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Response regulator homologues have complementary, light-dependent functions in the *Arabidopsis* circadian clock

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Abstract *TIMING OF CAB EXPRESSION 1 (TOC1)* functions with *CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1)* in a transcriptional feedback loop that is important for the circadian clock in *Arabidopsis thaliana* (L.) Heynh. *TOC1* and its four paralogues, the *Arabidopsis PSEUDO-RESPONSE REGULATOR (PRR)* genes, are expressed in an intriguing daily sequence. This was proposed to form a second feedback loop, similar to the interlocking clock gene circuits in other taxa. We show that *prr9* and *prr5* null mutants have reciprocal period defects for multiple circadian rhythms, consistent with subtly altered expression patterns of *CCA1* and *TOC1*. The period defects are conditional on light quality and combine additively in double-mutant plants. Thus *PRR9* and *PRR5* modulate light input to the circadian clock but are neither uniquely required for rhythm generation nor form a linear series of mutual *PRR* gene regulation.

Keywords Circadian rhythm · Biological clock · *Arabidopsis* · Light regulation

Abbreviations B: blue light · *CCA1*: *CIRCADIAN CLOCK-ASSOCIATED 1* · *CCR2*: *COLD AND CIRCADIAN-REGULATED 2* · DD: constant dark-

ness · LD: light:dark · PRR: *PSEUDO-RESPONSE REGULATOR* · R: red light · *TOC1*: *TIMING OF CAB EXPRESSION 1*

Introduction

The circadian clocks of diverse organisms generate robust, 24-h biological rhythms, via gene regulatory circuits that include interlocking loops of transcriptional negative feedback (Young and Kay 2001). Light signalling pathways regulate a component(s) of the feedback loops, entraining the endogenous clock to the environmental day/night cycle. The plant circadian clock regulates leaf position and the expression of many genes, including expression of chlorophyll *a/b*-binding protein (*CAB*) genes that peak in the mid-morning and *COLD AND CIRCADIAN-REGULATED (CCR)* genes peaking late in the day. Rhythmic transcription can be monitored in vivo using bioluminescent luciferase (*LUC*) reporter genes. Genetic screens have thus identified *Arabidopsis* clock mutants, such as the 21-h-period mutant *toc1-1* (reviewed in Hayama and Coupland 2003). A regulatory loop involving *TIMING OF CAB EXPRESSION 1 (TOC1)* and *CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1)* is thought to play a central part in generating circadian rhythms in *Arabidopsis* (Hayama and Coupland 2003; Mas et al. 2003). *TOC1* and its four paralogues, the *PSEUDO-RESPONSE REGULATOR (PRR)* genes, were separately identified by their homology to the receiver domain of prokaryotic two-component signalling systems (Makino et al. 2000; Strayer et al. 2000). In canonical two-component systems, an environmental sensor regulates an associated histidine kinase, which signals to various effector proteins via a His-Asp phosphorelay mechanism. The receiver domains of *TOC1* and *PRR* proteins have substitutions at conserved residues including the phosphorylated aspartate. *PRR* transcripts accumulate

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rhythmically in the order *PRR9-PRR7-PRR5-PRR3-TOC1* with peak levels from 2 h (for *PRR9*) to 10 h (for *TOC1*, also known as *APRR1*) after dawn (Makino et al. 2000; Matsushika et al. 2000; Strayer et al. 2000). The sequence of gene expression has been proposed to result from the serial activation of *PRR7-PRR5-PRR3-TOC1*, initiated by *PRR9* (Matsushika et al. 2000). Repression of *PRR9* by overexpression of *TOC1* suggested that this linear series might be closed to form another circadian feedback loop (Makino et al. 2002). We now characterise *prr5* and *prr9* mutations and demonstrate that their circadian effects are complementary, light-dependent and inconsistent with the serial activation of *PRR* genes.

Materials and methods

Plant material and growth and conditions

All experiments were carried out in the *Arabidopsis thaliana* (L.) Heynh. Columbia (Col) ecotype. SALK lines 6280 and 7551 were generated at the Salk Genomic Analysis Laboratory Institute and identified using the Insert Watch facility (<http://www.nasc.nott.ac.uk/insertwatch>) at the Nottingham Arabidopsis Stock Centre (NASC, UK). Seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC, Ohio State University, Columbus, OH, USA) and NASC. Homozygous mutants were identified from segregating F3 by PCR amplification of the T-DNA flanking regions. Seedlings were entrained at 22 °C to 12 h light/12 h dark (LD) under cool-white light, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 8 days prior to measurements except for the etiolated seedlings, which were grown in darkness for 4 days while entraining to 12 h 18 °C/12 h 24 °C. Circadian period was measured for luminescence rhythms imaged, and leaf movement as described by Doyle et al. 2002. Hypocotyl-growth assays were done as referred to in Doyle et al. (2002).

Constructs

Luciferase constructs reporting the activity of *CCA1*, *CAB2* and *COLD AND CIRCADIAN-REGULATED 2 (CCR2)* promoters (Doyle et al. 2002), were introduced into *prr5*, *prr9* and Col using *Agrobacterium*. Multiple independent transformants were analysed for each gene and genotype.

Results

We selected SALK lines 6280 and 7551 that carried a T-DNA insertion in the *PRR5* or *PRR9* gene in the Columbia-0 background (Col). *PRR5* shares the highest sequence identity with *PRR9* (Matsushika et al. 2000). PCR analysis showed that the T-DNAs were inserted within the second exon to second intron (data not shown), consistent with publicly available data (<http://signal.salk.edu/cgi-bin/tdnaexpress>). Reverse transcription (RT)-PCR assays detected no cognate RNA from mutant seedlings, when wild types showed strong, rhythmic expression (data not shown). *prr5-1* and *prr9-1* are likely null mutations, abbreviated as *prr5* and *prr9*.

We assayed circadian output rhythms and the expression of clock-associated genes in homozygous *prr*

mutant seedlings. Figure 1a, b shows that circadian period relative to Col was 1.0–2.4 h shorter in *prr5* and 1.0–1.4 h longer in *prr9*, for *CCA1*, *CAB2* and *CCR2* expression under simulated white (red + blue, RB) light and for rhythms of leaf movement in white fluorescent light. The altered period of leaf movement co-segregated with homozygosity for the *prr* mutation (data not shown), indicating that the T-DNA insertions caused these recessive phenotypes. *CCA1* and *TOC1* RNA levels were robustly rhythmic (Fig. 1c), so neither *PRR5* nor *PRR9* is uniquely required for the expression or rhythmicity of *TOC1*. The rising and/or falling phases of these transcripts were slightly advanced in *prr5* compared to Col and slightly delayed in *prr9*. Peak levels of *TOC1* RNA were lower in *prr5* and higher in *prr9*, supporting a correlation between period and *TOC1* expression levels (Mas et al. 2003). Leaf movements in *prr5;prr9* double mutants remained robustly rhythmic with a period indistinguishable from Col (Fig. 1a).

Mutants with aberrant light signalling can alter the circadian period in a light-dependent manner (reviewed in Hayama and Coupland 2003). We therefore measured the periods of *CCA1* and/or *CCR2* expression under 10–15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ constant red light (R), blue light (B) or darkness (DD). The *prr* mutations altered the period of *CCA1* expression in B by 2 h, affected the period of *CCR2* expression in R and B to a lesser extent, but had no effect on the period of *CCA1* expression in R or of *CCR2* expression in DD (Fig. 1a, b). Etiolated *prr* seedlings also had periods that differed from Col by only 0.6 h or less. Both *prr5* and *prr9* seedlings showed mild long-hypocotyl phenotypes when grown under constant R or B at a range of fluence rates (Fig. 1d).

Discussion

The opposite period phenotypes of *prr5* and *prr9* show that *PRR5* and *PRR9* are not functionally equivalent, consistent with the differing phenotypes of plants that overexpress these genes (Matsushika et al. 2002; Sato et al. 2002). The period phenotypes were light-dependent, suggestive of a function in light input to the clock. Strong *toc1* mutations abolish circadian rhythms in conditions lacking blue light (Mas et al. 2003), whereas *PRR5* and *PRR9* had their greatest effect in conditions containing blue light. The opposite period phenotypes of *prr5* and *prr9* are not due to opposite effects on general phototransduction, because both mutants have similar effects on light-regulated hypocotyl elongation. A phase-specific mechanism can explain the period phenotypes. *PRR9* is expressed early in the subjective day, when light treatment advances the circadian phase (Covington et al. 2001). Abrogation of this phase advance in *prr9* is consistent with the long period of *prr9* under constant light (Fig. 1a); the converse applies to *prr5* and *toc1*.

The rhythms of *CCR2* expression had consistently different periods, in multiple transgenic lines, from those of *CAB* or *CCA1* expression under some conditions. The

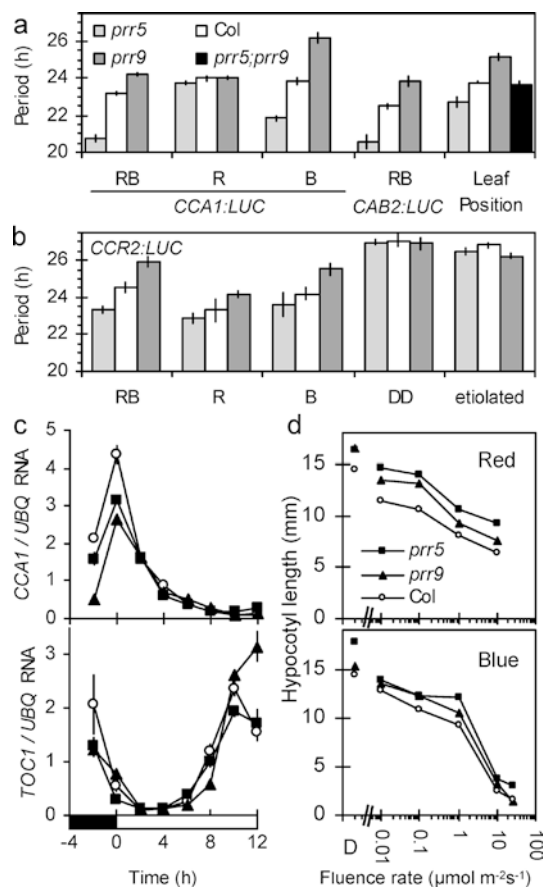


Fig. 1 a, b *prr* mutations conditionally alter the circadian period in *Arabidopsis thaliana*. *prr* mutant and wild-type seedlings carrying the *LUC* reporter genes indicated were grown for 8 days in 12 h L:12 h D cycles, and released into constant light at time 0. **a** Periods for *CCA1* and *CAB2* under constant R+B (RB), R or B, and for leaf movements under white light. **b** Periods for *CCR2* in R+B (RB), R, or B; also in darkness, in LD-entrained seedlings (DD) and in seedlings grown in darkness with 12 h 18°C:12 h 24°C temperature cycles (*etiolated*). Periods for *CCR2* in DD were measured from peak times over two cycles. Data are means \pm SE, $n=15-33$ seedlings or (*CCR2* etiolated) 8-16 groups of 20-40 seedlings. Leaf movement periods were identical in lines with and without *LUC* markers (data not shown). **c** *CCA1* (above) and *TOC1* (below) RNA levels were assayed by real-time RT-PCR relative to a ubiquitin control (*UBQ*), in extracts of *Arabidopsis* plants harvested at the times indicated during an LD cycle. Open circles, Col; filled triangles, *prr9*; filled squares, *prr5*. PCR primers and conditions are available from the authors on request. Data are means \pm SE of triplicate determinations, normalised to the mean of all measurements for each gene. Filled bar on time axis, dark interval; open bar, light interval. **d** Hypocotyl elongation was tested (Doyle et al. 2002) in *Arabidopsis* plants grown for 4 days in darkness (D) or the R (upper) or B (lower) fluence rates indicated. Data are means, $n=26-36$, SE within symbols, which are as indicated in c. For each physiological assay, 3-4 progeny families or independently transformed lines were tested with similar results in 2-5 independent experiments

effects of light and *prr* mutations also differed. Many, if not all, plant cells maintain a circadian system with a qualitatively similar molecular mechanism, so the *prr* mutations have similar effects on all rhythms. However, there are quantitative differences among rhythms in different cell types (Thain et al. 2002; Michael et al. 2003

and references therein). *CCR2* is expressed in a different spatial pattern (for example, in roots) than the other markers, so *CCR2* rhythms reflect a set of slightly different cellular clocks.

The small effects of the mutants on *TOC1* RNA levels and their opposite effects on period suggest that the *PRR* genes do not activate each other in a linear daily sequence that ultimately activates *TOC1*. Such serial activation would result in an epistatic genetic interaction between the *prr* mutants. The additive interaction that we observed in *prr5;prr9* confirms that *PRR5* and *PRR9* affect the circadian clock by largely independent mechanisms. We conclude that *PRR5* and *PRR9* participate in the complex interaction of light signalling with the circadian clock but are not required for rhythm generation.

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