that is involved in the photoperiodic control of flowering (Onouchi et al. 2000). Several lines of circumstantial evidence have already been provided to support the view that, not only PRR1/TOC1, but also other PRR members are important for a better understanding of the molecular links between circadian rhythm, control of flowering time and light signal transduction (Makino et al. 2001, Sato et al. 2002, Matsushika et al. 2002b, Ito et al. 2003, Yamamoto et al. 2003, Murakami et al. 2004). This view was supported further by the recent studies of several other laboratories (Eriksson et al. 2003, Kaczorowski and Quail 2003, Michael et al. 2003). In short, misexpression (or overexpression) and/or mutational lesions of any one of the five PRR1/TOC1 family genes disrupts normal circadian function, including free-running rhythms, control of flowering time and photomorphogenic responses (for a summarized view, see Murakami et al. 2004).

In this study, by employing mutant lines carrying severe lesions in both the PRR5 and PRR7 genes, evidence will be provided to support the view that PRR5 and PRR7 coordinately play essential roles at very close to the central oscillator.

**Results**

**Isolation of homozygous mutant lines carrying double T-DNA insertions in the PRR5 and PRR7 genes**

We previously characterized a set of T-DNA insertion mutants, each of which has a lesion in one of the PRR genes (e.g. prr9-10, prr7-11 and prr5-11) (Ito et al. 2003, Yamamoto et al. 2003). We already reported that each single mutant showed each characteristic phenotype with regard to circadian-associated biological events, including control of flowering time and light signal transduction (see below). Nevertheless, these genetic analyses with single mutants are obviously not sufficient to understand the epistatic interactions amongst these homologous genes. To extend such genetic analyses further, we isolated homozygous prr5-11 prr7-11 double mutant lines. Together with each single mutant, the prr5-11 prr7-11 double mutant plants (1) were grown for 20 d under light and dark (LD) cycles (12 h light and 12 h dark). Then, RNA samples were prepared from leaves at intervals (3 h), as schematically indicated (filled rectangles, night). They were analyzed by Northern blot hybridization with appropriate probes (PRR5 and PRR7). The content of rRNA in each lane was analyzed as an internal and loading reference, as indicated. On the gels for Δ5 and Δ5/7, the RNA samples from Col were loaded in the lanes denoted by PC (position control) to detect the intact transcript of PRR5, which is indicated by asterisks.

Fig. 1 Isolation of prr5-11 prr7-11 double mutant lines. (A) Pictures showing morphological characteristics of the established homozygous prr5-11 prr7-11 lines. Plants were grown for 28 d under 12 h light/12 h dark on gellan gum (0.3%) plates containing MS salts with 2% sucrose: wild-type (Col), prr5-11 (Δ5), prr7-11 (Δ7) and prr5-11 prr7-11 (Δ5/7, two independent lines, 1 and 2). (B) Northern blot hybridization detecting the transcripts of PRR5 and PRR7 in the prr5-11 prr7-11 double mutant. Together with wild-type plants (Col) and each single mutant, the prr5-11 prr7-11 double mutant plants (1) were grown for 20 d under light and dark (LD) cycles (12 h light and 12 h dark). Then, RNA samples were prepared from leaves at intervals (3 h), as schematically indicated (filled rectangles, night). They were analyzed by Northern blot hybridization with appropriate probes (PRR5 and PRR7). The content of rRNA in each lane was analyzed as an internal and loading reference, as indicated. On the gels for Δ5 and Δ5/7, the RNA samples from Col were loaded in the lanes denoted by PC (position control) to detect the intact transcript of PRR5, which is indicated by asterisks.

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mutant. Having these mutant Δ5/7 plants, we first examined the expression profiles of the transcripts in question (i.e. PRR5 and PRR7). It may be noteworthy here that the prr5-11 allele contains T-DNA segments at position 1213/1235, whereas the prr7-11 allele contains T-DNA segments at position 203/247 (the first nucleotide of each inferred ATG initiation codon was taken as 1) (Yamamoto et al. 2003). The results of Northern blot hybridization showed that the isolated homozygous Δ5/7 double mutant indeed has both the prr5-11 and prr7-11 alleles (Fig. 1B). In Col, the diurnal oscillation profiles were observed for both PRR5 and PRR7, as reported previously (Matsushika et al. 2000). As compared with Col, a shorter transcript of PRR5 was detected in Δ5, whereas no transcript of PRR7 was detected in Δ7, as characterized previously (Yamamoto et al. 2003). The truncated gene product in Δ5 is assumed to be non-functional (or severely defective), whereas Δ7 most probably represents a null mutation. When we inspected the profiles of Δ5/7, it was concluded that the established double mutant indeed lacks both the intact PRR5 and PRR7 genes.

Light signal transduction in Δ5/7

Some circadian-associated genes, if not all, are implicated in certain light signal transduction pathways (or photomorphogenic responses) (Quail 2002). A visible and biological hallmark of such photosensory signal transduction is the inhibition of hypocotyl elongation during de-etiolation under light. It was suggested previously that the PRR1/TOC1 family members play certain roles in a red light-dependent photosensory signal transduction pathway (Sato et al. 2002, Kaczorowski and Quail 2003, Mas et al. 2003a, Yamamoto et al. 2003, Fujimori et al. 2004). It was thus of interest to address this issue with regard to Δ5/7. When the set of seeds (Col, Δ5, Δ7 and Δ5/7) was germinated in the dark, the hypocotyl lengths of these etiolated seedlings were similar to each other (Fig. 2A, upper panel). When they were germinated in continuous red light (fluence rate, 12 μmol m⁻² s⁻¹), the average hypocotyl lengths of Δ5 and Δ7 were considerably longer than that of Col (Fig. 2A, lower pane). It was then noticed that the average hypocotyl length of Δ5/7 was even greater. When the fluence rate response curves were statistically examined, these events were observed over a broad range of red light fluence rates (Fig. 2B). These results were best explained by assuming that the mutational lesions of both the PRR5 and PRR7 genes commonly resulted in a pheno-type of hyposensitivity to red light, and that the double Δ5/7 mutant showed an additive phenotype in this respect, thereby giving rise to much longer hypocotyls. This view is consistent with the characteristic morphology of Δ5/7 grown under white light (see Fig. 1A). In this assay of photomorphogenesis, it may also be noted that the double mutant seedlings were also slightly hyposensitive to far-red light, but not to blue light (data not shown).

Control of flowering time in Δ5/7

The circadian clock is crucially involved in the control of flowering time in the long-day plant Arabidopsis thaliana (for reviews, see Koornneef et al. 1998, Carre 2001). The clock-controlled photoperiodic signaling pathway is important for proper regulation of floral pathways so as to promote flowering time in the long-day conditions (Mouradov et al. 2002, Yanovsky and Kay 2002). We and others previously suggested that the PRR1/TOC1 family members somehow play roles in control of flowering time (Somers et al. 1998a, Sato et al. 2002, Michael et al. 2003, Yamamoto et al. 2003, Murakami et al. 2004). It was thus of interest to examine the phenotype of Δ5/7 with reference to the flowering time. The set of seeds (Col, Δ5, Δ7 and and Δ5/7) was sown on soil, and then they were grown under long-day (16 h light /8 h dark cycle) conditions. In each case, they grew well and flowered eventually (Fig. 3A). As reported previously (Yamamoto et al. 2003), the Δ5 and Δ7 plants tended to flower slightly later in the long-day conditions, as compared with the wild-type plants. In the case of Δ5/7, it took a much longer time (or many days) to set the visible primary inflorescence, as also seen (Fig. 3A). The flowering times of these mutants were monitored statistically by measur-
showed a more severe phenotype in this respect, thereby setting photoperiodic conditions. Interestingly, the double plants showed a phenotype of late flowering in the long-day number at the onset of flowering (Fig. 3C) in the long-day con-
ing the time of bolting (Fig. 3B), and also by counting the leaf number at the onset of flowering (Fig. 3C) in the long-day conditions. The results indicated that both the Δ5 and Δ7 mutant plants showed a phenotype of late flowering in the long-day photoperiodic conditions. Interestingly, the double Δ5/Δ7 mutant showed a more severe phenotype in this respect, thereby setting flower much later in the long-day conditions. More importantly, such a late-flowering phenotype of Δ5/Δ7 was not evident under the short-day (10 h light /14 h dark cycle) conditions (Fig. 3D, E).

Free-running circadian rhythm in Δ5/Δ7
To see if Δ5/Δ7 is defective in clock function per se, we then examined the circadian rhythms at the level of transcription of certain clock-controlled genes under continuous light (LL) conditions (Fig. 4). The genes first examined were CCA1, LHY and PRR1/TOC1 (Fig. 4A, B, C). The transcripts of these genes clearly oscillated in Δ5 and Δ7, as robustly as in Col (as will be discussed further later). Interestingly, such robust free-running rhythms of CCA1 (Fig. 4A), LHY (Fig. 4B) and PRR1/TOC1 (Fig. 4C) were not seen (or less evident) in Δ5/Δ7 (blue lines for Col, red lines for Δ5/Δ7). In other words, when Δ5/Δ7 plants were released into LL, the free-running rhythms of these clock-component genes were rapidly dampened, and the amplitudes of their rhythms were greatly reduced. When other clock-controlled genes, PRR3 and CAB2 (CHLOROPHYLL a/b-BINDING PROTEIN 2), were also examined under the same conditions, essentially the same phenomena were seen in Δ5/Δ7 (Fig. 4D). Other clock-controlled genes tested were GI (GIGANTEA) and PRR9, the transcripts of which showed very narrow and sharp peaks in Col (Fig. 4E). Although the rhythms of GI and PRR9 were also markedly dampened in Δ5/Δ7, the levels of transcripts apparently fluctuated (the peaks in Col are indicated by a blue arrowhead, and the apparent peaks in Δ5/Δ7 by red arrowheads). Therefore, the results of Northern blot hybridization of GI and PRR9 were analyzed by using the FFT-NLLS method (fast Fourier transform-non-linear least squares) (Straume et al. 1991). The profiles of GI and PRR9 in Δ5/Δ7 were interpreted to display markedly shortened periods, as compared with those in Col. The estimated periods are: GI in Col, 24.0 ± 2.8, GI in Δ5/Δ7, 17.6 ± 2.6, PRR9 in Col, 21.7 ± 3.5, PRR9 in Δ5/Δ7, 19.6 ± 4.7. The same FFT-NLLS analyses were then applied to analyze the profiles of CCA1, LHY and PRR1/TOC1 (see Fig. 4A, B, C), but it was not possible to obtain any statistically meaningful values with regard to relative amplitude and period. In any case, the circadian rhythms of many clock-controlled genes in LL were severely perturbed in Δ5/Δ7.

It was also critical to examine such free-running circadian rhythms in darkness (DD) (Fig. 5). In wild-type plants (Col) in DD, the free-running (or sustainable) first and second peaks were clearly seen for CCA1 (Fig. 5A) and LHY (Fig. 5B). However, such robust free-running rhythms of CCA1 and LHY in DD disappeared in Δ5/Δ7. The CCR2 (COLD AND CIRCA-DIAN REGULATED2) gene is a hallmark in that the rhythm is robustly sustained in DD, as indeed demonstrated in Fig. 5C. This robust free-running rhythm of CCR2 in DD also disappeared rapidly in Δ5/Δ7. Essentially the same events were observed for other genes, including PRR1/TOC1 (Fig. 5D) and GI (Fig. 5E). Taken together (Fig. 4, 5), it was suggested that the clock function appears to be severely perturbed in Δ5/Δ7.

Fig. 3 Flowering time of the prr5-11 and prr7-11 mutant plants. (A) Characterized plants were wild type (Col), prr5-11 (Δ5), prr7-11 (Δ7) and prr5-11 prr7-11 (Δ5/Δ7), as indicated. After being grown for 33 d under the long-day conditions (16 h light/8 h dark), the photographs were taken of each representative plant. (B) Days to visible inflorescence (about 1 cm) were defined as the time at which a given plant possessed the flower primordia (with the naked eye). (C) For the number of leaves at flowering, the leaf count was taken on the day when the flower primordia were first observed on a given plant. The numbers of plants examined in these experiments (B and C) were: wild type (n = 12), Δ5 (n = 15), Δ7 (n = 15) and Δ5/Δ7 (n = 14). (D and E) Essentially the same examinations were also carried out under the short-day conditions (10 h light/14 h dark); wild type (n = 15), Δ5 (n = 13), Δ7 (n = 15), and Δ5/Δ7 (n = 15).
These clock-associated phenotypes of ∆5/7 were interpreted as ‘arrhythmic in both LL and DD’ and/or ‘very short period with reduced amplitude’. It should be emphasized that these phenotypes of ∆5/7 were more remarkable, as compared with those of each single mutant, ∆5 and ∆7 (this issue will be addressed later).

Diurnal oscillation of expression of clock-controlled genes under entrainment conditions

In general, altered clock rhythms with longer/shorter periods under free-running conditions would result in later/earlier (or delayed/advanced) phases under entrainment (or natural) conditions (Yanovsky and Kay 2002). It was thus assumed that...
diurnal rhythms under entrainment conditions in this double mutant would be significantly altered in phase and/or amplitude. Typically, the plant clock can be entrained to light/dark (LD) cycles. Thus, the set of plants (Col, Δ5, Δ7 and Δ5/7) was grown under 12 h light/12 h dark cycles, and RNA samples were analyzed for 72 h (i.e. three cycles) with reference to diurnal oscillation profiles of certain clock-associated genes (Fig. 6). When the transcripts of CCA1 were examined in the entrainment conditions, a diurnal oscillation profile with a high amplitude was observed not only in Col, but also in Δ5 and Δ7 (Fig. 6A, see the three peaks in the morning in the gel profiles). In the case of Δ5/7, such a robust oscillation of CCA1 was not observed. The transcripts of CCA1 were expressed more or less constitutively (or with a very low amplitude). The quantitative measurements of these gel profiles supported this view (Fig. 6A, lower panel). Similar events were also seen for the homologous LHY gene, which showed a diurnal fluctuation, but with a very low amplitude (Fig. 6B). When the same examinations were conducted for PRR1/TOC1, an alerted oscillation profile with a very low amplitude was again observed in Δ5/7 (Fig. 6C). A similar event was also seen for the downstream output CAB2 gene (Fig. 6F). The transcripts of PRR3 and GI also showed altered profiles in the entrained Δ5/7, as compared with those in Col, Δ5 and Δ7. In these oscillation profiles of PRR3 and GI, the phases were clearly advanced in Δ5/7 (it may be noted that the amplitudes were also reduced). In short, even under the light entrainment conditions, Δ5/7 plants displayed anomalous oscillation profiles with altered amplitude and/or phase with regard to many clock-controlled genes, including CCA1, LHY and PRR1/TOC1.

To gain more insight into this event, we next adopted temperature entrainment conditions by growing plants under 22°C/12°C cycles in LL. Briefly, the set of plants (Col, Δ5, Δ7 and Δ5/7) was grown in LL at 22°C for 14 d, and then they were

![Fig. 6](https://academic.oup.com/pcp/article-abstract/46/4/609/1872970/fig6)
Plant circadian clock

entrained to the temperature cycles (22°C for 12 h and 12°C for 12 h) for 96 h (i.e. three cycles). During the next two cycles, RNA samples were prepared at intervals (3 h) to see the resulting oscillation profiles under the temperature entrainment conditions with regard to CCA1, LHY, PRR1/TOC1, PRR3, GI and CAB2 (Fig. 7). All these genes showed each typical oscillation profile in the temperature-entrained wild-type (Col) plants: namely, the transcripts of CCA1 displayed peaks at the onset of transition from 12 to 22°C (corresponding to dawn), while the transcripts of PRR1/TOC1 have peaks at the onset of transition from 22 to 12°C (corresponding to dusk), in a manner very similar to that observed under the light entrainment conditions (compare the profiles in Fig. 6 and 7, both of which were plotted against ‘zeitgeber time’). It was then found that, even under the temperature entrainment conditions, Δ5/7 plants displayed anomalous oscillation profiles with altered amplitude and/or phase with regard to the clock-controlled genes tested. The altered profiles of CCA1 in Δ5/7 under the temperature entrainment conditions were very similar to those observed under the light entrainment conditions (Fig. 6A, Fig. 7A). The same view was also applicable for the results of other genes, including PRR3 and GI (Fig. 7E). These results are compatible with the idea that the Δ5/7 mutations result in a severe perturbation of clock function. More importantly, the observed Δ5/7 lesions could not simply be attributed to a defect in the light signal input pathway, because the consequences of lesions were seen even in the temperature entrainment conditions.

Fig. 7  Northern blot hybridization analyses of the transcripts of certain clock-controlled genes in the prr5-11 prr7-11 double mutants under temperature entrainment conditions. The transcripts analyzed in these experiments are as indicated: (A) CCA1; (B) LHY; (C) PRR1; (D) PRR3; (E) GI; (F) CAB2. The set of plants (Col, Δ5, Δ7 and Δ5/7) was grown in LL at 22°C for 14 d, and then they were entrained to the temperature cycles (22°C for 12 h and 12°C for 12 h) for 96 h (i.e. three cycles). During the next two cycles, RNA samples were prepared at intervals (3 h), as indicated (see the schematic rectangles). These RNA samples were analyzed by Northern blot hybridization with each indicated probe (upper panels). Others details are essentially the same as those given in the legend to Fig. 4. The relative amounts of mRNA (or transcript) were quantified for each of two cycles. The intensities of each band were normalized, in which the maximum level of the transcript in Col was taken as 10 arbitrarily. These two values for each time point were directly plotted against ‘zeitgeber time’. The experiments were repeated twice, and the representative data are shown.
Discussion

It is currently believed that the *Arabidopsis* central oscillator is composed of CCA1 (and LHY) and PRR1/TOC1, which together create a positive/negative transcriptional regulatory circuitry (or feedback loop) that generates a fundamental rhythm (Alabadi et al. 2001, see also Introduction). However, this proposed feedback loop is only a framework onto which other factors must be incorporated intensively. Indeed, many other circadian-associated factors have also been identified, although it is not easy to assign precisely each of their molecular roles either in input pathways, as clock components, or in output pathways (Eriksson and Millar 2003). In any case, they include certain photoreceptors (PHYA/B and CRY1/2) (Somers et al. 1998b), SRR1 (SENSITIVITY TO RED LIGHT REDUCED) (Staiger et al. 2003), ELF3 (EARLY FLOWERING3) (Covington et al. 2001, Hicks et al. 2001), ELF4 (EARLY FLOWERING4) (Doyle et al. 2002), GI (Fowler et al. 1999, Huq et al. 2000), and a family of flavin-binding proteins, ZTL/AD01 (ZEITLUPE/ADAGIO1) (Somers et al. 2000, Jarillo et al. 2001, Somers et al. 2004), FKF1 (FLAVIN-BINDING KELCH F BOX1) (Nelson et al. 2000) and LKP2 (LOV KELCH PROTEIN2) (Schultz et al. 2001). The molecular functions of these factors are not yet clear, except for the photoreceptors and the ZTL family of F-box proteins that are involved in protein degradation (Somers et al. 1998b, Mas et al. 2003b, Somers et al. 2004). Certain enzymes were also implicated as such clock-associated factors, which include a regulatory subunit (named CKB3) of casein kinase II (CK2) that is involved in CCA1 phosphorylation (Sugano et al. 1999), and poly (ADP-ribo) glycohydrolase (named TEJ) (Panda et al. 2002). Mutational lesions in any of these clock-associated components somehow affect clock-controlled biological events. Nevertheless, molecular links between these factors and the proposed central feedback loop have not yet been fully clarified. Furthermore, a number of as yet-unidentified factors appear to be missing from the current list of clock-associated factors.

Regarding these other putative clock-associated factors, we have been particularly interested in the PRR family of proteins, to which TOC1 (PRR1) belongs. We have reported that misexpression and/or mutational lesions of any one of the five PRR family genes result in disruption of normal circadian function, including control of flowering time, and photomorphogenic responses (for a summarized view, see Murakami et al. 2004). Nevertheless, such circadian-associated phenotypic alterations in a given single prr mutant were subtle in some instances (Yamamoto et al. 2003), as indeed observed in this study for the Δ5 and Δ7 single mutants (see Fig. 2–7). Also, we always considered that the results from overexpression experiments might be indirect events (or artifacts) (Sato et al. 2002). Therefore, we previously did not conclude that the PRR family members other than PRR1/TOC1 are directly involved in clock function per se, although there were many pieces of circumstantial evidence, as discussed earlier. The primary objective of this study was to address this issue further. The results of this study employing the prr5-11 prr7-11 double mutant now led us to propose for the first time that PRR5 and PRR7 coordinately play essential roles very close to the central oscillator.

In general, mutational lesions in any one of the clock-associated components (e.g. *eccl*, *elf3*, *gi*, *toc1* and *ztl*), if not all, affect not only circadian rhythms at the level of transcription of clock-controlled genes, but also photomorphogenic responses and/or control of flowering (see the references cited above). Indeed, here we demonstrated that the prr5-11 prr7-11 mutant showed remarkable phenotypes with respect to these circadian-associated biological events, including the regulation of elongation of hypocotyls in response to a light signal (Fig. 2, extremely hyposensitive to red light) and the photoperiodic control of flowering time (Fig. 3, very late flowering under the long-day conditions, but not under the short-day conditions). Each single mutant (prr5-11 or prr7-11) showed essentially the same phenotypes, as reported previously (Yamamoto et al. 2003). However, the phenotypes of the double mutant were very remarkable. These striking phenotypes of prr5-11 prr7-11 with regard to photomorphogenic responses and photoperiodic control of flowering time may result primarily from severe defects in circadian regulation, as has been generally considered (Deng and Quail 1999, Dowson-Day and Millar 1999, Yanovsky and Kay 2002). In any event, it is clear that PRR5 and PRR7 act together coordinately (or synergistically) in these biological aspects.

We previously showed that the prr5-11 single mutant exhibits a phenotype of short period (and/or advanced phase), whereas the prr7-11 single mutant displays a phenotype of long period (and/or delayed phase), as far as the free-running rhythms of certain genes in LL were concerned (Yamamoto et al. 2003). Other groups independently reported consistent observations with regard to other prr5 and prr7 single mutant alleles (Eriksson et al. 2003, Michael et al. 2003), although Kaczorowski and Quail (2003) reported that a prr7 mutant showed a phenotype of phase advance. In any case, the values of altered periods in these single mutants were too small to estimate precisely by the rough Northern blot hybridization analyses at 3 h intervals, as discussed previously (Yamamoto et al. 2003). Indeed, these phenotypes of each single mutant of prr5-11 and prr7-11 were not apparent in this study (see Fig. 4A, Δ5 and Δ7), when the first and second CCA1 peaks in LL were inspected (however, note that we previously analyzed the CCA1 rhythm in LL up to the fourth and fifth peaks to detect the significant changes in period) (Yamamoto et al. 2003). Surprisingly, the observed phenotype of the double mutant was much more striking, and they were best interpreted as ‘arrhythmic in both LL and DD’ and/or ‘very short period with low amplitude’ (Fig. 4, 5). In these events, interestingly, the level of PRR1/TOC1 was reduced to the trough level, while those of others increased to the peak level. In any case, it should be mentioned here that a loss-of-function mutant of PRR1/TOC1
(toc1-2) also shows a phenotype of 'very short period in LL (white light)'. Furthermore, the effects of the toc1-2 lesion on free-running rhythms are dependent on the light conditions, showing a phenotype of 'arrhythmic under red light or constant darkness' (Alabadi et al. 2001, Mas et al. 2003a). In short, the prr5-11 prr7-11 double lesions resulted in even more severe defects in circadian rhythms, as compared with the case of the toc1 clock lesion (toc1-2).

How do we explain such a synergistic effect of the prr5-11 prr7-11 double lesions on circadian rhythms? First of all, it may be noted that the PRR family genes are all subjected to circadian rhythms in such a manner that each PRR transcript starts accumulating after dawn sequentially in the order PRR9, PRR7, PRR5, PRR3 and PRR1/TOC1 with approximately 2 h intervals (Matsushika et al. 2000). In other words, the diurnally rhythmic expression of PRR7 occurs considerably earlier than that of PRR5, implying that these protein products accumulate at certain times different from each other. It is then tempting to speculate that both PRR5 and PRR7 might act as period-affecting factors, but they might also each have a distinct role, for instance, through interacting with different proteins, so that disruption of both the functions has a strongly additive effect. Considering the phenotypes of each single mutant mentioned above, PRR5 might primarily act as a period-affecting factor that lengthens the period, whereas PRR7 might function conditionally so as to shorten or lengthen the period, depending on entrainment conditions. It may thus be of interest to examine the set of mutant plants (Δ5, Δ7 and Δ5/7) closely under the conditions of different light intensities and/or light qualities.

It has been somewhat puzzling that even severe clock mutants (e.g. toc1-2 mutant and lhy cca1-1 double mutant) still retain the ability to show robust diurnal rhythms under light entrainment conditions (LD cycles) (Mizoguchi et al. 2002, Mas et al. 2003a). In this respect, the prr5-11 prr7-11 lesions resulted in marked alterations of diurnal oscillation profiles of clock-controlled genes in LD (Fig. 6). These observations are seemingly consistent with the general view that altered clock rhythms with longer/shorter periods under free-running conditions would result in later/earlier (or delayed/advanced) phases under entrainment conditions, thereby affecting the clock function in nature. In light entrainment (LD) conditions, the amplitudes of the diurnal rhythms of CCA1, LHY and PRR1/TOC1 were markedly reduced in the double mutant (Fig. 6). Under these conditions, the phases of GI and PRR3 were markedly advanced in the double mutant, as compared with those in the wild-type plants. Such advanced phases of certain clock-controlled evening genes (e.g. GI and PRR1/TOC1) in LD cycles were also observed previously for the clock mutant (carrying the lhy cca1-1 double lesions) (Mizoguchi et al. 2002). More importantly, essentially the same events were observed even when the prr5-11 prr7-11 mutant was entrained to temperature (low and high) cycles (Fig. 7). Thus, the observed prr5-11 prr7-11 lesions in LD could not simply be attributed to a defect in the light signal input pathway. Together with the fact that the double mutant plants show the severe phenotype in DD (Fig. 4), these results strongly supported the view that PRR5 and PRR7 act close to the central oscillator, not just in a light signal input pathway.

As pointed out previously (Somers et al. 2004), the promoting/repressing transcription cycle through CCA1 (and LHY) and PRR1/TOC1 is clearly only a framework onto which other period-affecting factors must be incorporated in order to make it possible for the central oscillator to incorporate time lags of many hours to culminate in circadian 24 h rhythm. In this respect, PRR5 and PRR7 might coordinate and directly serve as such period-affecting factors at close to the central oscillator, as discussed above. Furthermore, one can envisage a priori that the molecular functions of PRR5 and PRR7 must be similar to that of the PRR1/TOC1 clock component, because their amino acid sequences (or protein structural designs) are very similar to each other (Matsushika et al. 2000). For instance, ZTL interacts with PRR1/TOC1 and promotes the degradation of this clock component (Mas et al. 2003b), and LKP2 (another homologous F-box protein) interacts with not only PRR1/TOC1 but also PRR5 (Yasuhara et al. 2004). In future, clarification of the common molecular function of PRRs, and clarification of the molecular interactions within PRRs and/or with other factors remain major challenges. Are PRRs DNA-binding transcription factors? Do they directly interact with each other in plant cells? To address these general and specific issues, it would be of interest to conduct further combinatorial genetic studies by employing other appropriate prr alleles, prr1, prr3 and prr9.

Materials and Methods

Plant growth conditions and related materials

Arabidopsis thaliana (Col) were mainly used as wild-type plants. Seeds were imbibed and cold treated at 4°C for 3 d in the dark before germination under light, and then plants were grown at 22°C. Note that the imbibed seeds were exposed to white light for 30 min before incubation in the dark. Plants were grown in a chamber with light from fluorescent lights (70–80 µmol m⁻² s⁻¹) at 22°C on soil and/or agar plates containing MS salts and 2% sucrose. Light/dark conditions used were either 16 h light/8 h dark or 12 h light/2 h dark, as specifically noted for each experiment in the text. To entrain plants to temperature cycles, they were grown under continuous light (LL) for 14 d at 22°C, and they were entrained to 12 h 22°C/12 h 12°C (four cycles) in LL.

Preparing RNA, and Northern blotting

Total RNA was isolated from appropriate organs (mainly leaves) of Arabidopsis plants by the aurantricarboxylic acid (ATA) method (Taniguchi et al. 1998). For Northern blot hybridization, RNA was separated in agarose gels (1%) containing 0.67 M formaldehyde, then separated in agarose gels (1%) containing 0.67 M formaldehyde, then 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...
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0.5% SDS for 30 min at 65°C, and then with 0.2x SSPE and 0.5% SDS for 15 min at 65°C. The washed membranes were exposed and analyzed on a phosphoimage analyzer (BAS-2500ID) (FujiXerox, Tokyo, Japan).

Probes for Northern blot hybridization
Several double-stranded 32P-labeled DNA probes were used to detect each specific mRNA. The probes were used and amplified by polymerase chain reaction with an appropriate set of primers, which were designed appropriately, including CCA1 LHY, CAB2, GI, PRR5/TOC1, PRR8, PRR5, PRR7 and PRR9, as described previously (Makino et al. 2002, Yamamoto et al. 2003). Each 32P-labeled probe was prepared with the MegaPrimer DNA Labeling System (TaKaRa Shuzo, Kyoto, Japan).

Examination of light response in early photomorphogenesis
To examine the light response in early photomorphogenesis of plants, seeds were sown on gellan gum (0.3%) plates containing MS salts without sucrose. They were then kept at 4°C for 48 h in the dark. Then, seeds were exposed to white light for 3 h in order to enhance germination, followed by incubation at 22°C for 21 h again in the dark. Plants were grown for 72 h under continuous light with a varied range of fluence rates or in the dark. As the light sources for continuous light rates or in the dark. Plants were grown for 72 h under continuous light with a varied range of fluence rates or in the dark. As the light sources for continuous light, far-red light, STICK-mFR (735 nm at 25 µmol m⁻² s⁻¹), and red light, STICK-mR (735 nm at 25 µmol m⁻² s⁻¹), as described previously (Sato et al. 2002).

Examination of flowering time
Seeds were imbibed directly on soil (110 ml), supplemented with 50 ml of 5,000 times diluted HYPOXEN (N : P : K = 5 : 10 : 5) (HYPOXEN-JAPAN, Osaka, Japan). They were cold-treated at 4°C for 3 d in the dark. They were then kept at 4°C for 48 h in the dark. Then, seeds were exposed to white light for 3 h in order to enhance germination, followed by incubation at 22°C for 21 h again in the dark. Plants were grown for 72 h under continuous light with a varied range of fluence rates or in the dark. As the light sources for continuous irradiation, light-emitting diodes (LEDs) were used: for red light, STICK-mR (1,250 nm at 30 µmol m⁻² s⁻¹ (Tokyorika, Inc., Tokyo, Japan)); and far-red light, STICK-mFR (735 nm at 25 µmol m⁻² s⁻¹), as described previously (Sato et al. 2002).

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References
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