Plant Cell Physiol. 46(5): 686–698 (2005)
doi:10.1093/pcp/pci086, available online at www.pcp.oupjournals.org

Rapid Paper

PSEUDO-RESPONSE REGULATORS, PRR9, PRR7 and PRR5, Together Play Essential Roles Close to the Circadian Clock of *Arabidopsis thaliana*

Norihito Nakamichi, Masanori Kita, Shogo Ito, Takafumi Yamashino and Takeshi Mizuno [1]

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-8601 Japan

In *Arabidopsis thaliana*, a number of clock-associated protein components have been identified. Among them, CCA1 (CIRCADIAN CLOCK-ASSOCIATED 1)/LHY (LATE ELONGATED HYPOCOTYL) and TOC1 (TIMING OF CAB EXPRESSION 1) are believed to be the essential components of the central oscillator. CCA1 and LHY are homologous and partially redundant Myb-related DNA-binding proteins, whereas TOC1 is a member of a small family of proteins, designated as PSEUDO-RESPONSE REGULATOR. It is also believed that these two different types of clock components form an autoregulatory positive/negative feedback loop at the levels of transcription/translation that generates intrinsic rhythms. Nonetheless, it was not yet certain whether or not other PRR family members (PRR9, PRR7, PRR5 and PRR3) are implicated in clock function per se. Employing a set of *prr9*, *prr7* and *prr5* mutant alleles, here we established all possible single, double and triple *prr* mutants. They were examined extensively by comparing them with each other with regard to their phenotypes of circadian rhythms, photoperiodicity-dependent control of flowering time and photomorphogenic responses to red light during de-etiolation. Notably, the *prr9 prr7 prr5* triple lesions in plants resulted in severe phenotypes: (i) arrhythmia in the continuous light conditions, and an anomalous phasing of diurnal oscillation of certain circadian-controlled genes even in the entrained light/dark cycle conditions; (ii) late flowering that was no longer sensitive to the photoperiodicity; and (iii) hyposensitivity (or blind) to red light in the photomorphogenic responses. The phenotypes of the single and double mutants were also characterized extensively, showing that they exhibited circadian-associated phenotypes characteristic for each. These results are discussed from the viewpoint that PRR9/PRR7/PRR5 together act as period-controlling factors, and they play overlapping and distinctive roles close to (or within) the central oscillator in which the relative, PRR1/TOC1, plays an essential role.

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

Abbreviations: CCA1, CIRCADIAN CLOCK-ASSOCIATED 1; Col, Columbia ecotype; LHY, LATE ELONGATED HYPOCOTYL; PRR, PSEUDO-RESPONSE REGULATOR; TOC1, TIMING OF CAB EXPRESSION 1.

Introduction

Recent intensive studies on the model plant *Arabidopsis thaliana* have begun to shed light on the molecular nature of the plant biological clock, and also on the mechanisms underlying a variety of circadian-controlled biological events (for reviews see McClung 2000, Eriksson and Millar 2003, Yanovsky and Kay 2003, Salome and McClung 2004, and references therein). The clock (or oscillator) is central to such circadian rhythms (Somers 2001, Young and Kay 2001). The current candidates for *Arabidopsis* clock components are CCA1 (CIRCADIAN CLOCK-ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), which are homologous Myb-related transcription factors (Schaffer et al. 1998, Wang and Tobin 1998, Green and Tobin 1999, Alabadi et al. 2002, Mizoguchi et al. 2002). TOC1 (TIMING OF CAB EXPRESSION 1) 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 negative/positive feedback loop (Alabadi et al. 2001, Alabadi et al. 2002, Mas et al. 2003a). However, this proposed feedback loop at the level of transcription is only a framework onto which other factors must be intensively incorporated.

Such circadian-associated factors include: photoreceptors (PHYA/B and CRY1/2) (Somers et al. 1998b), ELF3 and ELF4 (EARLY FLOWERING 3 and 4) (Covington et al. 2001, Hicks et al. 2001, Doyle et al. 2002), GI (GIGANTEA) (Fowler et al. 1999, Huq et al. 2000) and a family of flavin-binding proteins including ZTL/ADO1 (ZEITLUPE/ADAGIO1) (Somers et al. 2000, Jarillo et al. 2001, Somers et al. 2004) and LKP2 (LOV KELCH PROTEIN2) (Schultz et al. 2001, Yasuhara et al. 2004). Mutational lesions in any one of these clock-associated components somehow (and more or less) affect clock-controlled biological events. However, it is also certain that a number

[1] Corresponding author: E-mail, tmizuno@agr.nagoya-u.ac.jp; Fax, +81-52-789-4091.

Table 1 Summary view of genetic results of the *PRR9*, *PRR7* and *PRR5* genes

Mutants	Period of rhythms (free-running in LL)	Flowering time (long-day conditions)	Red light sensitivity (elongation of hypocotyls)	References
cca1 lhy	Arrhythmic (short)	Early	Hypersensitive	Mizoguchi et al. (2002)
prr9	Slightly long	Slightly late	Slightly hyposensitive	Eriksson et al. (2003); Ito et al. (2003); Michael et al. (2003)
prr7	±	Late	Hyposensitive	Kaczorowski and Quail (2003); Michael et al. (2003); Yamamoto et al. (2003)
prr5	Slightly short	Slightly late	Hyposensitive	Eriksson et al. (2003); Michael et al. (2003); Yamamoto et al. (2003)
prr9 prr7	Extremely long	**? (LATE)**	**? (HYPOSENSITIVE)**	Farre et al. (2005); Salome and McClung (2005); this study
prr9 prr5	±	**? (SLIGHTLY LATE)**	Hyposensitive	Eriksson et al. (2003); this study
prr7 prr5	Extremely short	Late	Hyposensitive	Nakamichi et al. (2005); this study
prr9 prr7 prr5	**? (ARRHYTHMIC)**	**? (EXTREMELY LATE)**	**? (EXTREMELY HYPOSENSITIVE)**	This study
toc1-2	Short	ND	Hyposensitive	Alabadi et al. (2001); Mas et al. (2003a)

A T-DNA insertion mutant (SALK-007551) was mainly characterized for *prr9*, a T-DNA insertion mutant (SALK-030430) was mainly characterized for *prr7*, T-DNA insertion mutants (KAZUSA-KG24599, SALK-006280 and SALK-064538) were characterized for *prr5*. These are derivatives of Col. Note that *toc1-2* is a derivative of C24.
The answers in bold upper case were obtained in this study.
ND, not determined; ±, no significant alteration, or marginal if any.

of as yet unidentified factors are still missing from the current list of these clock-associated factors (Onai et al. 2004).

As such putative clock-associated factors, we have been particularly interested in the PRR (PSEUDO-RESPONSE REGULATOR) family of proteins, which consists of five members (PRR9, PRR7, PRR5, PRR3 and PRR1), among which PRR1 is identical to TOC1 (Makino et al. 2000, Matsushika et al. 2000, Makino et al. 2001, Makino et al. 2002, Matsushika et al. 2002a, Matsushika et al. 2002b, Murakami-Kojima et al. 2002, Sato et al. 2002, Ito et al. 2003, Murakami et al. 2003, Nakamichi et al. 2003, Yamamoto et al. 2003, Murakami et al. 2004, Nakamichi et al. 2004, Nakamichi et al. 2005). The PRR family proteins are unique to plants (Mizuno 2004), and these homologous 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 found also in the CO (CONSTANS) transcription factor that is involved in the photoperiodic control of flowering (Onouchi et al. 2000). These PRRs are nuclear-localized proteins, but no evidence has been provided that they serve as transcriptional factors. Therefore, the common molecular function of PRRs is not clear. Furthermore, it is not believed that the PRR family members other than PRR1/TOC1 are components of the central clock (Eriksson and Millar 2003, Salome and McClung 2004). However, several lines of circumstantial evidence have been accumulating 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 (see Table 1 and references therein). In short, misexpression (or overexpression) and/or mutational lesions of any one of the PRR family genes perturb the normal circadian-associated functions to some extent.

To address the relevant issues with regard to the PRR family members, here we extensively characterized a set of *prr* mutants that included *prr9 prr7* double, *prr9 prr5* double, *prr7 prr5* double and *prr9 prr7 prr5* triple mutant lines. The consistent results from genetic examinations in this study led us to propose that PRR9/PRR7/PRR5 together serve as period-controlling factors, and they coordinately play essential roles within (or close to) the central oscillator containing the relative, PRR1/TOC1.

Results

T-DNA insertion mutants of PRR9, PRR7 and PRR5

We previously characterized a set of homozygous T-DNA insertion mutants, each of which has a severe lesion in one of the *PRR* genes. They are all derivatives of the Columbia eco-

type (Col). The *prr9-10* allele (SALK-007551) has T-DNA segments at position +441/+451 (the first nucleotide of the inferred initiation codon of *PRR9* was taken as +1) (Ito et al. 2003). No transcript of *PRR9* was detected in the homozygous mutant plants. The *prr5-11* allele (KAZUSA-KG24599) contains T-DNA segments at position +1,213/+1,235, whereas *prr7-11* (SALK-030430) contains T-DNA segments at position +203/+247 (Yamamoto et al. 2003). A truncated transcript of *PRR5* was detected in the *prr5-11* plants, while no transcript of *PRR7* was detected in *prr7* (Nakamichi et al. 2005). It was assumed that the truncated transcript (or gene product) in *prr5-11* is non-functional (or severely defective), whereas the *prr9-10* and *prr7-11* alleles most probably represent null mutations. In this study, the circadian-associated phenotypes of these *prr* mutant alleles were characterized further by concomitantly establishing a set of mutant lines carrying double and triple *prr* mutant alleles. To clarify this text, we will use the abbreviated designations, as follows: wild-type Columbia plants, Col; *prr9* single mutant, *d9* (defective *prr9*); *prr7* single mutant, *d7*; *prr5* single mutant, *d5*.

Reinvestigation of circadian rhythms in a set of single prr mutants

With regard to these homozygous single *prr* mutants (*d9*, *d7* and *d5*), we previously reported that each of them showed characteristic phenotypes with regard to circadian-associated biological events, including (i) rhythmic expression of circadian-controlled genes; (ii) control of flowering time; and (iii) light sensitivity during early photomorphogenesis (Table 1, and see Ito et al. 2003, Yamamoto et al. 2003). Each single mutational lesion (*d9*, *d7* or *d5*) appeared to affect the period (and/or phase) of rhythms of certain circadian-controlled genes in the continuous light (LL) conditions. Previously, we interpreted their phenotypes as follows (see Table 1): *d9* shows a weak phenotype of long period, whereas *d5* exhibits a weak phenotype of short period, while the phenotype of *d7* was less evident (seemingly long period, but see Yamamoto et al. 2003). However, it was difficult to interpret the phenotypes of these mutants conclusively and quantitatively with special reference to the circadian rhythm, because they were roughly characterized by Northern blot hybridization of mRNA prepared from plants at 3 h intervals.

To address this issue critically, here we newly employed a *CCA1*::*LUC* fusion gene as an alternative reporter. This real-time bioluminescence reporter gene was previously characterized successfully in an *Arabidopsis* cultured cell (T87) line (Nakamichi et al. 2004). We introduced the *CCA1*::*LUC* fusion gene into these *prr* mutants, and the resultant transgenic plants were characterized with a bioluminescence monitoring system (Fig. 1). The bioluminescence intensities of *CCA1*::*LUC* in LL were robustly oscillated not only in wild-type plants (Col), but also in other mutants (*d9*, *d7* and *d5*). To estimate the free-running periods in each mutant statistically, the bioluminescence intensities were repeatedly analyzed (data not shown), and the lengths of periods (h) of free-running rhythms of *CCA1*::*LUC* were estimated for each: Col, 24.3 ± 0.1; *d9*, 26.2 ± 0.3; *d7*, 24.3 ± 0.2; *d5*, 22.6 ± 0.3 (Fig. 1). The results were fully consistent with the view that *d9* (red line) showed a phenotype of long period, whereas *d5* (green line) exhibited a phenotype of short period, while the rhythmic profile of *d7* (yellow line) was not distinguishable from that of Col (blue line) under the conditions tested (see Materials and Methods).

These views are in good agreement with those reported previously (Eriksson et al. 2003, Michael et al. 2003, Farre et al. 2005, Salome and McClung 2005). It may be noted that our *prr5* allele (KAZUSA-KG24599) expressing a truncated form of mRNA showed essentially the same phenotype as that of the *prr5* null allele (SALK-064538, Michael et al. 2003), suggesting that ours is also a loss-of-function allele. Taken together, it was suggested that these PRR family members are important for a better understanding of the molecular mechanism underlying the circadian clock. Nevertheless, one can argue that the circadian-associated phenotypic alterations in a given single *prr* mutant are subtle (Fig. 1). In other words, PRR9, PRR7 and PRR5 are dispensable (or not essential) for the clock function per se. However, it is the current general view of *Arabidopsis* genetics that we should conduct extensive studies by employing double and triple mutants, in order to characterize a set of redundant and homologous genes.

Isolation of double and triple prr mutants

To this end, extensive genetic crosses were conducted with the *d9*, *d7* and *d5* single mutants, and then a set of double and triple *prr* mutants were isolated. Those established were all possible combinatorial lines (or seeds): *prr9-10 prr7-11* double mutant, *prr9-10 prr5-11* double mutant, *prr7-11 prr5-11*double mutant and *prr9-10 prr7-11 prr5-11* triple mutant. These homozygous lines will be hereafter referred to as *d97*, *d95*, *d75* and *d975*, respectively. These mutant plants grew as well as the wild type (Col) on MS agar plates under white light. Interestingly, the lengths of petioles and hypocotyls of *d97*, *d75* and *d975* were considerably longer than those of Col and other mutants (data not shown), as demonstrated previously for *d75* (Nakamichi et al. 2005). In every case, the plants eventually and normally set flowers and seeds. However, it may be noteworthy that the fully developed *d975* plants showed characteristic morphologies: they are very tall, the stems are thick, and the leaves are dark green, as compared with wild-type plants. At a glance, these morphologies are considerably similar to those of plants overexpressing the *CCA1* gene (Wang and Tobin 1998). These characteristics of *d975* were dependent on the light conditions, and they were more evident when plants were grown under the long-day conditions. Having these plants, we then confirmed that they are indeed homozygous lines carrying the double or triple mutational lesions in question, by Northern blotting hybridization analyses of each transcript of *PRR9*, *PRR7* and *PRR5* (data not shown). These established *d97*, *d95*, *d75* and *d975* mutants, together with the

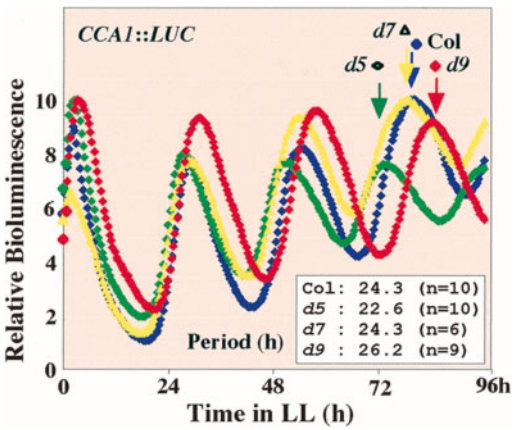


Fig. 1 Characterization of a set of single *prr* mutants with reference to the free-running rhythms of *CCA1*::*LUC*. A set of single *prr* mutants (*d9*, *d7* and *d5*) each carrying *CCA1*::*LUC* were grown in 12 h light/12 h dark (LD, white light 70 µmol m^{-2} s^{-1}) cycle conditions. They were then released into continuous light (LL, 70 µmol m^{-2} s^{-1}) conditions, and the bioluminescence intensities were monitored. The measured values (counts s^{-1} $sample^{-1}$) were normalized (the maximum value of each sample was taken as 10 to clarify the profiles). A number of samples were examined independently, and each representative is shown here: Col (blue), *d9* (red), *d7* (yellow), *d5* (green). The periods of free-running rhythms in LL were statistically measured, and they are indicated in the inset box (n = numbers of samples examined). With regard to the reproducibility of these data, see the references cited in Table 1.

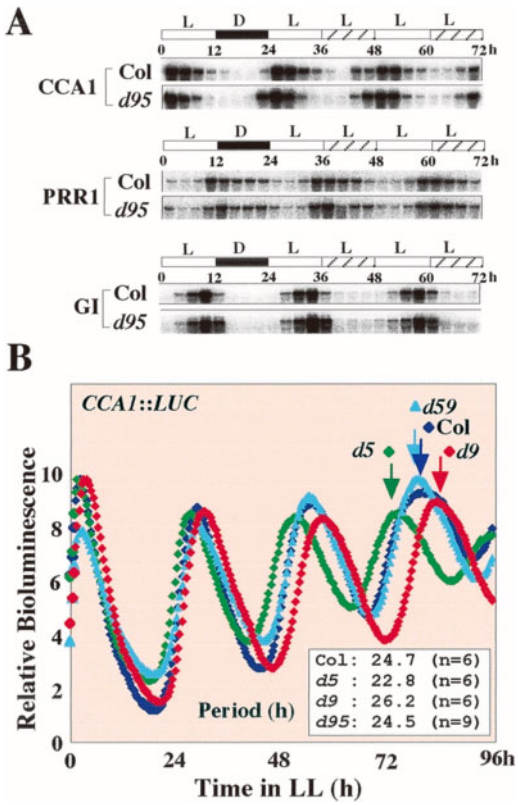


Fig. 2 Characterization of the *d95* double mutant. (A) Northern blot hybridization analyses of the transcripts of certain clock-controlled genes in the *d95* double mutants in LD and LL. The pair of plants (Col and *d95*) was grown in the 12 h light/12 h dark cycles (LD) for 20 d, and then they were released to LL (white light 70 µmol m^{-2} s^{-1}). RNA samples were prepared from leaves at the times indicated with appropriate intervals (3 h), as schematically indicated (see the top rectangles). Northern blot hybridization was carried out with probes specific to *CCA1*, *PRR1* and *GI*, respectively. In these experiments, the content of rRNA in each lane was analyzed as an internal and loading reference, but these reference data are not shown for clarity of the figures (as such examples, see Fig. 4). (B) Characterization of a set of single and double mutants with reference to the free-running rhythms of *CCA1*::*LUC*. A set of *prr* mutants (*d9*, *d5* and *d95*) each carrying *CCA1*::*LUC* was grown in the 12 h light/12 h dark (LD) cycle conditions, and then they were transferred into LL (white light 70 µmol m^{-2} s^{-1}). A number of samples were examined independently, and each representative is shown here: Col (blue), *d9* (red), *d5* (green), *d95* (light blue). Other details are the same as those given in Fig. 1. With regard to the reproducibility of these data, see the references cited in Table 1.

parental single mutants (*d9*, *d7* and *d5*), were characterized by comparing their circadian-associated phenotypes with each other.

A current summary of the results of genetic studies on the prr family genes.

Before describing the results as to these seven different *prr* mutants, it is worth summarizing the results of recent genetic studies on the PRR family genes because several independent groups have been studying certain *prr* mutants (Table 1). Eriksson et al. (2003) characterized *d9* (SALK-007551), *d5* (SALK-006280) and *d95*. Farre et al. (2005) and Salome and McClung (2005) independently reported the phenotypes of *d9* (SALK-007511), *d7* (SALK-030430) and *d97*. We have also characterized a set of mutants, including *d9* (SALK-007551), *d7* (SALK-030430), *d5* (KAZUSA-KG24599) and *d75* (Ito et al. 2003, Yamamoto et al. 2003, Nakamichi et al. 2005). In addition to these, Michael et al. (2003) and Kaczorowski and Quail (2003) characterized some *prr* single mutant alleles. Most of the results from these independent studies are very consistent with each other, if not perfectly. Therefore, these results allowed us to overview the characteristics of *prr* mutants with special reference to their circadian-associated phenotypes (Table 1): the changes in periods of circadian rhythms in LL, the effects on photoperiodicity-dependent flowering time and the changes in red light sensitivity of the elongation of hypocotyls during de-etiolation. To gain general ideas, the relevant phenotypes of the well-characterized clock mutants (the *cca1 lhy* double mutant together with the *toc1-2* mutant) were also incorporated into this list (Mizoguchi et al. 2002, Mas et al. 2003a).

Objective of this study

The content of Table 1 should provide us with the basis for a better understanding of the clock-associated functions of

the PRR family members. Nonetheless, Table 1 is not comprehensive enough because some crucial points are missing (see items in bold denoted by question marks in Table 1). For instance, the phenotypes of flowering time and light sensitivity have not been reported for *d97*. In turn, it is not known whether or not *d95* displays any phenotype with regard to flowering time. More importantly, no information is available with regard to the *d975* triple mutant. Furthermore, we wanted to examine the expression profiles of *CCA1* (and LHY) and *PRR1/TOC1* in a parallel manner in these mutant lines because these genes encode the essential clock components. For these multiple reasons, in this study, we carried out comprehensive studies by employing the *prr* mutant lines (*d97*, *d95*, *d75* and *d975*). In short, the objectives of this study are 4-fold (Table 1): (i) solid confirmation of previous results; (ii) supplementation of missing information; (iii) examination of the expression profiles of *CCA1* (and LHY) and *PRR1/TOC1* in every *prr* mutant; and, most importantly, (iv) characterization of the *d975* triple mutant. These results will be presented below (note that the answers are already given in Table 1).

Circadian rhythms in the prr9 prr5 double mutant

We first characterized our *d95* mutant in terms of its phenotype with special reference to the circadian rhythms of *CCA1*, *PRR1/TOC1* and *GI* (Fig. 2A). The expression of these genes in *d95* showed robust free-running rhythms in LL, the profiles of which were indistinguishable from those in Col. This was somewhat curious, when we considered the fact that the single *d9* mutant showed a phenotype of long period, whereas the single *d5* mutant showed a phenotype of short period (Fig. 1, Table 1). To address this issue critically, we constructed a transgenic *d95* line carrying *CCA1*::*LUC*. The phenotypes of *d9*, *d5* and *d95* were compared with each other by monitoring the bioluminescence intensities in LL (Fig. 2B). Indeed, the rhythmic profile of *CCA1*::*LUC* in *d95* was indistinguishable from that in Col, although each single mutant showed the anticipated phenotype (i.e. long period in *d9* and short period in *d5*). These results confirmed the previous notion that both *PRR9* and *PRR5* appear to act as period-controlling factors in such a way as to play interactive roles complementary to each other (Eriksson et al. 2003).

Circadian rhythms in the prr9 prr7 double mutant

Farre et al. (2005) and Salome and McClung (2005) independently characterized a set of *prr9* and *prr7* mutant lines, showing that the *d97* double mutant displayed a marked phenotype of long period in LL. We confirmed this view by employing the independently established *d97* line with special reference to circadian-controlled genes (e.g. *CCA1*, *PRR1/TOC1* and *GI*) (Fig. 3A–C). The free-running rhythms of these genes in *d97* were sustained in LL as in the case of Col. In every case, however, the periods between the first and second peaks in LL were considerably lengthened, as compared with in the case of Col. This event was particularly evident for *PRR1/TOC1* and *GI* (see bidirectional horizontal arrows in Fig. 3B, C). Our results also supported the view that the *d97* double mutant showed a phenotype of an extremely long period. We further confirmed this critical view by employing *d97* double mutant lines carrying *CCA1*::*LUC* (Fig. 4A). The results supported the view that the *d97* double lesions result in a marked phenotype of long period in LL (29.4 ± 0.7 h in LL). Considering the fact that the *d9* single mutant shows a weak phenotype of long period, the result of this study suggested the interesting view that a loss of the PRR7 function resulted in a marked exaggeration of the *d9* phenotype, despite the fact that the *d7* single mutant itself showed no clear phenotype with regard to the period under the conditions tested (Fig. 1).

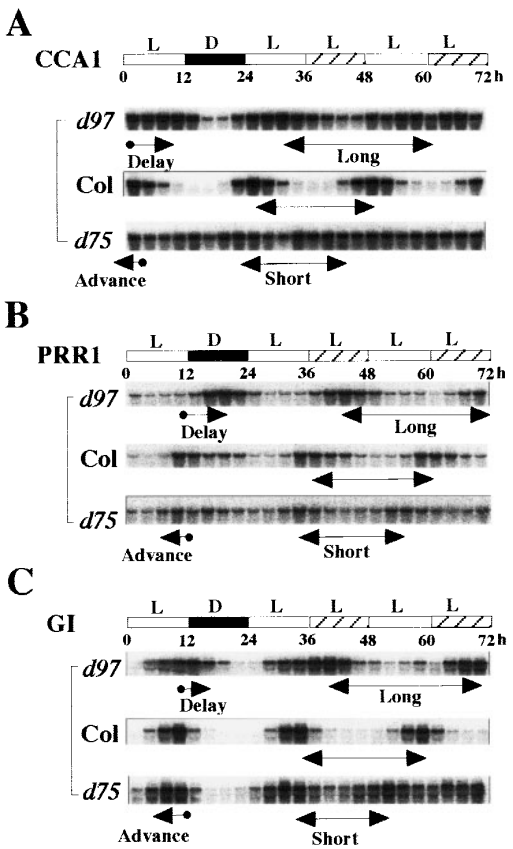


Fig. 3 Characterization of *d97* and *d75* double mutants. (A) Northern blot hybridization analyses of the transcripts of certain clock-controlled genes in both the *d97* and *d75* double mutants in LD and LL. A set of plants (Col, *d97* and *d75*) was grown in the 12 h light/12 h dark cycles (LD) for 20 d, and then they were released to LL (white light 70 μmol m^{-2} s^{-1}). Other details are given in Fig. 2. With regard to the two types of horizontal arrows, see the text. With regard to the reproducibility of these data, see the references cited in Table 1.

Circadian rhythms in the prr7 prr5 double mutant

We recently reported that the free-running rhythms of certain clock-controlled genes were severely perturbed in the *d75* double mutant plants (Nakamichi et al. 2005). The observed

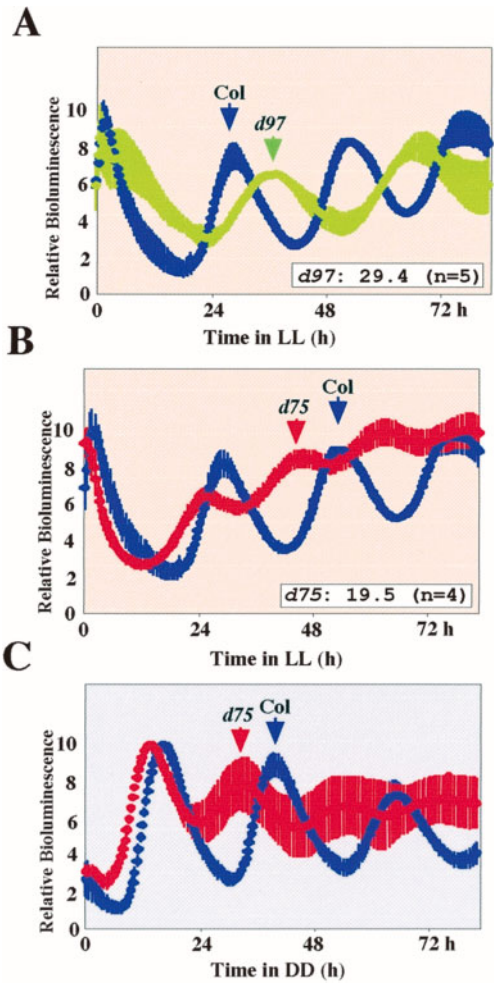


Fig. 4 Characterization of the *d97* and *d75* double mutants with reference to the free-running rhythms of *CCA1*::*LUC*. A number of samples were examined independently, and the monitored rhythms are collectively presented in a comparison with those of Col (blue): (A) *d97* in LL (green), (B) *d75* in LL (red), (C) *d75* in DD. Other details are the same as those given in Fig. 1.

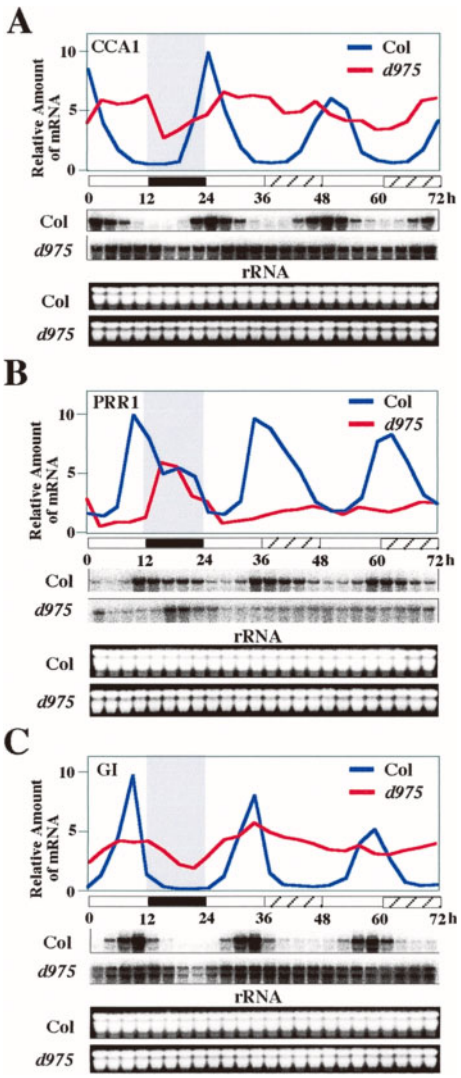


Fig. 5 Characterization of *d975* double mutants. Northern blot hybridization analyses of the transcripts of certain clock-controlled genes in the *d975* triple mutant in LD and LL. The pair of plants (Col and *d975*) was grown in 12 h light/12 h dark cycles (LD) for 20 d, and then they were released to LL (white light). Northern blot hybridization was carried out with probes specific to (A) *CCA1*, (B) *PRR1* and (C) *GI*. The hybridized bands were detected with a phosphoimage analyzer (BAS-2500, FujiXerox, Tokyo, Japan) (lower panels), and also quantified (blue lines for Col, red lines for Δ*5*/*7*) (upper panels). In these experiments, the content of rRNA in each lane was analyzed as an internal and loading reference, as indicated. These are representatives of several independent experiments.

phenotype was best interpreted as arrhythmia in LL and continuous darkness (DD) and/or markedly short period with reduced amplitude. Here we confirmed this view by examining the *d75* double mutant in comparison with the *d97* double mutant (Fig. 3A–C). In sharp contrast to *d97*, the *d75* mutant exhibited a phenotype of extremely short period (see bi-directional arrows). We again confirmed this event by employing *d75* double mutant lines carrying *CCA1*::*LUC* (Fig. 4B, 4C). The results supported the view that the *d75* double lesions result in a marked phenotype of short period with reduced amplitude in both LL and DD (19.5 ± 0.6 h in LL). Considering the fact that the *d5* single mutant shows a weak phenotype of short period, one can envisage that PRR7 coordinately (or synergistically) enhances the function of PRR5, again despite the fact that the *d7* single mutant itself showed no clear phenotype with regard to the circadian rhythms (Fig. 1).

Diurnal rhythms in d97 and d75 under light entrainment conditions

We previously noticed an interesting phenomenon that the *d75* mutant plants showed an anomaly of diurnal oscillation of

certain circadian-controlled genes even under the light entrainment (LD) conditions (Nakamichi et al. 2005). The observed anomaly for *d75* was an advanced phasing of certain clock-controlled genes (e.g. *PRR1/TOC1* and *GI*), as compared with in Col. Such events were reproducibly seen here (see unidirectional arrows in Fig. 3). Interestingly, it was also noticed that the *d97* mutant showed such an anomaly in LD; however, conversely, *d97* showed a delayed phasing (column-*d97*, see unidirectional arrows). These observations were consistent with the general idea that altered clock functions with longer/shorter periods under free-running (LL and DD) conditions per se would result in delayed/advanced (later/earlier) phases under entrainment (or natural LD) conditions. It should also be noted that this particular event in *d75* in LD was observed even under the temperature entrainment conditions (Nakamichi et al. 2005). This fact suggests that the *d75* phenotypes cannot simply (or only) be attributed to a defect in the light signal input pathway. Essentially the same idea was also proposed for the *d97* phenotypes (Salome and McClung 2005).

Circadian rhythms in the prr9 prr7 prr5 triple mutant

We finally characterized the *d975* mutant line (Fig. 5). In the *d975* triple mutant, the free-running rhythms of *CCA1*, *PRR1/TOC1* and *GI* were rapidly (or immediately) dampened in LL. The expression profile of *LHY* in *d975* was also examined (data not shown), and the results were very similar to those observed for *CCA1* (Fig. 5A). These phenotypes of *d975* (i.e. arrhythmia) were much more severe than those observed for the respective *d97* and *d75* double mutants (compare Fig. 3 and 5). The diurnal oscillations of the genes examined in LD were also markedly perturbed in the *d975* triple mutant (Fig. 5, see anomalous profiles in the mutant during the first LD 24 h). More interestingly, the expression of *CCA1* in *d975* was increased constitutively to a peak level in LL (Fig. 5A), while the expression of *PRR1/TOC1* was markedly decreased to a trough level (Fig. 5B). These results are entirely compatible with the idea that PRR9/PRR7/PRR5 together play an essential role(s) close to the central oscillator. It was also suggested that PRR9/PRR7/PRR5 together exert a negative effect on the transcription of *CCA1*, while they positively affect the transcription of *PRR1/TOC1*. In short, the clock in the plants lacking all the *PRR9/PRR7/PRR5* genes appears to be almost out of order.

Other circadian-associated phenotypes

In general, mutational lesions in any one of the clock-associated components, if not all, affect not only circadian rhythms at the level of transcription of clock-controlled genes, but also photomorphogenic responses (Deng and Quail 1999, Dowson-Day and Millar 1999, Quail 2002, Kaczorowski and Quail 2003) and/or control of flowering time (Carre 2001, Mouradov et al. 2002, Young and Kay 2001, Yanovsky and Kay 2002). For example, the *cca1 lhy* double mutant displays a marked phenotype of early flowering under the photoperiodic

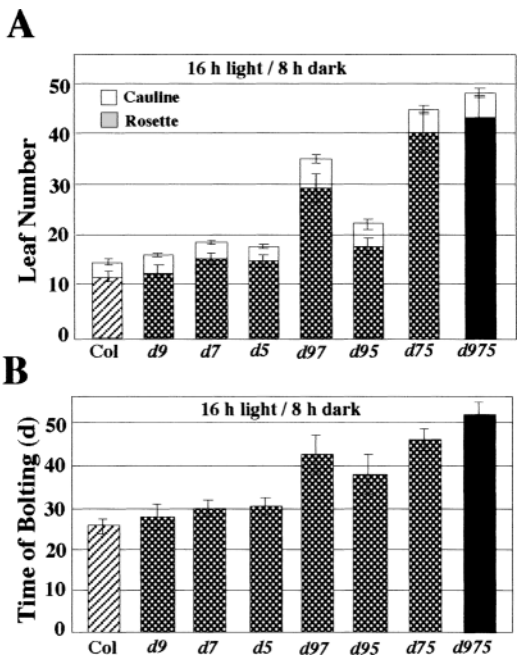
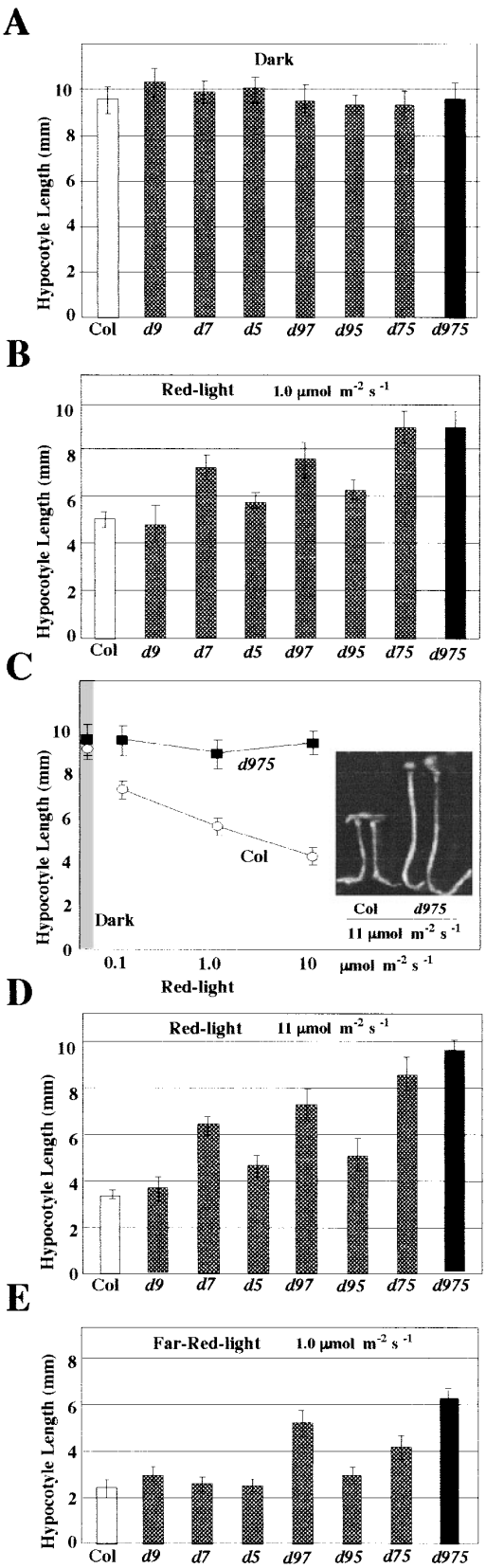


Fig. 6 Characterization of the flowering time of a set of *prr* mutants in the long-day photoperiodic conditions. Characterized plants were Col, *d9*, *d7*, *d5*, *d97*, *d95*, *d75* and *d975*, as indicated. Upper panel: for the number of leaves at flowering, the leaf count was taken on the day when the flower primordia were first observed on a given plant. Lower panel: days to visible inflorescence (about 1 cm) were defined as the time at which a given plant possessed the flower primordial (with the naked eye). The examinations were carried out statistically (n >8 each). Other details are given in Materials and Methods. With regard to the reproducibility of these data, see the references cited in Table 1.

short-day conditions, whereas the *toc1-2* de-etiolated seedlings are strikingly hyposensitive to red light (or far-red light), giving rise to longer hypocotyls than the wild type (C24) during photomorphogenic responses (Table 1). These pleiotropic phenotypes may be attributed primarily (or at least in part) to defects in the circadian clock per se, as has been generally thought. In fact, we previously reported that the *d75* line also showed such pleiotropic phenotypes of extremely late flowering in a manner independent of photoperiodicity, and marked hyposensitivity of seedlings to red light during de-etiolation (Table 1, Nakamichi et al. 2005). We then wanted systematically to characterize the set of combinatorial *prr* mutants with reference to these circadian-associated phenotypes, because such data for *d97*, *d95* and *d975* are currently not available (Table 1). These examinations were done (Fig. 6, 7), showing that the *d975* triple mutant is severely defective not only in the clock function, but also in the control of flowering time and the response to red light during de-etiolation.

Control of photoperiodicity-dependent flowering time

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, under



which the circadian-controlled *CO* gene plays a crucial role (Mouradov et al. 2002, Yanovsky and Kay 2002). We and others previously suggested that the PRR1/TOC1 family members also play roles in the control of flowering time (Somers et al. 1998a, Sato et al. 2002, Michael et al. 2003, Yamamoto et al. 2003, Murakami et al. 2004). The set of mutant seeds were sowed on soil, and then they were grown under the long-day (16 h light/8 h dark cycle) and short-day (10 h light/14 h dark cycle) conditions. The flowering times of these mutants were statistically monitored by counting the leaf number upon the onset of flowering under the long-day conditions (Fig. 6A), and also by measuring the time of bolting under the long-day (Fig. 6B) and short-day (data not shown) conditions. The late flowering phenotypes of *d7*, *d5* and *d95* were apparent, but rather subtle. A synergistic effect (i.e. marked phenotype of late flowering) was seen when the *d7* allele was combined with either *d9* or *d5* under the long-day photoperiod conditions. Such late-flowering phenotypes in these mutant plants were not evident under the short-day photoperiod conditions (data not shown). However, it may be noted that the flowering times of *d97* and *d975* plants were considerably delayed even in the short-day conditions, as compared with other mutants (data not shown). Thus, this interesting event remains to be carefully examined further (see Discussion). In any event, the results showed that the flowering time of *d975* was more or less insensitive to the photoperiodicity, suggesting that these three PRR proteins together play essential and positive roles in the photoperiodicity-dependent control of flowering time.

Light signal transduction that controls the length of hypocotyls during de-etiolation

Some circadian-associated genes are implicated in certain light signal transduction pathways (or photomorphogenic responses) (Quail 2002). A visible and biological hallmark of such light signal transduction is the inhibition of hypocotyl elongation during de-etiolation under light with a given spectrum. 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). When the set of mutant seeds were germinated in the dark, the hypocotyl lengths of these etiolated seedlings were similar to each other (Fig. 7A). When they were germinated in continuous red light (fluence rate,

Fig. 7 Characterization of red light responses of a set of *prr* mutant plants in early photomorphogenesis. The set of seedlings (Col, *d9*, *d7*, *d5*, *d97*, *d95*, *d75* and *d975*) were grown for 72 h in (A) darkness; (B) red light with a fluence rate of 1.0 μmol m^{-2} s^{-1}; (C) red light fluence rate response curves as to the lengths of hypocotyls were statistically examined for Col and *d975* seedlings, also showing representative pictures of seedlings; (D) red light with a fluence rate of 11 μmol m^{-2} s^{-1}; (E) far-red light with a fluence rate of 1.0 μmol m^{-2} s^{-1}. The resulting lengths of hypocotyls of seedlings were statistically examined (n >25 for each). Other details are given in Materials and Methods.

1 μmol m^{-2} s^{-1}), the average hypocotyl lengths varied considerably amongst the mutants (Fig. 7B). These events were observed consistently over a broad range of red light fluence rates (Fig. 7C). The typical results were best explained by assuming that the mutational lesion of the *PRR7* gene most severely affects the red light sensitivity of seedlings (Fig. 7D, fluence rate, 11 μmol m^{-2} s^{-1}). In other words, a synergistic effect (i.e. marked phenotype of hyposensitivity to red light) was seen when the *d7* allele was combined with either *d9* or *d5*. As a result, the *d975* triple mutant seedlings were almost blind to red light, as far as the de-etiolation under red light was concerned. Similar incidences of light sensitivity were seen under far-red light (Fig. 7E, fluence rate, 1 μmol m^{-2} s^{-1}). Unlike under red light, however, the *d975* mutant could respond to far-red light to a certain extent. When the seeds were germinated under blue light, such changes in the sensitivity were not evident for the set of *prr* mutants (data not shown), as has previously been documented (Yamamoto et al. 2003, Nakamichi et al. 2005). These results together suggested that PRR9/PRR7/PRR5 are crucially implicated as positive effectors in a branch of phytochrome-mediated red and/or far red light signal transduction pathways.

Discussion

Despite the fact that their amino acid sequences are highly homologous to each other, it is currently not believed that the PRR family members (PRR9, PRR7, PRR5 and PRR3) other than PRR1/TOC1 are components of the central clock. Here we would like to change this view slightly, based on the results of this study (also recent results summarized in Table 1). Several research groups independently and consistently provided evidence for the overview summarized in Table 1 (and references therein). Here we further showed that the clock function was severely perturbed in the mutant plants concomitantly lacking all the functions of PRR9, PRR7 and PRR5 (i.e. the *d975* triple mutant): (i) they showed a phenotype of arrhythmia in LL, and they also displayed an anomalous phasing in diurnal oscillation of certain circadian-controlled genes even in LD (Fig. 5); (ii) they showed a phenotype of extremely late flowering (Fig. 6); and (iii) the seedlings were blind to red light in the photomorphogenic responses during de-etiolation (Fig. 7). These results are compatible with the idea that PRR9/PRR7/PRR5 together play an essential clock-associated role(s).

Nevertheless, it is also true that PRR9, PRR7 and PRR5 are dispensable (or non-essential) in the strict sense that the circadian-associated phenotypes were subtle (or marginal) in the respective single mutant (*d9*, *d7* and *d5*) (Fig. 1) and even in the *d95* double mutant (Fig. 2). These genetic data could formally be explained by assuming that the circadian-associated functions of PRR9/PRR7/PRR5 are redundant. However, we needed to envisage a more complicated scenario, when we were then faced with the puzzling phenotypes of the *d97* and *d75* double mutants, respectively (Fig. 3, 4): the *d97* double mutant plants showed a marked phenotype of long period, whereas the *d75* double mutant showed a striking phenotype of short period. In other words, the mutational lesions of *d9* and *d5* were markedly and respectively exaggerated in the absence of PRR7, despite the fact that the *d7* single mutant itself showed no clear phenotype (if any) with regard to the period under the conditions tested. These results suggested that their roles are not simply redundant. The partially overlapping and clearly distinctive roles of PRR9/PRR7/PRR5 appear to be tightly coupled to each other, coordinately, complementarily and temporarily. As the results show, these PRR members together might constitute a period-controlling circuitry. In other words, the PRR9/PRR7/PRR5 circuitry might serve as a pacemaker that finely tunes the periods of rhythms by either shortening or lengthening depending on certain conditions. Furthermore, this presumed PRR9/PRR7/PRR5 circuitry must be tightly coupled to (or interlocked with) the main clock consisting of CCA1 and TOC1, because the circadian clock is almost out of order in plants lacking the PRR9/PRR7/PRR5 circuitry. These ideas are intriguing, when we consider the current view that the positive/negative transcription cycle through *CCA1* and *TOC1* is only a framework onto which other period-affecting factors must be incorporated in order to make the central oscillator capable of incorporating time lags of many hours to culminate in circadian (~24 h) rhythm. Indeed, we demonstrated here that the PRR9/PRR7/PRR5 circuitry could control the periods over a very wide range (from ~19 h in *d75* to ~30 h in *d97*) (Fig. 4).

The ideas hypothesized above as to the overlapping and distinctive functions of PRR9/PRR7/PRRR5 must be considered more specifically. To do so, first we would like to schematically summarize the crucial genetic results of this study, because they are apparently complicated (Table 1 and Fig. 8). In Fig. 8, it should be first pointed out that the transcripts of *CCA1* (and *LHY*) start accumulating late at night and peak in the morning (see the middle rectangle). The transcription of *PRR1*/*TOC1* starts in the evening and continues to midnight. Therefore, the transcription of *CCA1* (and *LHY*) and *PRR1*/*TOC1* (or the existence of these transcripts) appears to be mutually exclusive. This is the basis of the autoregulatory feedback model of the central clock. According to this timetable, the transcript of *PRR9* starts accumulating immediately after dawn. It was then followed by the sequential transcription of *PRR7* and *PRR5* at 2–3 h intervals (as also indicated in the circle in Fig. 8, Matsushika et al. 2000). We should also remember that both the *cca1-1* and *toc1-2* loss-of-function mutants display the similar phenotype of short period in LL (Table 1). In this context, it is generally considered that the period is the fundamental parameter of the clock function per se (the upper box in Fig. 8), implying that CCA1 and TOC1 also act independently as period-controlling factors that lengthen the period.

Based on these fundamental views, the first new result of this study is: the mutational lesion in the *PRR9* gene results in a weak phenotype of long period, whereas the mutational lesion

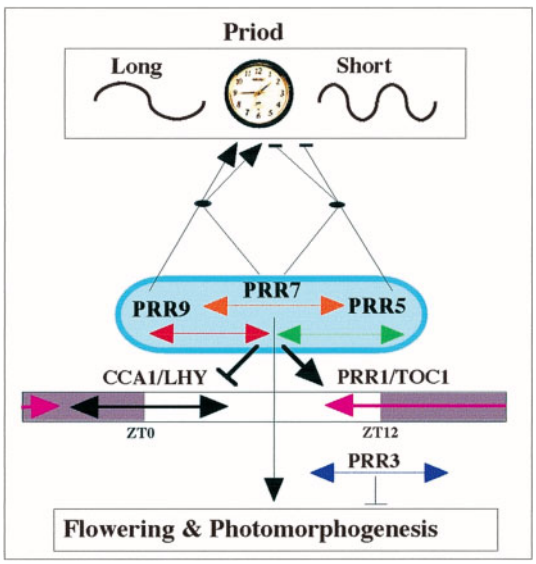


Fig. 8 A schematic and summarized view of the findings of this study with regard to the phenotypes of a set of *prr* mutants. This illustration was intended solely to summarize schematically the consistent findings of this study (and also of other groups, see Table 1) in relation to the relevant characteristics of the central clock components, CCA1 (and LHY) and PRR1/TOC1. In other words, this was not intended to propose any molecular model. We would like to emphasize only the plausible view that PRR9/PRR7/PRR5 together play essential roles close to the main (or central) clock, and that the PRR9/PRR7/PRR5 circuitry interlocks with the central clock loop through negative/positive linkages at the level of transcription, as schematically illustrated. Other details are discussed in the text.

in the *PRR5* gene results in a weak phenotype of short period, while the effect of the single mutational lesion of *PRR7* on the circadian rhythm is marginal, if any (Fig. 1). This suggests that PRR5 serves as a factor that lengthens the period (like a 'brake' indicated by T-bars in Fig. 8). In contrast, PRR9 is unique because this appears to shorten the period (like an 'accelerator' indicated by arrows in Fig. 8). PRR7 is puzzling in that this factor itself seemingly has no ability to modulate the period, because the *d7* single mutant showed a very marginal phenotype with regard to circadian rhythms (Fig. 1). This might mean that PRR7 indeed has nothing to do with the period. Alternatively, PRR7 might in fact be a sophisticated period-affecting factor, the mutational lesion of which has been genetically masked in the presence of both the *PRR9* and *PRR5* genes. We would like to favor the latter view, because the puzzling phenotypes of both the *d97* and *d75* double mutants could reasonably be explained on the basis of the latter view, as follows. In this respect, the second important result of this study is: on the one hand, PRR7 coordinately (or synergistically) enhances the function of PRR9, on the other hand, PRR7 coordinately (or synergistically) enhances the function of PRR5 (Fig. 3, 4), resulting in the quite different consequences in *d97* and *d75* (extremely long or short period, respectively). These findings led us to envisage that PRR7 might act as a bi-functional (or bi-directional) period-controlling factor: PRR7 might be able to shorten and also to lengthen the period, depending on the partners (PRR9 or PRR5) and/or the diurnal conditions (e.g. changes in light quality and intensity from morning to evening), as schematically shown in Fig. 8. This idea is consistent with the proposal that PRR7 appears to be involved in the red light intensity-dependent circadian entrainment (Farre et al. 2005). Based on these hypothetical views, one can a priori explain each phenotype of the *prr* mutants with regard to the circadian period (see Fig. 8): *d9* (slightly long, i.e. strong brake and a soft accelerator), *d7* (compromised phenotype), *d5* (slightly short, i.e. strong accelerator and a soft brake), *d97* (markedly long period, i.e. no accelerator), *d95* (compromised phenotype), *d75* (enhanced short period, i.e. no brake). Considering the fact that the *d75* double mutants showed the altered rhythms with reduced amplitudes in LL (see *CCA1* in Fig. 3, 4), it is possible that these PRR members might also be involved in the stabilization of amplitude. These explanations are quite superficial, and they do not provide us with any molecular bases underlying the functions of these PRRs. However, at least one can conclude that the PRR9/PRR7/PRR5 circuitry together play overlapping and distinctive roles very close to (or within) the central clock.

Regardless of whether the above hypothetical views are correct or not, the third conclusion of this study is that these PRR members together are essential for the clock function per se, because the circadian clock is almost out of order in the plants lacking these proteins (Fig. 5). This suggests that there is a tight linkage between the PRR9/PRR7/PRR5 circuitry and the central clock. In other words, the PRR9/PRR7/PRR5 circuitry might somehow be tightly interlocked with (or integrated into) the central clock. In this respect, the fourth new finding of this study is that the expression of *CCA1* was constitutively derepressed in *d975*, whereas the expression of *PRR1/TOC1* was severely attenuated (Fig. 5). It is thus tempting to speculate that the mode of linkage between the PRR9/PRR7/PRR5 circuitry and the central clock might be a negative/positive transcriptional regulation (as also indicated in Fig. 8). This view is consistent with the model proposed recently for the interaction between CCA1/LHY and PRR9/PRR7 (Farre et al. 2005). These issues will be addressed more intensively elsewhere (Mizuno and Nakamichi 2005).

Finally, we should discuss other circadian-associated phenotypes of the set of *prr* mutants. With regard to the photoperiodicity-dependent control of flowering time and the red light sensitivity during de-etiolation, the phenotypes of the set of *prr* mutants could be interpreted rather straightforwardly (Fig. 6, 7). PRR9/PRR7/PRR5 together are crucially involved in these circadian-associated signaling pathways, and they act as positive factors. Interestingly, PRR7 plays a prominent role in these processes, despite the fact that the *d7* single mutant itself showed no clear phenotype with regard to the circadian rhythms (Fig. 1). In any case, the *d975* mutant exhibits striking

phenotypes: late flowering that was no longer sensitive to the photoperiodicity, and hyposensitivity (or blind) to red light in the photomorphogenic responses. Nonetheless, in this study, we were faced with several new puzzles. (i) It should be noted that the flowering time of *d97* and *d975* plants is slightly delayed even in the short-day conditions, as compared with other mutants (e.g. Col and *d75*). Thus, it would be of interest to examine *d975* in terms of other flowering pathways (gibberellin and/or autonomous pathways) (Yanovsky and Kay 2002, Yanovsky and Kay 2003). (ii) The phenotype of *d975* is very similar to the *phyB* null mutants, as far as the red light sensitivity during de-etiolation is concerned (see Fig. 7C). However, the *d975* plants are extremely late flowering, while the *phyB* mutant plants are early flowering. (iiii) More puzzlingly, both the *d97* and *d75* double mutants showed essentially the same phenotypes (late flowering and hyposensitivity), suggesting that there is no simple correlation between the periods of intrinsic rhythm in LL (or the apparent phases in LD, see Fig. 4) and the consequences of these particular output pathways. This event is puzzling but not surprising, because it is well known that the *cca1-1* mutant shows the phenotypes of short period and hypersensitivity, whereas the *toc1-2* mutant displays the phenotype of short period and hyposensitivity (Table 1). Therefore, clarification of these general and apparent problems emerging from the *d975* triple mutant might provide us with new insights into the molecular linkages between the circadian rhythms, control of flowering time and light signal transduction. Meanwhile, it may be noted that the phenotypes of *d975* are essentially the same as those (late flowering and hyposensitivity) of *CCA1*-overexpressing transgenic lines (Green and Tobin 1999). In fact, the morphologies of *d975* plants, grown under the long-day conditions, were also considerably similar to those of CCA1-overexpressing transgenic plants (e.g. tall with a thick stem, data not shown). Together with the fact that *CCA1* is misexpressed and the expression of *PRR1*/*TOC1* is severely repressed in *d975* (as discussed above), it is reasonable to assume that the PRR9/PRR7/PRR5 circuitry might indirectly affect these output regulatory pathways through the interactions with CCA1 (and LHY), PRR1/TOC1 and/or some other interacting factors (e.g. ZTL, LKP2 and PIF3). There is indeed a piece of evidence that PRR1/TOC1 physically interacts with PRR9 as well as PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Yamashino et al. 2003, Ito et al. 2003, Fujimori et al. 2004), and also that ZTL (and LKP2) interacts with PRR5 as well as PRR1 (Mas et al. 2003b, Yasuhara et al. 2004). These issues also remain to be addressed in the context discussed above.

In short, the results of this study and the hypothetical views proposed here provide new insight into the mechanisms underlying circadian-associated events in plants. Nonetheless, we know nothing about the molecular modes of actions of the PRR family members. We must conduct further genetic and biochemical examinations, which should include the examination of the least characterized *PRR3* gene, the transcript of which appears in the evening more or less with the same timing as that of *PRR1/TOC1* (Matsushika et al. 2000). As an approach within this context, here we finally characterized the rhythmic expression profiles of *PRR3* in the set of double and triple mutant (Fig. 9). The results were fully consistent with the notions documented already for the phenotypes with reference to the rhythms of *CCA1*, *PRR1*/*TOC1* and *GI*. More importantly, the expression of *PRR3* was constitutive in *d975* (Fig. 9), which is in sharp contrast to the case of *PRR1*/*TOC1* (Fig. 5). We recently characterized PRR3-overexpressing transgenic plants, showing that they displayed interesting phenotypes (late flowering and hyposensitivity) (Murakami et al. 2004), which were in contrast to those (early flowering and hypersensitivity) observed for PRR1-overexpressing transgenic lines and PRR5-overexpressing lines (Makino et al. 2002, Sato et al. 2002). Therefore, PRR3 appears to serve uniquely as a negative regulator in the relevant signaling pathways (as also indicated in Fig. 8). PRR3 might play a role distinctive from (or antagonistic to) other PRR members, and such a presumed unique role for PRR3 must also be incorporated into the framework of Fig. 8 eventually.

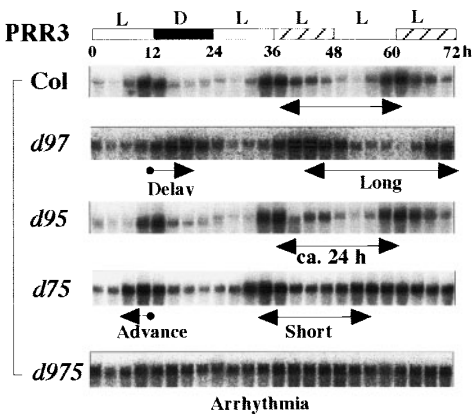


Fig. 9 Characterization of a set of *prr* double and triple mutants with special reference to the expression of *PRR3*. Northern blot hybridization analyses of the transcript of PRR3 were carried out for the indicated plants (Col, *d97*, *d95*, *d75* and *d975*) grown in LD and LL. Other details are the same as those given in Fig. 5.

Materials and Methods

Plant growth conditions and related materials

Arabidopsis thaliana (Columbia accession, Col) was mainly used as the wild-type plant. 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, 12 h light/12 h dark or 10 h light/14 h dark, as specifically noted for each experiment in the text.

Preparing RNA, and Northern blotting

Total RNA was isolated from appropriate organs (mainly leaves) of *Arabidopsis* plants by the aurintricarboxylic acid (ATA) method. For Northern blot hybridization, RNA was separated in agarose gels (1%) containing 0.67 M formaldehyde, then transferred to Hybond-N+ membranes. The fixed membranes were hybridized with ^{32}P-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 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 phosphoimage analyzer (BAS-2500II) (FujiXerox, Tokyo, Japan).

Probes for Nothern blot hybridization

Several double-stranded ^{32}P-labeled DNA probes were used to detect each specific mRNA. The probes used were amplified by polymerase chain reaction (PCR) with appropriate sets of primers, which were designed appropriately, including *CCA1*, *GI*, *PRR1/TOC1*, *PRR3*, *PRR5*, *PRR7* and *PRR9*, as described previously (Makino et al. 2002, Yamamoto et al. 2003). Each ^{32}P-labeled probe was prepared with the Megaprime DNA Labeling System (TaKaRa Shuzo, Kyoto, Japan).

Biomuminescence assays

The *CCA1*::*LUC* reporter gene was constructed previously (the construct-c corresponding to a protein fusion, Nakamichi et al. 2004). Appropriate lines of plants were transformed with a binary vector carrying the *CCA1*::*LUC* gene by means of the *Agrobacterium tumefaciens*-mediated DNA delivery method (Makino et al. 2002). In every case, several independent transgenic (T1) seedlings were obtained by monitoring the bioluminescence activities detected by photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan). Then, transgenic plants (from T2 seeds) were grown for 10 d in LD (white light of 70 μmol m^{-2} s^{-1}) on MS plates which contained luciferin (8 μg ml^{-1}) and cefotaxime (100 μg ml^{-1}) (note that each single plant was grown in a separate plate). The plate containing the young seedling was transferred into LL (white light, 70 μmol m^{-2} s^{-1}) (on the 11th d, ZT0), and then the plant was analyzed by the real-time bioluminescence monitoring and the auto-calculating system, as described previously (Kondo et al. 1993, Nakamichi et al. 2004). In every case, several independent transgenic plants were examined to obtain consistent and reproducible results (see Fig. 1, 2, 4).

Examination of light response in early photomorphogenesis.

To examine the light response in early photomorphogenesis of plants, seeds were sowed on gellangum (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 at 22°C 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 [λ_{max} = 660 nm at 30 μmol m^{-2} s^{-1} (TOKYO RIKA, Japan)]; far-red, STICK-mFR (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 HYPONEX (N : P : K = 5 : 10 : 5) (HYPONEX-JAPAN, Osaka, Japan). They were cold-treated at 4°C for 3 d in the dark. They were grown in chambers (22°C) under the conditions of long day (16 h light/8 h dark, 80 μmol m^{-2} s^{-1}) or short day (10 h light/4 h dark, 120 μmol m^{-2} s^{-1}).

Acknowledgments

For *Arabidopsis* mutant seeds, we would like to thank The Salk Institute Genomic Analysis Laboratory (California, USA), The Arabidopsis Biological Resource Center (Columbus, OH, USA) and Kazusa DNA Research Institute (Chiba, Japan). This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan, and also the Ministry of Agriculture, Forestry and Fisheries of Japan. N.N. was supported by the Japanese Society for Promotion of Science Fellowships for Young Scientists (16005828).

References

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, F.G., Mas, P. and Kay, S.A. (2001) Reciprocal regulation between *TOC1* and *LHY/CCA1* within the *Arabidopsis* circadian clock. *Science* 293: 880–883.

Alabadi, D., Yanovsky, M.J., Mas, P., Harmer, S.L. and Kay, S.A. (2002) Critical role for CCA1 and LHY in maintaining circadian rhythmicity in *Arabidopsis*. *Curr. Biol.* 12: 757–761.

Carre, I.A. (2001) Day-length perception and the photoperiodic regulation of flowering in *Arabidopsis*. *J. Biol. Rhythms* 16: 415–423.

Covington, M.F., Panda, S., Liu, X.L., Strayer, C.A., Wagner, D.R. and Kay, S.A. (2001) ELF3 modulates resetting of the circadian clock in *Arabidopsis*. *Plant Cell* 13: 1305–1316.

Deng, X.W. and Quail, P.H. (1999) Signaling in light-controlled development. *Semin. Cell Dev. Biol.* 10: 121–129.

Dowson-Day, M.J. and Millar, A.J. (1999) Circadian dysfunction causes aberrant hypocotyl elongation patterns in *Arabidopsis*. *Plant J.* 17: 63–71.

Doyle, M.R., Davis, S.J., Bastow, R.M., McWatters, H.G., Kozma-Bognar, L., Nagy, F., Millar, A.J. and Amasino, R.M. (2002) The ELF4 gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. *Nature* 419: 74–77.

Eriksson, M.E., Hanano, S., Southern, M., Hall, A. and Millar, A.J. (2003) Response regulator homologues have complementary, light-dependent functions in the *Arabidopsis* circadian clock. *Planta* 218: 159–162.

Eriksson, M.E. and Millar, A.J. (2003) The circadian clock. A plant's best friend in a spinning world. *Plant Physiol.* 132: 732–738.

Farre, M.E., Harmer, S.L., Harmon, F.G., Yanovsky, M.J. and Kay, S.A. (2005) Overlapping and distinct roles of PRR7 and PRR9 in the *Arabidopsis* circadian clock. *Curr. Biol.* 15: 47–54.

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. and Putterill, J. (1999) *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *EMBO J.* 18: 4679–4688.

Fujimori, T., Yamashino, T., Kao, T. and Mizuno, T. (2004) Circadian-controlled basic/helix–loop–helix factor, PIL6, implicated in light-signal transduction in *Arabidopsis thaliana*. *Plant Cell Physiol.* 45: 1078–1086.

Green, R.M. and Tobin, E.M. (1999) Loss of the circadian clock-associated protein 1 in *Arabidopsis* results in altered clock-regulated gene expression. *Proc. Natl Acad. Sci. USA* 96: 4176–4179.

Hicks, K.A., Albertson, T.M. and Wagner, D.R. (2001) *Early flowering3* encodes a novel protein that regulates circadian clock function and flowering in *Arabidopsis*. *Plant Cell* 13: 1281–1292.

Huq, E., Tepperman, J.M. and Quail, P.H. (2000) GIGANTEA is a nuclear protein involved in phytochrome signaling in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* 97: 9789–9794.

Ito, S., Matsushika, A., Yamada, H., Sato, S., Kato, T., Tabata, S., Yamashino, T. and Mizuno, T. (2003) Characterization of the APRR9 pseudo-response regulator belonging to the APRR1/TOC1 quintet in *Arabidipsis thaliana*. *Plant Cell Physiol.* 44: 1237–1245.

Jarillo, J.A., Capel, J., Tang, R.H., Yang, H.Q., Alonso, J.M. and Cashmore, A.R. (2001) An *Arabidopsis* circadian clock component interacts with both CRY1 and phyB. *Nature* 410: 487–490.

Kaczorowski, K.A. and Quail, P.H. (2003) *Arabidopsis PSEUDO-RESPONSE REGULATOR7*is a signaling intermediate in phytochrome-regulated seedling deetiolation and phasing of the circadian clock. *Plant Cell* 15: 2654–2665.

Kondo, T., Strayer, C.A., Kulkarni, R.D., Taylor, W., Ishiura, M., Golden, S.S. and Johnson, C.H. (1993) Circadian rhythms in prokaryotes: luciferase as a reporter of circadian gene expression in cyanobacteria. *Proc. Natl Acad. Sci. USA* 90: 5672–5676.

Makino, S., Kiba, T., Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Ueguchi, C., Sugiyama, T. and Mizuno, T. (2000) Genes encoding pseudo-response regulators: insight into His-to-Asp phosphorelay and circadian rhythm in *Arabidopsis thaliana*. *Plant Cell Physiol.* 41: 791–803.

Makino, S., Matsushika, A., Kojima, M., Oda, Y. and Mizuno, T. (2001) Light response of the circadian waves of the APRR1/TOC1 quintet: when does the quintet start singing rhythmically in *Arabidopsis*? *Plant Cell Physiol.* 42: 334–339.

Makino, S., Matsushika, A., Kojima, M., Yamashino, T. and Mizuno, T. (2002) The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: I. Characterization with APRR1-overexpressing plants. *Plant Cell Physiol.* 43: 58–69.

Mas, P., Alabadi, D., Yanovsky, M.J., Oyama, T., Kay, S.A. (2003a) Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. *Plant Cell* 15: 223–236.

Mas, P., Kin, W.Y., Somers, D.E. and Kay, S.A. (2003b) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. *Nature* 426: 567–570.

Matsushika, A., Imamura, A., Yamashino, T. and Mizuno, T. (2002b) Aberrant expression of the light-inducible and circadian-regulated *APRR9* gene belonging to the circadian-associated APRR1/TOC1 quintet results in the phenotype of early flowering in *Arabidopsis thaliana*. *Plant Cell Physiol.* 43: 833–843.

Matsushika, A., Makino, S., Kojima, M., and Mizuno, T. (2000) Circadian waves of expression of the APRR1/TOC1 family of pseudo-response regulators: insight into the plant circadian clock in *Arabidopsis thaliana*. *Plant Cell Physiol.* 41: 1002–1012.

Matsushika, A., Makino, S., Kojima, M., Yamashino, T. and Mizuno, T. (2002a) The APRR1/TOC1 quintet implicated in circadian rhythms of *Arabidopsis thaliana*: II. Characterization with CCA1-overexpressing plants. *Plant Cell Physiol.* 43: 118–122.

McClung, C.R. (2000) Circadian rhythms in plants: a millennial view. *Physiol. Plant.* 109: 359–371.

Michael, T.P., Salome, P.A., Yu, H.J., Spencer, T.R., Sharp, E.L., McPeek, M.A., Alonso, J.M., Ecker, J.R. and McClung, C.R. (2003) Enhanced fitness conferred by naturally occurring variation in the circadian clock. *Science* 302: 1049–1053.

Mizoguchi, T., Wheatley, K., Hanzawa, Y., Wright, L., Mizoguchi, M., Song, H.-R., Carre, I.A. and Coupland, G. (2002) *LHY* and *CCA1* are partially redundant genes required to maintain circadian rhythms in *Arabidopsis*. *Dev. Cell* 2: 629–641.

Mizuno, T. (1998) His–Asp phosphotransfer signal transduction. *J. Biochem. (Tokyo)* 123: 555–563.

Mizuno, T. (2004) Plant response regulators implicated in signal transduction and circadian rhythm. *Curr. Opin. Plant Biol.* 7: 499–505.

Mizuno, T. and Nakamichi, N. (2005) *Pseudo* response regulators (PRRs) or *true* oscillator components (TOCs). *Plant Cell Physiol.* 46: 677–685.

Mouradov, A., Cremer, F. and Coupland, G. (2002) Control of flowering time: interacting pathways as a basis for diversity. *Plant Cell* 14: S111–S130.

Murakami, M., Ashikari, M., Miura, K., Yamashino, T. and Mizuno, T. (2003) The evolutionarily conserved *Os*PRR quintet: rice pseudo-response regulators implicated in circadian rhythm. *Plant Cell Physiol.* 44: 1229–1236.

Murakami, M., Yamashino, T. and Mizuno, T. (2004) Characterization of circadian-associated APRR3 pseudo-response regulator belonging to the APRR1/TOC1 quintet in *Arabidopsis thaliana*. *Plant Cell Physiol.* 45: 645–650.

Murakami-Kojima, M., Nakamichi, N., Yamashino, T. and Mizuno, T. (2002) The APRR3 component of the clock-associated APRR1/TOC1 quintet is phosphorylated by a novel protein kinase belonging the WNK family, the gene for which is also transcribed rhythmically in *Arabidopsis thaliana*. *Plant Cell Physiol.* 43: 675–683.

Nakamichi, N., Ito, S., Oyama, T., Yamashino, T., Kondo, T. and Mizuno, T. (2004) Characterization of plant circadian rhythms by employing *Arabidopsis* cultured cells with bioluminescence reporters. *Plant Cell Physiol.* 45: 57–67.

Nakamichi, N., Kita, M., Ito, S., Sato, E., Yamashino, T. and Mizuno, T. (2005) The *Arabidopsis* pseudo-response regulators, PRR5 and PRR7, coordinately play essential roles for circadian clock function. *Plant Cell Physiol.* 46: 609–619.

Nakamichi, N., Matsushika, A., Yamashino, T. and Mizuno, T. (2003) Cell autonomous circadian waves of the APRR1/TOC1 quintet in an established cell line of *Arabidopsis thaliana*. *Plant Cell Physiol.* 44: 360–365.

Onai, K., Okamoto, K., Nishimoto, H., Morioka, C., Hirano, M., Kami-Ike, N. and Ishiura, M. (2004) Large-scale screening of *Arabidopsis* circadian clock mutant by a high-throughput real-time bioluminescence monitoring system. *Plant J.* 40: 1–11.

Onouchi, H., Igeno, M.I., Perilleux, C., Graves, K. and Coupland, G. (2000) *Mutagenesis* of plants overexpressing CONSTANS demonstrates novel interactions among *Arabidopsis* flowering-time genes. *Plant Cell* 12: 885–900.

Quail, P.H. (2002) Phytochrome photosensory signaling networks. *Nat. Rev. Mol. Cell. Biol.* 3: 85–93.

Salome, P.A. and McClung, C.R. (2004) The *Arabidopsis thaliana* clock. *J. Biol. Rhythms* 19: 425–435.

Salome, P.A. and McClung, C.R. (2005) Pseudo-response regulator 7 and 9 are partially redundant genes essential for the temperature responsiveness of the *Arabidopsis* circadian clock. *Plant Cell* 17: 1–14.

Sato, E., Nakamichi, N., Yamashino, T. and Mizuno, T. (2002) Aberrant expression of the *Arabidopsis* circadian-regulated APRR5 gene belonging to the APRR1/TOC1 quintet results in early flowering and hypersensitiveness to light in early photomorphogenesis. *Plant Cell Physiol.* 43: 1374–1385.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A. and Coupland, G. (1998) The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93: 1219–1229.

Schultz, T.F., Kiyosue, T., Yanovsky, M., Wada, M. and Kay, S.A. (2001) A role for LKP2 in the circadian clock of *Arabidopsis*. *Plant Cell* 13: 2659–2670.

Somers, D.E. (2001) Clock-associated genes in *Arabidopsis*: a family affair. *Philos. Trans. R. Soc. Lond. Ser. B* 356: 1745–1754.

Somers, D.E., Devlin, P.F. and Kay, S.A. (1998b) Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* 282: 1488–1490.

Somers, D.E., Kim, W.-Y. and Geng, R. (2004) The F-box protein ZEITLUPE confers dosage-dependent control on the circadian clock, photomorphogenesis, and flowering time. *Plant Cell* 16: 769–782.

Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.A. (2000) ZEITLUPE encodes a novel clock-accociated PAS protein from *Arabiopsis*. *Cell* 101: 319–329.

Somers, D.E., Webb, A.A.R., Pearson, M. and Kay, S.A. (1998a) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. *Development* 125: 485–494.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R. Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000) Cloning of the *Arabidopsis* clock gene *TOC*1, an autoregulatory response regulator homolog. *Science* 289: 768–771.

Wang, Z.-Y. and Tobin, E.M. (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED1*(*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93: 1207–1217.

Yamashino, T., Matsushika, A., Fujimari, T., Sato, S., Kato, T., Tabata, S. and Mizuno, T. (2003) A link between circadian-controlled bHLH factors and the APRR1/TOC1 quintet in *Arabidopsis thaliana*. *Plant Cell Physiol.* 44: 619–629.

Yamamoto, Y., Sato, E., Shimizu, T., Nakamich, N., Sato, S., Kato, T., Tabata, T., Nagatani, A., Yamashino, T. and Mizuno, T. (2003) Comparative genetic studies on the *APRR5* and *APRR7* genes belonging to the APRR1/TOC1 quintet implicated in circadian rhythm, control of flowering time, and early photomorphogenesis. *Plant Cell Physiol.* 44: 1119–1130.

Yanovsky, M. and Kay, S.A. (2003) Living by the calendar: how plants know when to flower. *Nat. Rev. Mol. Cell. Biol.* 4: 265–276.

Yanovsky, M.J. and Kay, S.A. (2002) Molecular basis of seasonal time measurement in *Arabidopsis*. *Nature* 419: 308–312.

Yasuhara, M., Mitsui, S., Hirano, H., Takanabe, R., Tokioka, Y., Ihara, N., Komatsu, A., Seki, M., Shinozaki, K. and Kiyosue, T. (2004) Identification of ASK and clock-associated proteins as molecular partners of LKP2 (LOV kelch protein2) in *Arabidopsis*. *J. Exp. Bot.* 55: 2015–2027.

Young, M.W. and Kay, S.A. (2001) Time zones: a comparative genetics of circadian clocks. *Nat. Rev. Genet.* 2: 702–715.

(Received February 23, 2005; Accepted March 10, 2005)