Planta (2003) 218: 159–162
DOI 10.1007/s00425-003-1106-4

RAPID COMMUNICATION

Maria E. Eriksson · Shigeru Hanano
Megan M. Southern · Anthony Hall · Andrew J. Millar

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

Received: 11 June 2003 / Accepted: 23 July 2003 / Published online: 4 September 2003

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

M. E. Eriksson · S. Hanano · M. M. Southern
A. Hall · A. J. Millar (✉)
Department of Biological Sciences,
University of Warwick,
Coventry, CV4 7AL, UK
E-mail: andrew.millar@warwick.ac.uk
Fax: +44-024-76523701

Present address: M. E. Eriksson
Umeå Plant Science Centre,
Department of Plant Physiology,
Umeå University, 901 87 Umeå, Sweden

Present address: S. Hanano
Max Planck Institute for Plant Breeding Research,
Carl-von-Linné-Weg 10, 50829 Köln, Germany

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

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 μmol m^{-2} 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 μmol photons m^{-2} 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

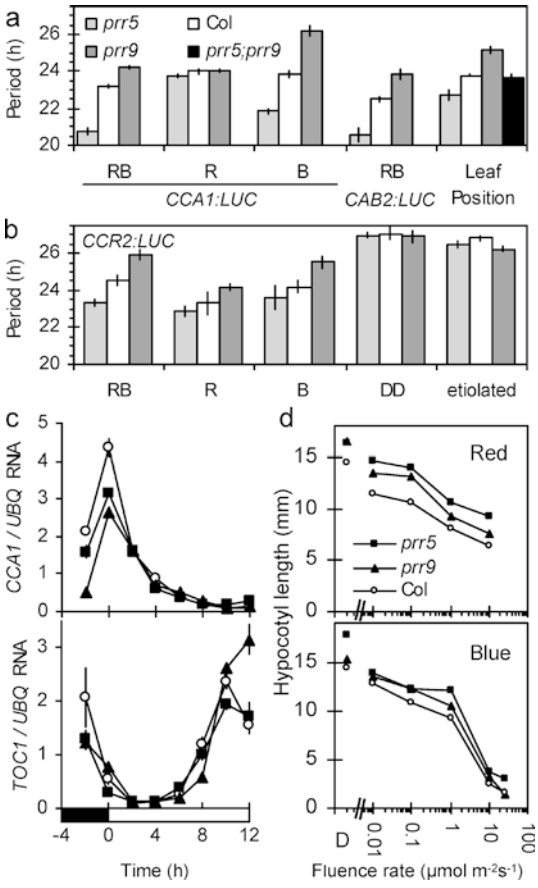

Fig. 1 a, b *prr* mutations conditionally alter the circadian period in *Arabidopsis thaliana*. *prr* mutant and wild-type seedlings carrying the *LUC* reporter genes indicated were grown for 8 days in 12 h L:12 h D cycles, and released into constant light at time 0. **a** Periods for *CCA1* and *CAB2* 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 ± 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 ± 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.

Acknowledgements M.E. Eriksson, S. Hanano and M.M. Southern contributed equally to this work. We thank Victoria Hibberd and Nazir Shariff for technical assistance, Seth Davis for discussions and preliminary data, the Salk Institute Genomic Analysis Laboratory for sequence-indexed T-DNA insertion mutants, TAIR (Huala et al. 2001) for database integration, NASC for the Insert Watch facility, and ABRC and NASC for seeds. We acknowledge a Marie Curie Fellowship from the European Union (M.E.E.), a postdoctoral Fellowship from the Japan Society for the Promotion of Science (S.H.), and a postgraduate studentship (M.M.S.) and grant G15231 from the Biotechnology and Biological Sciences Research Council (A.J.M.).

References

Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, Kay SA (2001) ELF3 modulates resetting of the circadian clock in *Arabidopsis*. Plant Cell 13:1305–1315

Doyle MR, Davis SJ, Bastow RM, McWatters HG, Kozma-Bognar L, Nagy F, Millar AJ, Amasino RM (2002) The ELF4 gene controls circadian rhythms and flowering time in *Arabidopsis thaliana*. Nature 419:74–77

Hayama R, Coupland G (2003) Shedding light on the circadian clock and the photoperiodic control of flowering. Curr Opin Plant Biol 6:13–19

Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, LaFond F, Hanley D, Kiphart D, Zhuang MZ, Huang W, Mueller LA, Bhattacharyya D, Bhaya D, Sobral BW, Beavis W, Meinke DW, Town CD, Somerville C, Rhee SY (2001) The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic Acids Res 29:102–105

Makino S, Kiba T, Imamura A, Hanaki N, Nakamura A, Suzuki T, Taniguchi M, Ueguchi C, Sugiyama T, 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, Yamashino T, 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 MJ, Oyama T, Kay SA (2003) Dual role of TOC1 in the control of circadian and photomorphogenic responses in *Arabidopsis*. Plant Cell 15:223–236

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

Matsushika A, Imamura A, Yamashino T, Mizuno T (2002) 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

Michael TP, Salome PA, McClung CR (2003) Two *Arabidopsis* circadian oscillators can be distinguished by differential temperature sensitivity. Proc Natl Acad Sci USA 100:6878–6883

Sato E, Nakamichi N, Yamashino T, 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

Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. Science 289:768–771

Thain SC, Murtas G, Lynn JR, McGrath RB, Millar AJ (2002) The circadian clock that controls gene expression in *Arabidopsis* is tissue specific. Plant Physiol 130:102–110

Young MW, Kay SA (2001) Time zones: a comparative genetics of circadian clocks. Nat Rev Genet 2:702–715