CHAPTER NINETEEN

Characterization of Pseudo-Response Regulators in Plants

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Contents

Abstract

A small family of clock-regulated pseudo-response regulators (PRRs) plays a number of critical roles in the function of the plant circadian clock. The

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regulation of the PRRs is complex and entails both transcriptional and post-translational regulation. PRR proteins engage in a number of important protein–protein interactions, some of which are modulated by modifications including phosphorylation. PRR stability is also tightly controlled. This chapter provides methods for studying both the *PRR* genes and their encoded proteins.

1. The *Arabidopsis* Circadian Clock

Circadian rhythms, the subset of rhythms with a period of approximately one solar day, are widespread in nature. These rhythms are driven by endogenous, self-sustaining clocks. All eukaryotic circadian oscillators studied to date are based on multiple interlocked negative feedback loops (Bell-Pedersen *et al.*, 2005) and the Arabidopsis clock is no exception (Harmer, 2009; McClung, 2008).

Five pseudo-response regulators (PRRs) play important roles in clock function. *TIMING OF CAB EXPRESSION1* (*TOC1*, also known as *PSEUDO-RESPONSE REGULATOR1*, *PRR1*) was the first clock gene identified in plants on the basis of a mutant circadian phenotype, altered period length (Millar *et al.*, 1995). *TOC1/PRR1* is the founding member of a small family of five *PRR* genes whose transcript abundance oscillates with circadian period; *PRR9*, *PRR7*, *PRR5*, *PRR3*, and finally *PRR1/TOC1* expression peaks in succession at ~2-h intervals, with *PRR9* peaking early in the morning and *TOC1* peaking in the early evening (Matsushika *et al.*, 2000; Strayer *et al.*, 2000). Like *TOC1*, the other four *PRR* genes lack the conserved aspartic acid found in the receiver domain of classical response regulators and so are unlikely to function via a conventional phosphorelay (Mizuno and Nakamichi, 2005). The modified receiver domain is termed a pseudo-receiver (PsR) domain. Reverse genetic analysis established that each *PRR* gene is important for clock function because loss of *PRR* function alters period length (McClung, 2006; Mizuno and Nakamichi, 2005). In addition, several *PRRs* (*PRR5*, *PRR7*, and *PRR9*) are positive regulators of flowering time (gain of function leads to early flowering) and are positive regulators of light sensitivity as measured by hypocotyl elongation (Matsushika *et al.*, 2007; Mizuno and Nakamichi, 2005; Nakamichi *et al.*, 2005). However, there is no good model explaining the biochemical function of the PRR proteins (Harmer, 2009; McClung, 2008; Mizuno and Nakamichi, 2005).

The Arabidopsis clock has multiple loops, with PRRs involved in each (Fig. 19.1). In the central loop, TOC1 is a positive regulator of two partially redundant single Myb-domain transcription factors, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) (Alabadí *et al.*, 2001). Although TOC1 lacks defined

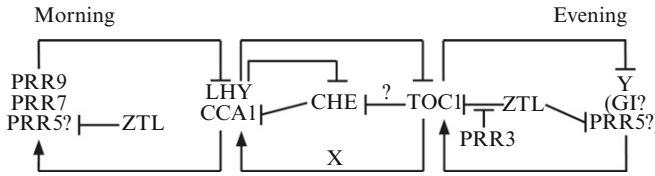

Figure 19.1 Model of the *Arabidopsis* circadian clock. PRR proteins play prominent roles in the *Arabidopsis* circadian clock, which consists of multiple interlocked negative feedback loops. This model is oversimplified to emphasize the basic architecture and the prominence of the PRRs; not all known components are shown and more components remain to be identified. This model includes some speculations, indicated with question marks, as positions of and interactions among some components remain inconclusively determined.

DNA-binding domains, it is recruited to the *CCA1* promoter, possibly through interaction with another DNA-binding protein(s). CCA1 HIKING EXPEDITION (CHE), a TCP transcription factor, binds to a canonical TCP binding site in the *CCA1* promoter, and negatively regulates *TOC1* (Pruneda-Paz *et al.*, 2009). TOC1 and CHE interact, but the significance of this interaction remains incompletely known (Pruneda-Paz *et al.*, 2009). CCA1 and LHY form the negative arm of this feedback loop, binding to the *TOC1* promoter to inhibit expression (Alabadí *et al.*, 2001, 2002; Mizoguchi *et al.*, 2002).

In the second interlocked loop, also called the "evening" loop because of the time of day at which the expression of loop members is maximal, TOC1 represses a component, Y, that may include GIGANTEA (GI), which activates TOC1 (Locke *et al.*, 2005, 2006). In the third interlocked loop, also called the "morning" loop, CCA1 and LHY are positive regulators of two TOC1 relatives, *PRR7* and *PRR9* (Harmer and Kay, 2005; Mizuno and Nakamichi, 2005). The *prr7prr9* double mutant exhibits dramatic period lengthening and is conditionally arrhythmic (Farré *et al.*, 2005; Nakamichi *et al.*, 2005; Salomé and McClung, 2005). PRR5 is also implicated in this loop; the *prr5prr7prr9* triple mutant is completely arrhythmic and PRR5/7/9 are considered to be negative regulators of *CCA1/LHY* because *CCA1* is constitutively transcribed in that triple mutant (Nakamichi *et al.*, 2005). Thus, each of these four PRRs (TOC1, PRR5, PRR7, and PRR9) regulate *CCA1* and *LHY* expression, with three (PRR5, PRR7, and PRR9) negative regulators and TOC1 a positive regulator.

Transcriptional regulation is important in clock function, but it is clear that posttranscriptional regulation is an essential constituent of the clock mechanism (Gallego and Virshup, 2007). In particular, the temporally regulated proteasomal degradation of specific clock proteins is necessary for progression through the oscillation. The stability of a number of plant clock proteins, including GI (David *et al.*, 2006), LHY (Song and Carré,

2005), ZEITLUPE (ZTL) (Kim *et al.*, 2003), and members of the TOC1/PRR family (Farré and Kay, 2007; Fujiwara *et al.*, 2008; Ito *et al.*, 2007; Kiba *et al.*, 2007; Más *et al.*, 2003; Para *et al.*, 2007) is clock regulated. Most is known about TOC1, which peaks in abundance at dusk. An E3 ubiquitin ligase SCF complex including the F-box protein ZTL is crucial for clock-regulated proteasomal degradation of TOC1 (Han *et al.*, 2004; Más *et al.*, 2003). ZTL also targets PRR5 for proteasomal degradation through direct interaction with the PsR domain of PRR5 (Kiba *et al.*, 2007). The mechanism(s) regulating the stability of PRR7 and PRR9 remain less fully described, although proteasome activity is implicated (Farré and Kay, 2007; Ito *et al.*, 2007). Unlike the other PRRs, which regulate *CCA1* and *LHY* expression, PRR3 regulates the stability of TOC1 via protein–protein interaction (Fujiwara *et al.*, 2008; Para *et al.*, 2007). This mechanism is notably similar to that by which ZTL protein abundance oscillations, despite noncycling mRNA abundance, are a consequence of periodic stabilization through interaction with GI protein, which cycles in abundance as a consequence of rhythmic transcription and mRNA accumulation (Kim *et al.*, 2007).

Critical to the investigation of PRR function is the ability to monitor PRR protein abundance and localization. We have taken advantage of PRR–GFP protein fusions, using the GFP moiety both as a fluorescent tag and as a protein tag for immunological detection.

2. Detection of PRR Proteins

2.1. Immunodetection of GFP-tagged PRR proteins from Arabidopsis extracts

To examine the circadian dynamics of the PRR proteins *in planta* and at endogenous levels we used stably transformed Arabidopsis plants expressing PRR:GFP fusions driven by the endogenous PRR promoter (Fujiwara *et al.*, 2008). Genomic fragments including the full promoter (starting at −1431, −5116, −1194, and −1541 bp for *PRR3*, *PRR5*, *PRR7*, and *PRR9*, respectively), 5′UTR and coding sequences up to the last codon before the STOP codon, were amplified by PCR with ExTaq (Takara Bio USA, Madison, WI) and subcloned first into pGEM-T Easy (Promega, Madison, WI), transferred into the Gateway Entry vector pENTR-1A, and finally placed upstream of the GFP variant mGFP6 in the binary vector pMDC206 (Curtis and Grossniklaus, 2003) by LR recombination (Invitrogen, Carlsbad, CA). pPRR:PRR:GFP constructs were transformed into Arabidopsis Columbia plants by floral dip (Bechtold *et al.*, 1993) via *Agrobacterium tumefaciens* strain GV3101. Analysis was performed in select stably transformed lines.

For TOC1–YFP, PRR3–GFP, PRR5–GFP, PRR7–GFP, and PRR9–GFP detection, Arabidopsis seedlings were grown under light–dark cycles (12 h light: 12 h darkness) for 8–10 days and harvested rapidly into prelabeled aluminum foil packets and placed immediately into liquid nitrogen. Seeds were sown directly onto filter paper, overlaying MS media (GIBCO BRL, Cleveland, OH) +3% sucrose solidified with 0.8% agar, to facilitate easy harvest. For transient expressions in *Nicotiana benthamiana* using Agrobacterium-mediated coinfiltration, all constructs were 35S cauliflower mosaic virus (CaMV) promoter-driven (Kim *et al.*, 2007; Voinnet *et al.*, 2003).

The following protocols also describe the techniques to detect phosphorylated forms of the PRR proteins (Fujiwara *et al.*, 2008). The critical feature is the adjustment of the acrylamide:bisacrylamide ratio from the "standard" 37.5:1 to a ratio of 149:1. This ratio may be used for any percentage gel, and we find that an 8% acrylamide gel works best for the PRR proteins.

2.2. Protein extract preparation for PRR family members

(1) Resuspend frozen ground tissues in extraction buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 0.5% Nonidet P-40, 1 m*M* EDTA, 3 m*M* DTT, 1 m*M* phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml antipain, 5 μg/ml chymostatin, 2 m*M* Na_3VO_4, 2 m*M* NaF, 2 m*M* glycerol phosphate, 50 μM MG132, 50 μM MG115, 50 μM ALLN; all reagents were purchased from Sigma) by vortexing for 10 s (v/v, 1:1). Keep the tubes on ice.
(2) Centrifuge the extracts at 16,000×*g* for 5 min at 4 °C.
(3) Keep the tubes on ice and quickly transfer the supernatant to prechilled new tubes.
(4) Add 6× sample buffer (SDS Reducing buffer; 125 m*M* Tris–HCl, pH 6.8, 50% glycerol, 4% (w/v) SDS, 0.02% (w/v) Bromophenol blue, and freshly added 10% (v/v) β-mercaptoethanol) to the supernatant, warm the sample to room temperature for 2 min and centrifuge at 1000×*g* for 1 min at 4 °C before loading. Heating to higher than room temperature may cause severe degradation of PRR proteins in the total lysate. Do not boil samples.

2.3. PRR protein detection: immunoblots

(1) We use 8% SDS–PAGE (acrylamide:bisacrylamide, 149:1) gels to detect phosphorylated PRR proteins (GFP or YFP tagged) migrating in the range of approximately 80–106 kDa. It is best to run the gel with low voltage at 4 °C. Running at a higher voltage (e.g., 120 V)

may cause dumbbell-like band patterns making it difficult to detect closely spaced bands, especially with larger proteins. We use 60 V for 30 min until samples go into the lower gel, and then change to 90 V to get the best result.

(2) Equilibrate the gel and soak the polyvinylidene difluoride (PVDF) membrane [(Bio-rad) briefly pretreated with 100% methanol)], filter paper, and fiber pads in transfer buffer for 10 min. Always wear gloves when handling membranes to prevent contamination.

(3) Arrange gel sandwich for blotting. It is important to remove any air bubbles between the gel and the membrane to ensure complete and even transfer. Use a glass tube or roller to gently roll air bubbles out.

(4) Transfer proteins at 100 V for 1 h at 4 °C.

(5) To prepare 5% blocking solution, dissolve 5 g of nonfat dried milk in 100 ml of 1× TBST (10 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 0.05% Tween-20) for 30 min.

(6) Soak the membrane in 5% blocking solution and incubate at room temperature for 1 h with agitation.

(7) Incubate with primary anti-GFP antibody (Abcam, ab6556, 1:2000) in the blocking solution for 1–2 h at room temperature or overnight at 4 °C.

(8) Wash the membrane three times with 1× TBST for 10 min each.

(9) Incubate with anti-rabbit secondary IgG, HRP-Linked (GE Healthcare, NA934, 1:3000) in the blocking solution for 1 h at room temperature.

(10) Wash the membrane three times for 10 min each with 1× TBST.

(11) Detect signals with Pierce West Pico solution (Pierce, 34080) using standard X-ray film (MIDSCI, St Louis, MO) or a chemiluminescence detector.

2.4. Immunoprecipitation/coimmunoprecipitation

(1) To prepare antibody-bound resin, mix 30 μl of protein A-agarose 50% slurry (Invitrogen, 15918-014), anti-GFP antibody (mouse monoclonal, Molecular Probes, A11120,1:250), and 40 μl of 1× immunoprecipitation (IP) buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 0.5% Nonidet P-40, 1 m*M* EDTA, 3 m*M* DTT).

(2) Incubate at least 1 h at 4 °C with gentle agitation.

(3) Resuspend 1 ml of ground tissues in 1 ml IP buffer containing 1 m*M* phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 5 μg/ml antipain, 5 μg/ml chymostatin, 2 m*M* Na_3VO_4, 2 m*M* NaF, 2 m*M* Glycerol phosphate, 50 μ*M* MG132, 50 μ*M* MG115, and 50 μ*M* ALLN by vortexing for 10 s (v/v, 1:1) and spin at 16,000×*g* for 5 min at 4 °C.

(4) Incubate 1–2 mg of soluble protein extracts (ca. 950 μl) from #3 and the resin from #2 with gentle agitation for 1 h at 4 °C.
(5) Centrifuge at 1000×*g* at 4 °C for 1 min to collect immune complexes.
(6) Wash the protein A-agarose beads three times with 1 ml of ice-cold IP buffer by gently inverting the tube 10 times.
(7) Wash the pellet once more with 1× PBS (0.2 *M* phosphate, pH 7.4; 1.5 *M* NaCl).
(8) Suspend pellet in 30 μl of 2× SDS–PAGE sample buffer. Heat samples at 90 °C for 60–90 s prior to SDS–PAGE and immunoblotting. Many of the PRR proteins are quite labile and it is important to heat briefly and below 93 °C to minimize degradation. However, PRR proteins isolated by immunoprecipitation are less labile than in crude protein extracts, so higher temperatures at this stage are both necessary (to dissociate the immuncomplexes) and not as damaging.
(9) To prepare a positive control (pre-IP input), mix 90 μl of the protein extract from #3 and mix with 30 μl of 6× sample buffer, warm the sample at room temperature for 2 min and centrifuge at 1000×*g* for 1 min at 4 °C before loading. As noted earlier, heating higher than room temperature may cause severe degradation of PRRs in the total lysate.

2.5. Determination of phosphorylation states of PRR family members

(1) Prepare protein extracts either in 1× λ-phosphatase buffer (New England Biolabs; supplied by company) supplemented with 2.5 m*M* $MnCl_2$, 0.5% Triton X-100, and 0.4% Nonidet P-40 for λ-phosphatase treatment or in New England Biolabs buffer 3 supplemented with 0.5% Triton X-100 and 0.4% Nonidet P-40 for calf intestinal alkaline phosphatase (CIP) treatment. Modified IP buffer without EDTA supplemented with 2.5 m*M* $MnCl_2$ for λ-phosphatase or 10 m*M* $MgCl_2$ for CIP can be used. In all cases supplement with protease inhibitors (2.5 μg/ml Antipain, 2.5 μg/ml Chymostatin, 1 μg/ml Pepstatin, 5 μg/ml Leupeptin, 5 μg/ml Aprotinin, 1 m*M* phenylmethylsulfonyl fluoride) and proteasome inhibitors (50 μ*M* MG132, 50 μ*M* MG115, and 50 μM ALLN) to prohibit nonspecific degradation during extraction. Depending on the protein, λ-phosphatase may work better than CIP, or *vice versa*.
(2) Incubate 50-μl aliquots of the resulting protein extracts with 400 U of λ-protein phosphatase (New England Biolabs) with or without phosphatase inhibitors (NaF/Na_3VO_4) at 30 °C for 15 min or with 10–20 U of CIP (New England Biolabs) with or without phosphatase inhibitors (NaF/Na_3VO_4) at 37 °C for 15 min.

(3) Stop the reaction by adding phosphatase inhibitors cocktail (2 m*M* Na_3VO_4, 2 m*M* NaF, 2 m*M* glycerol phosphate).
(4) Add 20 μl of 6× loading buffer to the reaction, heat at room temperature for 2 min and centrifuge at 16,000×*g* for 1 min at room temperature.
(5) Load samples into 8% SDS–PAGE (acrylamide:bisacrylamide, 149:1) and analyze by immunoblotting as described above. As noted earlier, running the gel at low voltage and at 4 °C will help to improve the resolution of closely migrating forms of the protein. Maximum resolution is usually observed when the protein of interest has migrated to the center of the gel.

3. Localization of PRR Proteins

3.1. Localization of GFP-tagged PRR proteins in leek cells

One of the first steps to elucidate protein function is to determine in which subcellular compartment the protein resides and acts. CCA1 and LHY, two Myb-domain transcription factors, localize to the nucleus (Carré and Kim, 2002; Yakir *et al.*, 2009), where they bind to the promoters of their target genes. The genetic interactions of the PRR family members with known nuclear-localized clock components such as CCA1 and LHY suggest a role in the nucleus if these interactions are direct. Consistent with this hypothesis, protein localization programs such as PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) predict nuclear localization for each of the PRRs. TOC1/PRR1 has been known for some time to be nuclear-localized. Overexpression of a TOC1:GFP fusion demonstrated a clear nuclear location of TOC1 in tobacco BY-2 cells (Strayer *et al.*, 2000). Similarly, a TOC1:GUS fusion protein showed nuclear localization in transgenic Arabidopsis (Para *et al.*, 2007). Most recently, TOC1 was localized to DNA at the *CCA1* (but not *LHY*) promoter by chromatin IP (Pruneda-Paz *et al.*, 2009).

Despite the evident importance of PRR localization, experimental evidence has only recently been forthcoming for other family members. We set out to localize GFP fusions of the four remaining circadian PRR family members, first in leek and then in Arabidopsis (Fujiwara *et al.*, 2008). All constructs examined in leek used the constitutive CaMV 35S promoter to drive expression of PRR fused in frame to a N-terminal GFP. *PRR* cDNAs were cloned into the entry vector pENTR-2B (Invitrogen) and recombined with the Gateway™ destination vector pK7WGF2 (Karimi *et al.*, 2002). Each construct was introduced into leek cell by biolistics (Sanford *et al.*, 1993).

We found that PRR3, PRR5, PRR7, and PRR9 proteins localized to the nucleus of leek cells (Fig. 19.2), and we detected differences in the subcellular localization of some PRR family members. For instance,

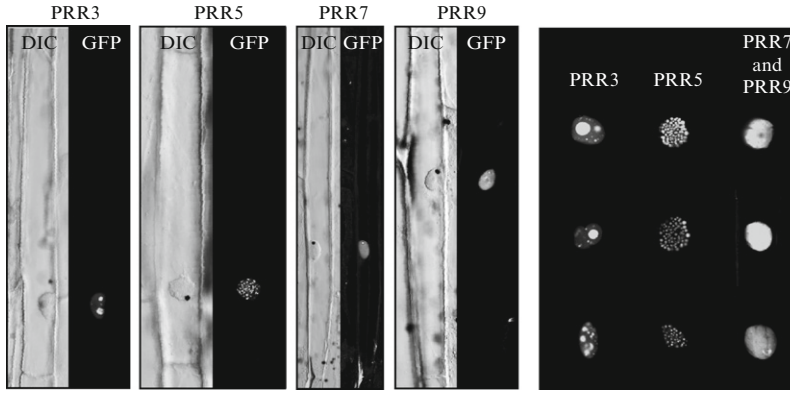

Figure 19.2 Nuclear localization of PRR family members in transient assays in leek cells. Leek epidermal cells were bombarded with gold particles coated with DNA for binary constructs expressing a N-terminal GFP fusion to the PRR, placed under the control of the 35S promoter (*p35S:GFP:PRR*). Left panel: examples of individual cells expressing the PRR:GFP fusions are shown on the left; the position of the nucleus can be clearly seen by differential interference contrast microscopy (DIC). Right panel: individual nuclei from three cells expressing *p35S:GFP:PRR3*, *p35S:GFP:PRR5*, or *p35S:GFP:PRR9*. The localization of TOC1 is very similar to PRR5 (Strayer *et al.*, 2000); as shown in the left panels, PRR7 and PRR9 show the same evenly distributed nuclear localization pattern.

PRR3 is found most strongly in the nucleolus of leek cells, while PRR7 and PRR9 appear evenly distributed in the nucleus. PRR5 offered an interesting case in that, although clearly nuclear-localized, the GFP fusion tends to form aggregates, oftentimes called subnuclear foci. The number and size of these foci are in direct proportion with the GFP signal, suggesting that accumulation of PRR5 to subnuclear foci might be an artifact from high expression.

3.1.1. Protocol: transient expression of 35S:GFP:PRR clones in leek cells

(1) Prepare 100 mm diameter petri plates containing MS medium supplemented with 1–2% sucrose.
(2) Buy leeks at your local food store. Wider leeks are preferred as the epidermis will not curl as much once on the plate.
(3) Cut a 3 cm section from the inner, white parts of the leek, discarding green leaves.
(4) Gently peel off the single-cell layer on the inner side of the leek white section, and deposit on the surface of the medium, inner (adaxial) side against the medium to prevent curling.

(5) Mix 2 μg of DNA per *p35S:GFP:PRR* fusion with 10 μl gold particles (1.5–3 μm; ~60 mg/l), 50 μl 2.5 *M* $CaCl_2$, and 20 μl 0.1 *M* spermidine and vortex for 1 min.
(6) Quickly spin gold particles down (5–10 s, at most, in a microfuge operated below 4000 rpm).
(7) Wash gold particles once in 70% ethanol, then twice in 100% ethanol.
(8) Resuspend gold particles in 10–20 μl of 100% ethanol, and apply to the macrocarrier disk.
(9) Bombard leek peels at 1000 psi under 27 in. of Hg vacuum. If using a PDS-1000/He instrument (Bio-Rad, Hercules, CA), gap distance should be at 3 mm.
(10) Incubate bombarded tissue in the dark overnight, but no longer than 16–20 h, as strong overexpression of the proteins might lead to artifactual localization.
(11) Mount tissue in water for visualization on a confocal microscope (GFP excitation: 488 nm; GFP emission 498–561 nm). Look for gold particles under back-lighting to find bombarded zones, then switch to GFP settings for GFP detection.

3.2. Immunolocalization of GFP-tagged PRR proteins in Arabidopsis plants

To confirm the nuclear localization of the PRR proteins under endogenous conditions, we turned to stably transformed Arabidopsis plants expressing PRR:GFP fusions driven by the endogenous *PRR* promoter (Fujiwara *et al.*, 2008). Because PRR proteins are inabundant, the GFP signal was generally weak and, in our hands, disappeared rather quickly due to GFP photobleaching. We therefore decided to use the GFP moiety as an epitope for immunolocalization, rather than as a fluorescent molecule, taking advantage of the signal amplification provided by the primary and secondary antibodies. A number of protocols exist for immunolocalization of proteins in Arabidopsis seedlings and ours, detailed below, is adapted from two protocols described earlier (Guo *et al.*, 2001; Zachgo *et al.*, 2000). The most critical part of the procedure is to partially digest away the cell wall and revert the cross-linking so that primary and secondary antibodies are allowed access to the epitopes. Proteinase K digestion proved effective for our own purposes. Although others have employed a heat treatment of fixed seedlings rather than proteinase digestion (Vitha *et al.*, 2001), we did not compare the effectiveness of the two treatments. It is likely that another protein (other than GFP) will behave differently when subjected to paraformaldehyde fixation.

(1) Grow seedlings under light–dark cycles (12 h light: 12 h darkness) for 8–10 days.

(2) Fix seedlings for 3 h in 4% paraformaldehyde in 1× PBS at 4 °C. Eppendorf tubes (1.5 ml) work well for young seedlings; alternatively, 12- or 24-well plates can be used.
(3) Wash three times in 1× PBS, 0.1% Triton X-100 for 15 min, and three times in water for 15 min.
(4) Digest cell walls in 1% cellulase, 1% macerozyme in 1× PBS for 45 min with shaking. Wash three times in 1× PBS, 0.01% Triton X-100.
(5) Incubate seedlings in blocking buffer (5% BSA in 1× PBS) for 2 h at room temperature, followed by incubation with the primary antibody (rabbit anti-GFP, Abcam, Cambridge, UK) diluted at 1:250 in 2% BSA, 1× PBS with gentle shaking overnight at 4 °C.
(6) Wash seedlings four times, 15 min each, in 1× PBS, 0.01% Triton X-100.
(7) Incubate with secondary antibody for 2 h at room temperature (in this case: goat antirabbit, Alexa Fluor-488 conjugated, Molecular Probes, Eugene, OR), diluted to 1:400 in 2% BSA, 1× PBS.
(8) Wash four times 15 min in 1× PBS, 0.01% Triton X-100.
(9) Mount seedlings in Mount Quick (Electron Microscopy Sciences, Washington, PA) and detect signal on confocal microscope using same GFP settings as for transient expression in leek. Note that, when dry, Mount Quick will become autofluorescent under the conditions used for GFP localization. For long-term storage and retrieval of slides, a good choice of mounting medium is ProLong antifade reagent.

4. Exploring the Circadian Phenotypes of *PRR* Mutants

4.1. Cotyledon movement analysis

High-throughput forward genetic mutant screens based on rhythmic luciferase expression are very powerful tools for the isolation of novel genes involved in circadian rhythms (Southern and Millar, 2005; Welsh *et al.*, 2005). Such a screen in Arabidopsis made use of the *LHCB1*1* (*CAB2*) promoter, and led to the identification of, a number of mutants, including *timing of cab expression1* (*toc1*) and *zeitlupe* (*ztl*) (Millar *et al.*, 1995; Somers *et al.*, 2000).

The ability to perform reverse genetics in which one might wish, for example, to assess sets of targeted T-DNA insertion for alleles that confer circadian defects would be greatly facilitated by an assay that did not require the introduction of a transgene. One obvious class of mutants warranting investigation includes loss of function alleles of homologues to known clock genes like *CCA1*, *LHY*, *TOC1*, and *ZTL*. Another class of mutants includes those identified from screens not based on circadian phenotypes,

such as flowering time, hypocotyl elongation, or hormone signaling. For many years the only circadian rhythm studied was leaf movement in plants (McClung, 2006). Such studies emphasized pulvinar movements in many legume species, but species that lack pulvini, like Arabidopsis, often express a circadian rhythm in the movements of cotyledons (Millar *et al.*, 1995).

In Arabidopsis, cotyledon movement presumably is driven by daily rhythmic changes in cell elongation in the petiole. An inexpensive surveillance camera is sufficient to record cotyledon movement, coupled to a computer system to control image capture. Seeds are surface-sterilized and sown on MS medium supplemented with sucrose. After 3–4 days of stratification at 4 °C in the dark, plates are released under the desired entraining conditions: light–dark cycles of any photoperiod or hot–cold cycles for thermoentrainment. The number of days in entraining cycles before recording movement will depend on the genotype and growth conditions (thermocycles slow down growth), but averages 4–6 days. Seedlings reach the optimal stage for transfer when cotyledons are well expanded, and primary leaves are just starting to emerge at the apex. We then cut out a cube of solid medium around the seedling, and gently transfer the seedling in agar to the wells of clear 24 square-well plates. We tape Whatmann paper to the back of the plate to increase contrast between the seedling and the plate. The lid of the plate is held into place with 1-in. wide surgical tape to allow gas exchange. Recording can be started immediately with your favorite program, such as Kujamorph (http://millar.bio.ed.ac.uk/JnlPage.htm) or NKTRACE (Onai *et al.*, 2004). We routinely assay seedlings for 1 week. We analyze circadian data with fast Fourier transform-nonlinear least-squares (FFT-NLLS) analysis (Plautz *et al.*, 1997; Straume *et al.*, 1991) and with BRASS (http://millar.bio.ed.ac.uk/PEBrown/BRASS/BrassPage.htm).

A number of variations can be applied during seedling growth. For mutants with long hypocotyls, it might be necessary to first release them into continuous light for 3–4 days before starting an entraining regime (Hicks *et al.*, 1996). The first day in continuous light will inhibit hypocotyl elongation and allow seedlings to fit much more easily in the wells of the plates. Seedlings can then be entrained for 2 days before recording movement. Thermocycles can also be applied while cotyledon movement is being recorded; differences in the two temperatures should be kept between 0 and 6 °C to limit the amount of condensation on the lid of the plate around transitions from one temperature to the other. Temperatures that are too low (below 12 °C) will stall cotyledon movement, although we know that the underlying circadian clock is still rhythmic (Gould *et al.*, 2006). Finally, the temperature during the assay need not be 22 °C, but can be set lower or higher. It is important to adjust the timing of transfer of the plates to the new temperature. Ideally, a step up in temperature should be made to coincide with subjective dawn, while a step down in temperature should follow subjective dusk.

Cotyledon movement is of course not limited to Arabidopsis. For example, we are now measuring cotyledon movement from *Brassica rapa* seedlings grown directly on soil (P. Liu, Q. Xiu, X. Xu, and CRM, unpublished); cotyledon movement is likely to be applied successfully to many dicotyledonous species.

4.2. Generating versatile LUCIFERASE (LUC) fusion constructs

Although recording movement of cotyledons is fast and does not require the introduction of a transgene into the genotype to be tested, it also suffers from being several levels removed from the central oscillator controlling the rhythm. To more directly measure clock gene expression, the firefly luciferase has become the gold standard for circadian biologists (see Welsh *et al.*, 2005) in Neurospora, mammalian systems and plants (Gooch *et al.*, 2008; Millar *et al.*, 1992a, 1995; Morgan *et al.*, 2003; Stanewsky, 2007; Welsh *et al.*, 2004; Wilsbacher *et al.*, 2002). Circadian research in green algae *Synechococcus elongatus* PCC 7942 has opted for a bacterial version of the gene, encoded by the *luxAB* operon from *Vibrio harveyi* (Kondo *et al.*, 1993; Liu *et al.*, 1995); the necessary genes for the synthesis of the substrate have been introduced into the Synechococcus genome to circumvent lethality issues with exogenous substrates. How easily transgenic seedlings can be selected determines whether primary transgenic plants can be assayed. The original firefly LUC construct, in the pPZP series backbone (Hajdukiewicz *et al.*, 1994), conferred resistance to gentamycin and required several weeks growth on selective media to allow unambiguous determination of resistance. To make selection of transgenic plants easier and more unambiguous, we replaced the gentamycin resistance cassette with either neomycin phosphotransferase II (derived from pBI101, Jefferson *et al.*, 1987) or phosphinotricine phosphotransferase (from 35SpBARn, LeClere and Bartel, 2001) for kanamycin and BASTA selection, respectively. Then, to facilitate the cloning of promoter fragments, we introduced a Gateway™ recombination cassette (comprised of the chloramphenicol resistance gene and the ccdB gene for counter-selection of intact destination vector molecules, derived from the Gateway™ destination vector pK7WGF2, Karimi *et al.*, 2002) upstream of luciferase. The resulting clones, PS517 (for kanamycin selection) and PS643 (for BASTA selection) were recombined with entry clones bearing the promoters of the clock genes *CCA1*, *LHY*, *TOC1*, *PRR7*, and *PRR9*. Maps for the PS517 and PS643 vectors are shown in Fig. 19.3.

Primary transformants can now be selected unambiguously within 7–10 days. Seeds are first sterilized 1–2 h in 95% ethanol, air-dried, and then plated out on MS medium *not* supplemented with sucrose, and containing either 50 μg/ml kanamycin and 500 μg/ml carbenicillin (for PS517-derived constructs) or 10 μg/ml glufosinate ammonium and 500 μg/ml carbenicillin (for PS643 derivatives).

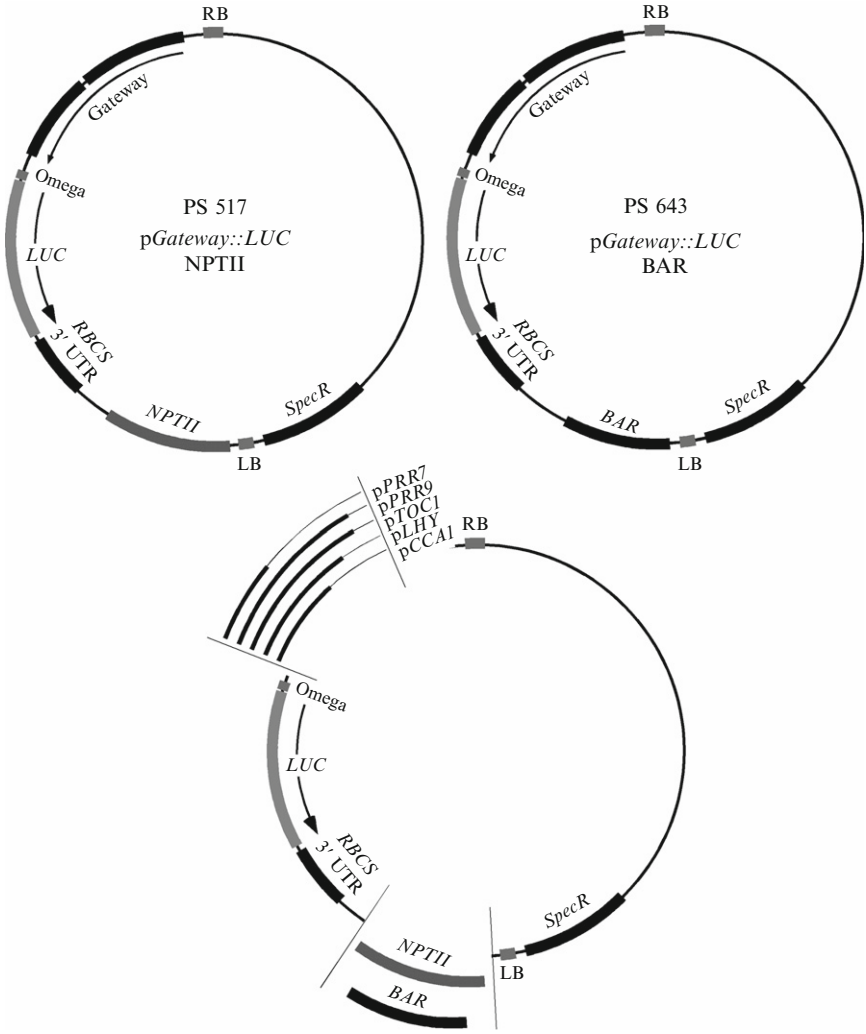

Figure 19.3 A set of versatile vectors to characterize the expression pattern of the *PRR* family members. PS517 was constructed from pPZΩLUC+ by replacing the plant selection marker (gentamycin) with kanamycin resistance. The same gentamycin selection marker was replaced with BASTA resistance (from 35SpBARn) to create PS643. The Gateway™ recombination cassette was PCR-amplified from pK7WG2D and cloned between the *Bam*HI and *Hin*dIII sites upstream of luciferase. Promoters from the clock genes *CCA1*, *LHY*, and *TOC1*, as well as the *TOC1*-related genes *PRR7* and *PRR9*, were subcloned into the entry vector pENTR11 or pENTR-2B, and placed upstream of luciferase by LR recombination with either PS517 or PS643.

4.3. Transient expression in *Nicotiana benthamiana*

The generation and selection of transgenic plants is time-consuming, and one does not wish to discover after months of wait that the circadian reporter expressed by one's transgenic seedlings does not actually exhibit rhythmic expression. Likewise, in the context of a promoter resection study, a quick look at the behavior of the constructs made thus far can accelerate the identification of sequences of interest (for rhythmicity or high amplitude expression) and direct further cloning while awaiting phenotypic confirmation in stable transgenic lines. Arabidopsis cell suspensions can in theory be transfected, but handling and maintaining the cultures can turn into a time-consuming effort. In addition, many plant cell lines are not intrinsically rhythmic, greatly limiting their use for quick assays.

A convenient option is the use of *N. benthamiana*. *Nicotiana benthamiana* plants grow quickly, and a few pots can be sown every week until needed without requiring much growth space. An Agrobacterium suspension bearing the construct of interest is infiltrated into leaves from the abaxial (bottom) side of the leaves. The bacterial suspension is made competent to transfer the T-DNA fragment of the construct by induction with acetosyringone the day before infiltration. Most transgenes expressed in this fashion tend to be silenced within 2–3 days as the plant generates small RNAs targeting the highly expressed transgenes, but coinfiltration with a viral suppressor gene like p19 or HC-Pro will sequester siRNAs away from the luciferase mRNA (Voinnet *et al.*, 2003). Quite surprisingly, luciferase activity can be readily detected for at least 1–2 weeks when leaf cuttings of the infiltrated regions are transferred to 96-well plates and provided with luciferin. With this method, we have tested the *CCA1*, *LHY*, and *TOC1* promoters (in the PS643 backbone, Fig. 19.4), as well as the *CCA1*, *LUX*, *GI*, *CCR2* (*GRP7*), and *CAT3* promoters in other vector backbones with great success. Coinfiltration with p19 or HC-Pro is not required to detect clear rhythms; it does however tend to help decrease variation between cuttings from different leaves. The fact that clock-regulated Arabidopsis promoters oscillate in Nicotiana argues that the regulatory modules found in these promoters are conserved among species, consistent with genomic and transcriptomic analyses (Michael *et al.*, 2008; Zdepski *et al.*, 2008).

4.4. Transient rhythmic assay in *Nicotiana benthamiana*

(1) Start a small overnight 10 ml culture of the agrobacterial strains carrying the luciferase constructs and the viral suppressor (p19 or HC-Pro). We currently use ASE1 with good success.
(2) In the morning, use the overnight culture to inoculate a 30-ml culture, diluting the agrobacteria 1:200. In the evening, induce cell culture with acetosyringone, applied to a final concentration of 150 μM. Allow formation of the necessary pilus overnight.

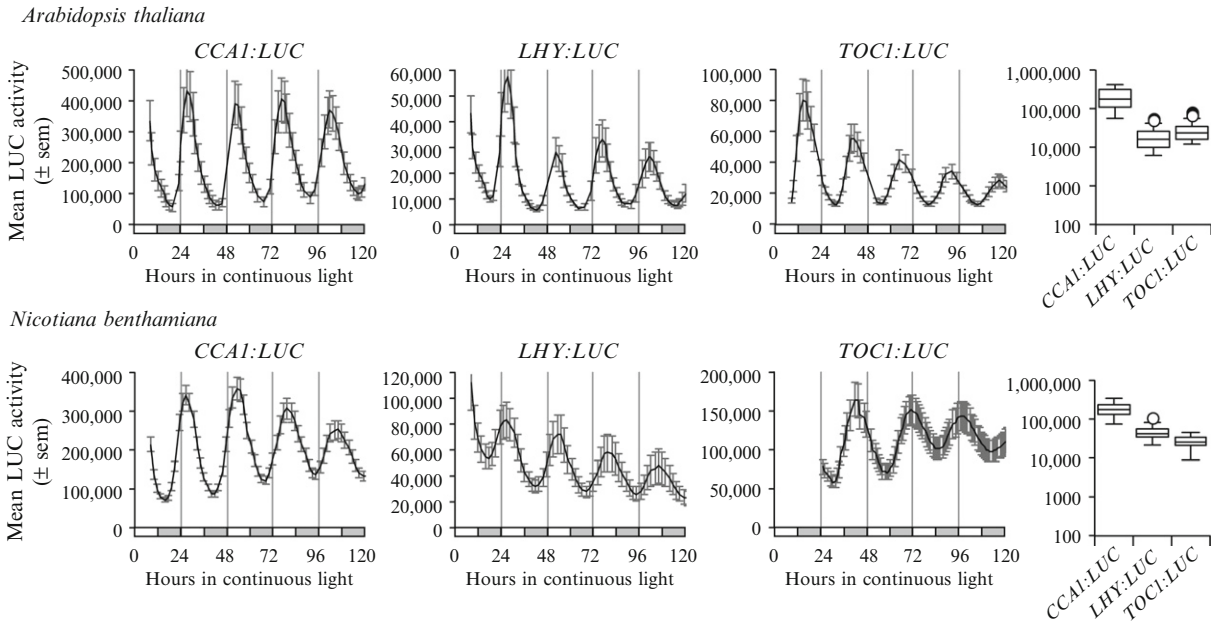

Figure 19.4 A transient expression assay in *Nicotiana benthamiana* for circadian experiments. Binary constructs bearing the luciferase fusions to the *CCA1*, *LHY*, or *TOC1* promoters were described previously (Salomé and McClung, 2005). They all share the same backbone (PS643), and confer BASTA resistance in plants. A typical circadian profile of the three constructs in stably transformed Arabidopsis seedlings is shown in the upper panels. The lower panels show representative mean traces for the same binary constructs, introduced into *Nicotiana benthamiana* leaves via Agrobacterium-mediated infiltration (Voinnet *et al.*, 2003).

(3) Collect induced cells by centrifugation (15 min, 2500 rpm, at room temperature). Wash pellet carefully with resuspension solution (10 m*M* $MgCl_2$, 10 m*M* MES, pH 5.7, 150 μM acetosyringone; filter sterilize), to remove all traces of antibiotics. Resuspend cells in 30 ml of resuspension solution, and add fresh acetosyringone at final concentration of 150 μM.
(4) Allow cells to incubate for 2–3 h at room temperature, without shaking.
(5) Mix cells bearing the luciferase construct with the viral suppressor, 3:1 (volume/volume), and proceed to infiltrate suspension from the abaxial side of leaves. Gently scrape the epidermis of the leaf (without cutting through the leaf) to ease entry of the suspension, and push the suspension into the leaves with a 5-ml syringe without needle. A good infiltration will generate a ring of macerated tissue 1–3 cm in diameter.
(6) Transfer infiltrated plants back to the plant room for 2–3 days. On the day of LUC assay, detach infiltrated leaves, and cut out small squares or circles of leaf tissue, and transfer to the well of a 96-well plate, already containing MS medium (with or without added sucrose) and luciferin.
(7) Keep plate under one more entraining cycle to allow burn off of early expression luciferase, and start recording luciferase activity as for a normal Topcount™ assay.

4.5. Luciferase assays on a Topcount™

Although many labs rely on imaging to measure luciferase activity (Southern and Millar, 2005; Welsh *et al.*, 2005), another powerful and high-throughput circadian assay is based on a 96-well plate format (Southern and Millar, 2005). We have been using a Topcount™ Microplate Scintillation and Luminescence Counter (Perkin Elmer) with six detectors. With a reading time for each well set to 10 s, one full 96-well plate can be read in about 2 min, and a stack of 11 plates in constant light will run through the machine in 90 min.

96-well plates are fed to the Topcount luminometer from "stackers," long towers made of aluminum that sit outside on the sampling chamber. Two models are available, for 20 or 40 plates. The current version of stackers has solid walls of aluminum surrounding the plates inside, for increased sturdiness; this however causes a problem when one wishes to assay plants in the light. To permit light to reach the plates, we have cut holes on all sides without compromising the physical integrity of the stacker columns. For each sample plate, we position three clear 96-well plates above (from Costar, catalog number 3795), to allow light to reach the seedlings. A "stop plate" must be placed after the last sample plate: this plate should have two barcode stickers on the right side, to be recognized by the luminometer

as the last of a stack of plates. Together with this protocol, we have put together a step-by-step guide with screenshots for setting up the assay on the Topcount computer; it is available on request.

Just as for cotyledon movement assays, seeds are surface-sterilized and plated on MS medium, which can be supplemented with 1–2% sucrose. After stratification for 3–4 days at 4 °C, plates are released into entraining conditions (light dark cycles or temperature cycles) for 7–10 days, or until primary leaves are just starting to show between cotyledons. In contrast to cotyledon movement, where we transfer both seedling and the medium onto which the seedling had germinated, only seedlings are transferred to the wells of the plate (opaque, white or black, to prevent light contamination between wells; white plates are purchased from Perkin Elmer, Optiplate-96 catalog number 6005299); it is therefore advantageous to make medium with only 0.6–0.8% agar, so as to facilitate the removal of seedlings with their roots intact, although robust rhythms will still be obtained when the root is snapped off. Each well of the 96-well plates contain 200 μl of the same medium used for seedling growth during entrainment, as well as 30 μl of a 2.5-m*M* D-luciferin solution (potassium salt, purchased from Biosynth or PJK). Plates are then sealed with adhesive sealant (Perkin Elmer, catalog number 6005185); 2–3 holes should be made above each plant with a needle to allow gas exchange. Luciferase protein is quite stable and accumulates prior to introduction of the substrate luciferin, which destabilizes luciferase activity. This allows luciferase activity to accurately represent *de novo* translation; because the transcript is unstable, luciferase activity accurately tracks *de novo* transcription (Millar *et al.*, 1992b; Welsh *et al.*, 2005). Therefore, introduction of luciferin results in a transient pulse of anomalously highlight production which should be allowed to dissipate prior to measurement of *de novo* activity: we therefore routinely entrain seedlings within 96-well plates for one more entraining cycle before release into constant conditions. For some experiments where the assay temperature is distinct from the temperature during entrainment, 1 or 2 more days of entrainment can be used to acclimate seedlings to the new conditions. For instance, for a temperature compensation experiment at 30 °C, one does not wish to shift seedlings directly from 22 to 30 °C. Rather, it is a good idea to expose seedlings within 96-well plates to the new temperature, while still enjoying light–dark cycles. The transition to the higher temperature should be timed with dawn; after 1–2 days under this new regime, plates can be moved to the Topcount for luciferase activity recordings. Conversely, for temperature compensation experiments run at 12–16 °C, the transition to the new temperature should coincide with dusk. These precautions ensure that one is looking at the effects of the new temperature on the pace of the clock, and not the combined effects of the new temperature and the temperature shift associated with the single transfer, which in and of itself would be similar to a temperature pulse for a phase response curve.

It is often necessary to rescue a seedling from a well of a 96-well plate at the end of a run, and we have found that seedlings can be readily transferred to soil. Often the cotyledons have stuck to the adhesive seal; one can scrape the leaves off the adhesive seal with a razor blade while gently pulling the seal off the wells. Some epidermal tissue will remain on the seal, but seedlings will survive. Another possibility is to cut out the seal above the well, and transfer the whole seedling, still attached to the seal. After a few days of growth on soil, seedlings will have grown enough to move their "plastic hat" away from the center of the rosette. We have had almost 100% success with either method, rendering rescue of critical seedlings a very easy process.

5. Concluding Remarks

The availability of increasingly sophisticated reagents and methods is facilitating mechanistic studies of the circadian clock. One can be optimistic that the next few years will yield much deeper understanding of the roles played by the PRR proteins in the circadian clock mechanism.

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