Pseudo-Response Regulator (PRR) Homologues of the Moss Physcomitrella patens: Insights into the Evolution of the PRR Family in Land Plants

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Abstract

The pseudo-response regulators (PRRs) are the circadian clock component proteins in the model dicot Arabidopsis thaliana. They contain a receiver-like domain (RLD) similar to the receiver domains of the RRs in the His–Asp phosphorelay system, but the RLDs lack the phosphoacceptor aspartic acid residue invariably conserved in the receiver domains. To study the evolution of PRR genes in plants, here we characterize their homologue genes, PpPRR1, PpPRR2, PpPRR3 and PpPRR4, from the moss Physcomitrella patens. In the phylogenetic analysis, PpPRRs cluster together, sister to an angiosperm PRR gene subfamily, illustrating their close relationships with the angiosperm PRRs. However, distinct from the angiosperm sequences, the RLDs of PpPRR2/3/4 exhibit a potential phosphoacceptor aspartic acid–aspartic acid–lysine (DDK) motif. Consistently, the PpPRR2 RLD had phosphotransfer ability in vitro, suggesting that PpPRR2 functions as an RR. The PpPRR1 RLD, on the other hand, shows a partially diverged DDK motif, and it did not show phosphotransfer ability. All PpPRRs were expressed in a circadian and light-dependent manner, with differential regulation between PpPRR2/4 and PpPRR1/3. Altogether, our results illustrate that PRRs originated from an RR(s) and that there are intraspecific divergences among PpPRRs. Finally, we offer scenarios for the evolution of the PRR family in land plants.

Key words: circadian clock; His–Asp phosphorelay system; response regulator; pseudo-response regulator; Physcomitrella patens

1. Introduction

Circadian rhythms are endogenous biological oscillations with a period of 1 day, and they are controlled by an autonomous oscillator, the circadian clock. This clock regulates the timing of metabolism, physiology and behaviour of organisms, coordinating them with environmental factors that cycle with the rotation of the earth. The mechanisms of the eukaryotic clocks are proposed to be founded on interlocked autoregulatory loops between genes that function as components of the clock machinery, whereas the identities of these genes are largely different between animals, plants and fungi. In the model dicot Arabidopsis thaliana, a representative set of such component genes is the pseudo-response regulator (PRR) gene family, which comprises five member genes, TOC1 (also called PRR1)/PRR3/PRR5/PRR7/PRR9, which play regulatory roles at multiple nodes in the interlocked loops of the A. thaliana circadian network. All the PRR genes are, largely based on phenotypic analyses of mutants, supposed to be functionally important in the A. thaliana circadian system. Although phenotypic changes in a single mutation of each PRR gene are not large, combinations of mutations of different PRRs often result in stronger...
phenotypes, e.g., essentially arrhythmic in an extreme case.\(^4\)

PRRs share a conserved domain, the receiver-like domain (RLD), along with another domain CONSTANS/CONSTANS-LIKE/TOC1 (CCT). The RLD is similar to the receiver domain of the RRs in the histidine to aspartic acid (His→Asp) phosphorelay, a versatile signal transduction system in organisms from bacteria to eukaryotes other than animals.\(^5,6\) In the His→Asp phosphorelay, a phosphate group is transferred from a histidine kinase (HK), via an intermediate signal transducer histidine-containing phosphotransmitter (HPT), down to a counterpart RR, thereby transducing various environmental and endogenous signals intracellularly.\(^6\) An aspartic acid residue in the receiver domain of RRs is conserved as the phosphoacceptor site, whereas the RLDs of all the \(A.\ thaliana\) PRRs lack this aspartic acid residue, and they carry a glutamic acid instead.\(^5,6\) Consistent with this, Makino \textit{et al.}\(^7\) showed that the RLD of TOC1 did not undergo phosphotransfer \textit{in vitro}.

Here, we hypothesize that the ancestors of PRRs were authentic RRs and they have lost the phosphorelay function through the course of evolution. If so, it is of particular interest to know in what evolutionary scenario PRRs lost the phosphorelay function, in order to understand the evolution of plant clock machineries. Homologue sequences of the \(A.\ thaliana\) PRR genes have recently been characterized in several other angiosperms.\(^8–15\) Importantly, Corellou \textit{et al.}\(^16\) recently showed that the green alga \textit{Ostreococcus tauri} has a PRR homologue sequence (\textit{Otoc1}) that functions as a master clock gene. The Otoc1 protein carries a potential phosphoacceptor aspartic acid in its RLD,\(^16\) indicating that the substitution of the aspartic acid to the glutamic acid is likely to date back to the period between the emergence of the green alga and the divergence of angiosperms. It would be informative, therefore, to characterize their homologues from non-angiosperm land plants, which cover a wide spectrum of phylogenetic groups.\(^17,18\)

\textit{Physcomitrella patens}, a species of Bryopsida (moss), one of the basal land plants,\(^17,18\) diverged from vascular plant lineages at least 450 million years ago.\(^18\) This moss is an attractive model plant because various molecular biology techniques such as targeted gene disruption have been well established.\(^19\) In a recent study, we isolated and characterized two \(P.\ patens\) cDNAs \(PpCCA1\) \(a\) and \(PpCCA1\) \(b\) encoding moss homologues of \(A.\ thaliana\) CCA1/LHY. CCA1/LHY is a pair of paralogous single myb proteins, which function as another type of important component proteins in the \(A.\ thaliana\) circadian network.\(^3,4\) Disruption experiments on \(PpCCA1\) \(a\) and \(PpCCA1\) \(b\) genes indicate that these two genes are functional counterparts of CCA1/LHY.\(^20\) We also identified four candidate genes that encode PRR homologues in the \(P.\ patens\) genome database.\(^20–22\) We isolated the full-length cDNA for one of these four moss PRR genes and showed that this gene is expressed in a circadian manner.\(^20\) Very recently, Holm \textit{et al.}\(^23\) reported a survey of clocks-associated genes on the \(P.\ patens\) genome database, including the four moss PRRs. They constructed an unrooted tree of plant PRR homologues, in which the four moss PRRs clustered independently from angiosperm PRR subfamilies.\(^23\)

In this study, we isolated and characterized cDNAs for the remaining three moss PRR genes, for which gene structures were so far predicted only from the genomic sequences. Using experimentally validated sequences of the moss PRR cDNAs, we constructed a rooted phylogenetic tree, thereby clearly defining the evolution of plant PRRs. We also conducted biochemical characterization and expression analyses of the moss PRRs; the results of these experiments suggest that at least one of the moss PRRs functions as an authentic RR and that there are intraspecific divergences among the moss PRRs. Finally, we will discuss scenarios of the evolution and divergence of PRR homologue genes in land plants.

2. Materials and methods

2.1. Plant materials, growth conditions and light treatment

\textit{Physcomitrella patens} ssp. \textit{patens}\(^24\) was maintained in 12-h light and 12-h dark cycles (12:12LD) under white fluorescent light (light intensity \(\sim 40\, \mu\text{mol m}^{-2}\, \text{s}^{-1}\)) at 25°C. Protonemal and gametophore tissues were grown on BCDAT medium and BCD medium, respectively, both supplemented with 1 mM CaCl\(_2\).\(^25\) Protonemal cells were collected every 5–7 days and were grown with a homogenizer before application to new BCDAT agar plates. For light sources of the light induction experiments, the light-emitting diodes (STICK LED, Tokyo Rikakikai) were used for blue light (\(\lambda_{\text{max}} = 470\) nm) and the red-emitting fluorescent tubes (FL20S-Re-66, Toshiba Lighting & Technology) filtered through a red plastic sheet (Acrylite102, Mitsubishi Rayon) for red light (\(\lambda_{\text{max}} = 660\) nm). White light was provided by fluorescence lumps (FL20SS-W/18, Toshiba Lighting & Technology).

2.2. Identification and isolation of cDNAs covering the entire coding regions of \textit{PpPRRs}

The 5′- and 3′-terminal portions of the \(PpPRR2\) and \(PpPRR4\) cDNAs were RACE-amplified using GeneRacer (Invitrogen) with primers based on the JGI database sequences. The amplified cDNA fragments were cloned into the pGEM-T Easy vector (Promega) and sequenced with DYEnamic ET terminator [GE
Healthcare (Former Amersham Biosciences)]. The middle region of each gene was amplified by RT-PCR with primers based on the RACE-amplified sequences. The entire regions of both cDNAs were amplified with KOD plus polymerase Ver.2 (TOYOBO), subjected to A-tailing by Taq polymerase (TAKARA Bio), cloned into the pGEM-T Easy vector and sequenced using a primer walking method. The PpPRR3 cDNA that spans its entire coding region was amplified with KOD plus polymerase Ver.2 using primers based on the JGI database sequences. Nucleotide sequences were assembled by DNAlis software (Hitachi software engineering). All primer sets used are described in Supplementary file 1.

2.3. Phylogenetic analyses

Amino acid sequences of PRR homologues were aligned using the ClustalW program and the numbers of amino acid substitution sites between each pair of PRR proteins were estimated by the Jones–Taylor–Thornton (JTT) model with the complete-deletion option. From the estimated numbers of amino acid substitutions, a phylogenetic tree was reconstructed using the minimum evolution (ME) method. The bootstrap values were calculated with 1000 replications. These procedures were all performed using MEGA4.1 software (http://www.megasoftware.net/index.html). We also reconstructed a phylogenetic tree by the maximum-likelihood method using PhyML (http://atgc.lirmm.fr/phyml/) applying the JTT model for amino acid substitutions. We obtained a similar phylogeny pattern with both the methods.

2.4. Semi-quantitative and quantitative RT-PCR analyses

Semi-quantitative RT-PCR (sqRT-PCR) analysis was performed as described previously. We conducted preliminary experiments to improve its quantitative nature as follows. First, thermal cycle numbers were optimized so that signals from PCR products did not reach a plateau. Next, we confirmed that when various known relative amounts of cDNA for each tested gene were used as PCR templates, the amount of the PCR product of each reaction showed a linear relationship with that of the input cDNA. The primers and optimal cycle numbers for PCR are described in Supplementary file 1. Quantitative real-time PCR (qRT-PCR) analysis was performed as described previously. The primers are same as those used in the qRT-PCR analysis (Supplementary file 1).

2.5. Construction of plasmids

To express the PpPRR1 and PpPRR2 RLD peptides in E. coli cells, a cold shock expression system (pCold-II vector; TAKARA Bio) was used as follows. The coding region for the RLD of each PpPRR protein was PCR-amplified with primers described in Supplementary file 1. The resultant fragment was cloned into the pGEM-T Easy vector, digested with KpnI and XbaI and inserted into KpnI–XbaI-cleaved pCold-II vector (pCold-II-His-PRR1 and pCold-II-His-PRR2 for PpPRR1 RLD and PpPRR2 RLD, respectively). The nucleotide sequence was determined by DYEnamic ET terminator. The RLD peptides are fused with a 6×His-tag, by expression from pCold-II vector, allowing their purification with a TALON™ metal affinity resin column as described below.

2.6. Expression and purification of the PpPRR1 and PpPRR2 RLD peptides

The E. coli strain BL21 (DE3) harbouring pG-KJE8 (TAKARA Bio), which encodes chaperone proteins DnaK, DnaJ, GroE, GroEL and GroES, was transformed with pCold-II-His-PRR1 or pCold-II-His-PRR2. To over-produce the RLD peptides of PpPRR1 and PpPRR2, each transformant was cultivated in 1 l of LB medium containing ampicillin (50 μg ml⁻¹), Chloramphenicol (25 μg ml⁻¹), tetracycline (20 ng ml⁻¹) and L-arabinose (10 mg ml⁻¹) at 37°C until the logarithmic phase of growth in a rotary shaker (at 110 r.p.m.). They were cold-shocked by standing at 15°C for 30 min after the addition of IPTG at the final concentration of 1 mM and cultured in a shaker (at 110 r.p.m.) at 15°C for 5 h. Cells were collected by centrifugation and suspended in 20 ml of a standard buffer [50 mM Tris–HCl (pH 8.0), 100 mM NaCl and 10% glycerol]. The cell suspension was mixed with DNase I (25 μg ml⁻¹), EDTA (1.25 mM) and lysozyme (250 μg ml⁻¹) at 4°C for 15 min, and then passed through a French Press at 100 MPa. The resultant cell lysate was centrifuged and separated from cell debris and then applied onto a TALON™ metal affinity resin (Clontech Laboratories) column. After washing the TALON™ column with 50 mM Tris–HCl (pH 8.0), 10% glycerol and 100 mM NaCl, each of 6×His-tagged PpPRR1 and PpPRR2 RLD peptides was eluted with the same buffer including 250 mM imidazole and finally dialysed against 50 mM Tris–HCl (pH 8.0) and 10% glycerol. Dialysed semi-purified protein was concentrated by loading on Amicon Ultra Filter (Ultraclel-10K, Millipore) and centrifuged for 15 min at 4700 r.p.m. at 4°C. The sample was quantified by the Lowry method and subjected to SDS–PAGE and detected with Coomassie Brilliant Blue. It was also analysed by western blotting with anti-6×His antibodies (Cat. No. A190-114A, Bethyl Laboratories).

2.7. Preparation of the E. coli ArcB-enriched cytoplasmic membrane

Escherichia coli DAC903/pIA001-ArcB and DAC903/pIN-III were used as an ArcB overproducer and a
negative control, respectively.34 From each strain, membrane vesicles were isolated and pelleted as an insoluble fraction of the cell lysate according to the method of Azuma et al.35 They were suspended in a small volume of a buffer comprising 50 mM Tris-acetate (pH 8.0), 1 mM DTT, 2% glycerol and 250 mM sucrose in order to isolate cytoplasmic membrane as follows. The suspended membrane vesicles were layered over a 21-mL five steps gradient of sucrose dissolved in the same buffer (3 mL of 50%, 9 mL of 45%, 3 mL of 40%, 3 mL of 35% and 3 mL of 30% from bottom to top) in a bottle assembly polycarbonate tube (part no. 355618, Beckman Coulter) and centrifuged at 47,000 r.p.m. for 2 h at 4 °C with the type 50.2 Ti rotor (Beckman Coulter) and centrifuged at 47,000 r.p.m. for 2 h at 4°C with the type 50.2 Ti rotor (Beckman Coulter). The cytoplasmic membrane was collected by taking the fraction between the layers of 35 and 40% sucrose and diluted with a large volume of a buffer (Tris-GS-buffer) containing 5 mM MgCl₂, 200 mM KCl and (50 mM Tris–HCl, pH 8.0, 0.35 mM EDTA, 10% glycerol) containing 5 mM MgCl₂, 200 mM KCl and 2 mM DTT at 25°C for 1, 5 and 10 min. The reaction was terminated by the addition of SDS–PAGE sample buffer. The samples were subjected to SDS–PAGE. The gel was dried and analysed with BAS-2500.

3. Results

3.1. Phylogenetic analyses of the moss PpPRR genes

Previously, we identified four PRR homologue genes, PpPRR1 (protein ID in the JGI P. patens database: 154145), PpPRR2 (165025), PpPRR3 (173125) and PpPRR4 (165029) in the JGI genome database.21 We characterized the PpPRR1 gene (and its corresponding cDNA) as ‘PpPRRa’ before,36 but in this paper, we use the name ‘PpPRR1’, not PpPRRa, to avoid confusion. Holm et al. also followed this nomenclature (PpPRR1–PpPRR4) deposited in the JGI genome database.21 Other than these four sequences, we found no gene models that are significantly similar to the angiosperm PRR genes in the moss genome. We isolated cDNAs that cover the entire coding regions for the PpPRR2, PpPRR3 and PpPRR4 genes and confirmed that all of their predicted proteins share, as do PpPRR1, an N-terminal RLD and a C-terminal CCT domain (Fig. 1a and b; Supplementary file 2). The receiver domains of authentic RRs share the aspartic acid–aspartic acid–lysine (DDK) motif, essential for the phosphotransfer activity,36,37 whereas the first two residues of this motif are diverged in the angiosperm PPR proteins (Fig. 1a; for review, see Mizuno and Nakamichi4 and Mizuno6). In all the available angiosperm PRR sequences, the second aspartic acid, conserved as the phosphoacceptor residue in the His–Asp phosphotransfer, is replaced by a glutamic acid (E) (Fig. 1a). In contrast, RLDs of moss PpPRR proteins are all predicted to retain the second aspartic acid; furthermore, PpPRR2, PpPRR3 and PpPRR4 also retain the first aspartic acid, hence exhibiting the complete DDK motif (Fig. 1a). PpPRR1 replaces the first aspartic acid residue with a tyrosine (Y) (Fig. 1a). These observations suggest that at least PpPRR2, PpPRR3 and PpPRR4 are potentially phosphorylated by a HK(s) as authentic RRs. The CCT domain is well conserved in all PpPRR proteins (Fig. 1b).

To examine phylogenetic relationships among PpPRR genes and other land plant PRRs, we constructed a rooted phylogenetic tree using PRR homologue
sequences from various plants (Fig. 2). We used the PRR homologue of the green alga *O. tauri* (*OtTOC1*)\(^{16}\) as the outgroup because: (i) *OtTOC1* is positioned relatively distant from any other PRR homologue sequences in an unrooted tree of PRRs\(^{23}\); and (ii) *OtTOC1* is sister to all the other PRR homologue sequences when an authentic RR sequence is used as the outgroup (Supplementary file 3). Also included were three algal sequences: two from *C. reinhardtii* and one from *Chlorella variabilis*. In the phylogenetic tree, the angiosperm sequences are divided, as previously reported,\(^{8,11,15}\) into three groups: TOC1, PRR7/3 and PRR9/5 (Fig. 2). The TOC1 group is basal to all the other sequences that include both PRR7/3 and PRR9/5 groups, indicating a more ancient origin of the TOC1 group than the other two groups. The four moss PpPRR sequences are grouped with one another, further forming a cluster with two lycophyte sequences, and this cluster is sister to the PRR7/5 group (Fig. 2). This indicates

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**Figure 1.** Alignment of conserved domains of PRR homologues from various species. RLDs (a) and CCT domains (b) of PRR homologue sequences from various plant species are aligned using ClustalW program.\(^{26}\) Shading was performed using BOXSHADE ver 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Amino acids identical or similar in more than 70% of the sequences are shaded by black or grey background, respectively. The number at the end of each line indicates the rightmost amino acid. Arrowheads show amino acids corresponding to the DDK motif. The PRR homologue sequences are as follows: PpPRR1, PpPRR2, PpPRR3 and PpPRR4 from *P. patens*; SmPRR7a (450934, protein ID in the JGI *Selaginella moellendorffii* database), SmPRR7b (450936), SmTOC1 (438647) from *S. moellendorffii*; OtTOC1 (AY740079 in DDBJ/EMBL/GenBank) from *O. tauri*; AtPRR3 (BAB13744), AtPRR5 (BAB13743), AtPRR7 (BAB13742) and AtPRR9 (BAB13741) from *A. thaliana.*
that the moss *PpRR* genes are more closely related to the *Prr7/3* group than to the two other groups. The four moss sequences are divided into two groups *PpPRR1/PpPRR3* and *PpPRR2/PpPRR4* (Fig. 2).

Next, we compared the distribution of intron insertion sites on the RLD and CCT coding regions between *PpPRR* genes (Fig. 3). In the RLD region, two insertion sites are conserved among all the *PRR* sequences examined (Fig. 3a; see Takata et al.\textsuperscript{15}). This distribution pattern of intron insertion sites is clearly different from those seen in the receiver domain of authentic *RR* genes,\textsuperscript{13} confirming the idea that *PRR* genes
diverged distinctly from authentic RR genes. In the CCT domain region, the TOC1 group sequences are unique in that they show no intron, whereas all sequences in the other two groups have a conserved single insertion site in the middle of the domain (Fig. 3b; see Takata et al. [15]). PpPRR sequences are divided into two groups, PpPRR1/PpPRR3 with one intron (like the PRR7/3 and PRR9/5 groups) and PpPRR2/PpPRR4 with no intron (like the TOC1 group; Fig. 3). This divergence is consistent with the results of the phylogenetic tree (Fig. 2), whereas the loss of an intron in PpPRR2/PpPRR4 should have occurred in the moss lineage independently from the TOC1 group (Fig. 2).

3.2. In vitro phosphotransfer from a HK to the RLD of PpPRR2

We examined whether RLDs of PpPRR1 and PpPRR2, both of which retain the potential phosphoacceptor residue while showing mutually diverged features (Figs 1–3), are phosphorylated by a His–Asp phosphorelay process in an in vitro phosphotransfer assay (Fig. 4). In this assay, ArcB, an E. coli HK, added in excess and hence lacking substrate specificity, transfers its phosphate to the phosphoacceptor site in a receiver domain. We purified the RLD peptides of PpPRR1 and PpPRR2 overexpressed in E. coli cells (Fig. 4b and c) and tested each of them to assess whether or not they undergo phosphotransfer. The PpPRR2 RLD peptide was phosphorylated within 5 min in the presence of ArcB (Fig. 4g). The phosphorylation levels of ArcB decreased concomitantly (Fig. 4g), indicating that the phosphorylation of PpPRR2 RLD is due to phosphotransfer from ArcB. On the other hand, we could not detect any phosphorylation signal with PpPRR1 RLD (Fig. 4f), consistent with its relatively diverged RLD sequence (Fig. 1a). When the PpPRR2 RLD peptide was incubated with the membrane fraction with no overexpressed ArcB, we could not detect the phosphorylation signal (Fig. 4h). This supports the interpretation that the increased levels of phosphorylation seen with the PpPRR2 RLD peptide (Fig. 4g) is due to phosphotransfer from ArcB, but not due to other types of kinases. These observations indicate that PpPRR2 presumably functions as an RR in an unknown His–Asp phosphorelay signal transduction pathway in P. patens. Moreover, PpPRRs likely diverged from one another based not only on their RLD sequences but also on their phosphotransfer ability.

3.3. Circadian regulation of PpPRR expression profiles

In a previous study, we reported that the PpPRR1 mRNA accumulation showed circadian variation in 12-h light and 12-h dark cycles (12:12LD) and in continuous darkness (DD), but not in continuous
light (LL), by conducting sqRT-PCR analysis. Here, we studied mRNA accumulation profiles for all four PpPRR genes with the same method (Fig. 5). In 12:12LD, all genes showed high-amplitude mRNA rhythms with a period of ≈1 day, which peaked in the latter half of the light phase (Fig. 5a). In DD, all genes showed endogenous rhythms with damping (Fig. 5b). These rhythms in 12:12LD or DD showed phase relationships roughly similar to A. thaliana PRR3, PRR5 or PRR7 genes.4,6 In LL, in contrast, all the genes exhibited no hint of circadian regulation and were arrhythmic as demonstrated for PpPRR1 (Fig. 5c; see Okada et al.20). The arrhythmic profiles in LL are consistent with our observations that the moss genes so far tested are, if clock gene homologues or clock-controlled genes, all arrhythmic in LL,38–40 and this is in contrast to angiosperm PRR genes, all of which show robust circadian rhythms in LL.4,6

The results of the sqRT-PCR experiments suggest that phases of the four genes seem to be differentially fine-tuned into two types: in 12:12LD, troughs of PpPRR1/PpPRR3 occurred 4 h before dawn, whereas those of PpPRR2/PpPRR4 just at dawn (Fig. 5a). We also measured mRNA accumulation for PpPRRs in one cycle of 12:12LD by the qRT-PCR analysis with a shorter sampling interval (Fig. 6). At ZT01 (ZT, zeitgeber time: time in a light–dark cycle, putting the light onset as ZT0), the levels of PpPRR1/PpPRR3 showed certain increase (30–40% of the maximum levels), whereas those of PpPRR2/PpPRR4 were still very close to zero (Fig. 6), confirming the distinction of expression profiles between PpPRR1/PpPRR3 and PpPRR2/PpPRR4. This differential fine-tuning appears to be conferred by the endogenous circadian clock, because the sampling of cells at dawn (Fig. 5a, hours 0 and 24)
was performed in the absence of light. This idea is also supported by the rhythms of PpPRRs in DD, where endogenous clock regulation is more clearly seen: the first peaks of PpPRR2/PpPRR4 lagged behind those of PpPRR1/PpPRR3 by around 4 h (Fig. 5b; Supplementary file 4).

3.4. Induction of PpPRR by light

We examined the effect of light on the accumulation of the PpPRR mRNAs by sqRT-PCR analyses. The mRNAs of all four PpPRRs were induced by a 2-h pulse of white, blue or red light (Fig. 7). The rates of induction by white light are 8.7 for PpPRR1, 2.6 for

![Figure 5. Changes in mRNA abundance for the PpPRR genes under 12:12LD, DD and LL conditions examined by the sqRT-PCR analysis. Physcomitrella patens protonemal cells were maintained in 12:12LD for more than 2 weeks after which cells were harvested in 12:12LD (a), DD (b) or LL (c) conditions at indicated times. From the top, changes in mRNA abundance for PpPRR1, PpPRR2, PpPRR3 and PpPRR4 are shown. Light conditions are overlaid with each graph: regions with no shade and those shaded with dark grey represent light and dark phases, respectively; regions shaded with light grey represent subjective light phases in (b) or subjective dark phases in (c). Graphs show the results of quantification of the mRNA levels for each gene after normalization to those for actin as a control. The maximum levels are set to 1.0. The photo below each graph shows the hybridized bands for each test gene or the control actin gene, detected as chemiluminescence signals. We obtained similar results in two independent experiments.](image-url)
The rates of induction for \(\text{PpPRR}_1\) and \(\text{PpPRR}_3\) are the highest and second highest, respectively, for any colour of light; this observation does not contradict the idea that there is an intraspecific divergence between \(\text{PpPRR}_1/\text{PpPRR}_3\) and \(\text{PpPRR}_2/\text{PpPRR}_4\). The rates of induction by blue light and red light are lower than those of white light for any \(\text{PpPRR}\) gene.

4. Discussion

The results of the current study help to understand the origins of the angiosperm \(\text{PRR}\) genes. In our \textit{in vitro} assay, the \(\text{PpPRR}_2\) RLD peptide underwent phosphotransfer (Fig. 4), consistent with its complete DDK motif (Fig. 1a), strongly suggesting that \(\text{PpPRR}_2\) functions as an RR. Besides, according to our phylogenetic tree (Fig. 2), \(\text{PpPRRs}\) are phylogenetically closely related to angiosperm \(\text{PRRs}\). Therefore, it is presumed that the similarity of RLDs of \(\text{PRRs}\) to the authentic receiver domain is not only superficial but that \(\text{PRRs}\) were certainly derived from an authentic RR. Most probably, angiosperm \(\text{PRRs}\) have lost their phosphorelay function through the course of evolution, largely due to the substitution of the phosphoacceptor aspartic acid to a glutamic acid in their RLDs, and other critical residues such as the first aspartic acid of the DDK motif should have concomitantly diverged. Importantly, the \(\text{O. tauri}\) OtTOC1 and the \(\text{C. reinhardtii}\) CrPRR2 also share not only the potential phosphoacceptor aspartic acid but also the entire DDK motif (Fig. 1a; see Corellou \textit{et al.}\textsuperscript{16}). Although there has so far been no report about whether or not these algal proteins undergo phosphotransfer, they are also assumed to function as RRs, representing prototypic proteins of the land plant \(\text{PRRs}\).

Our results also offer evolutionary explanations not only for the origin but also for the diversity of \(\text{PRRs}\) in land plants (Fig. 8). The pattern of our tree indicates that the TOC1 group first diverged, and then split the PRR9/5 group and the other branch, the latter containing the PRR7/3 group. Since \(\text{PpPRRs}\) are positioned inside the branch containing the PRR7/3 group, the origins of the TOC1 and PRR9/5 groups date back before the divergence of moss from higher plant lineages. The ancient origin of the TOC1 group is supported by the observation that \(\text{C. reinhardtii}\) and \(\text{C. variabilis}\) seem to have a TOC1 ortholog (Fig. 2). Furthermore, since the cluster of \(\text{PpPRRs}\) and lycophyte SmPRR7a/7b is sister to all the angiosperm PRR7/3 sequences (Fig. 2), the divergence between the PRR7/3 group and the ancestor of \(\text{PpPRRs}\) and SmPRR7a/7b also predate the divergence of moss from higher plants. Therefore, the common ancestor of moss and higher plants possessed TOC1, PRR7/3 and PRR9/5 orthologues in its genome, but these genes appear to have been lost later within the moss lineage (Fig. 8). In the lycophyte lineage, PRR7/3 and PRR9/5 orthologues have been lost and
Within each lineage of the TOC1, PRR7 acid residue must have been substituted, likely; however, if this were the case, the aspartic residue in these sequences has never been substituted. In the algal sequences; the phosphoacceptor residues of the PpPRRs and SmPRR7a/7b can be traced back to that found in the algal sequences; the phosphoacceptor residue in these sequences has never been substituted. This explanation might seem simple and more likely; however, if this were the case, the aspartic acid residue must have been substituted, according to the branching patterns of our tree, independently within each lineage of the TOC1, PRR7/3 and PRR9/5 groups (Figs 2 and 8a). In another scenario (Fig. 8b), simpler at least in terms of the frequency of substitutions, the aspartic acid was substituted only once to a glutamic acid before the divergence of all the land plant PRR subfamilies. In this case, an aspartic acid was regained by a second substitution in the ancestral lineage of PpPRRs and two lycophyte genes (Fig. 8b). If this were the case, PpPRR1 might be, again, in the process of divergence from authentic RR-type sequences. In order to know which hypothesis, or yet another scenario, is more plausible, we need more sequence data; in particular, PRR sequences should be characterized from other primitive plants, i.e. liverworts, hornworts, ferns and gymnosperms.

Our results demonstrate intraspecific divergences among the PpPRR genes. The phylogenetic tree (Fig. 2), intron insertion sites (Fig. 3) and expression profiles (Figs 5–7 and Supplementary file 4) suggest the divergence between PpPRR1/PpPRR3 and PpPRR2/PpPRR4. The A. thaliana PRR family members show differentially regulated expression profiles, reflecting the fact that they act at different nodes in the circadian network and functionally diverged from one another.4,6 Therefore, PpPRR1/PpPRR3 and PpPRR2/PpPRR4 are also predicted to be functionally diverged. It should be noted that Holm et al.23 did not detect any differential expression among the four genes in a light–dark cycle followed by DD. This may be because their light–dark regime is different from ours: they used a long day regime, a 16-h light and 8-h dark cycle, whereas ours is a 12-h light and 12-h dark cycle(s) (12:12LD; Figs 5 and 6). All PpPRRs are induced by light (Fig. 7), and this light responsiveness may have apparently synchronized the trough phases of PpPRRs with the earlier dawn in the short night of their long day regime, whereas difference in trough phases between PpPRR1/PpPRR3 and PpPRR2/PpPRR4 are obvious in our longer nights (Fig. 5a).

On the other hand, the results of the in vitro assay (Fig. 4) and detailed sequence comparison (Fig. 1a) suggest that PpPRR2/PpPRR3/PpPRR4 function as RRs, whereas PpPRR1 does not or its phosphotransfer ability is very weak. The moss PpPRRs, if not all of them, are anticipated to have some clock-associated functions as do A. thaliana PRRs, from the following facts: (i) the circadian networks of A. thaliana and P. patens are predicted to be at least partially conserved20; and (ii) O. tauri, which belongs to green algae, the closest relative of land plants, has a PRR homologue that functions as a clock gene.16 Since RRs are generally involved in signalling cascades responsive to environmental/endoogenous signals, PpPRR2/PpPRR3/PpPRR4 might function, rather than in the core clock circuitry, in an input pathway(s) that must be responsive to environmental cues such as light and temperature.

Figure 7. Light-induced expression of the PpPRR genes examined by the sqRT-PCR analysis. Physcomitrella patens protonemal cells were maintained under LL for 1 week, exposed to darkness for 24 h, and 2 h of white light, blue light, red light or dark period was administered before the cells were sampled. The fluence rate of each colour or light was 40 μmol m⁻² s⁻¹. Total RNAs were extracted, and the abundances of PpPRR mRNAs were measured and normalized to those for actin as a control. The graph shows the mRNA levels of PpPRRs induced by different colour of light (white light, open bars; blue light, bars with horizontal hatches; red light, bars with vertical hatches) relative to those from samples maintained in the prolonged darkness, the latter of which are set to 1.0. Values are the mean ± SD of three replications. The bottom photos show the hybridized bands for PpPRRs (upper four slips) or the control actin gene for each PpPRR (lower four slips), detected as chemiluminescence signals. We obtained similar results in two independent experiments.
In a future study, in parallel with gene knock out experiments, it should also be addressed whether PpPRRs are involved in phosphotransfer functions in planta. Moreover, a HK(s) and a HPt(s) that are partners to (at least some of) PpPRR proteins should be characterized. It might not be easy to identify a partner HK(s) because the P. patens genome contains as many as \( \approx 50 \) HK sequences, unlike A. thaliana, which contains only 8 HK genes. However, the availability of the entire genome sequence, many full-length cDNA clones and the tractability of gene functional analysis based on gene targeting techniques will support the identification of such HK and HPt genes in P. patens, possibly as novel clock genes.

### 4.1. Conclusion

Here, we demonstrated that the moss PpPRRs have close relationships with angiosperm PRRs and at least PpPRR2 can function as an RR, indicating that the plant clock-associated PRR families are derived from an authentic RR(s). Moreover, here, we offered evolutionary explanations for divergence of PRR genes in land plants. This study should be a foundation for understanding how a particular set of genes involved in regulation of the clock evolved their functions, in coordination with changes (or maintenance) of the clock mechanisms.

### 4.2. Accession numbers for the sequence data

The sequences reported in this paper have been submitted to the public databases [DDBJ]/EMBL/GenBank: AB558266 (PpPRR1), AB558268 (PpPRR2), AB558267 (PpPRR3) and AB558269 (PpPRR4).

### Supplementary Data:

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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