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The unique function of the *Arabidopsis* circadian clock gene *PRR5* in the regulation of shade avoidance response

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Abbreviations: S.A.R, shade avoidance response; PIF, phytochrome Interacting factor; CCA1, CIRCADIAN CLOCK-ASSOCIATED 1; LHY, LATE ELONGATED HYPOCOTYL; TOC1, TIMIMG OF CAB EXPRESSION 1; GI, GIGANTEA; PRR, PSEUDO RESPONSE REGULATOR; LD, 16 h light/8 h dark cycle; LL, continuous lighting; ZTL, ZEITLUPE

Shade avoidance response (S.A.R) is regulated by light and circadian clock. Circadian clock controls S.A.R by the transcriptional regulation of positive regulators of S.A.R, PIF4 and PIF5, to prevent plants from responding to 'light' of dark period. Thus, in many cases, deficits in circadian clock appear in abnormalities of hypocotyl and/or petiole elongation. Previously, interesting phenomena were reported that the triple mutants of *PSEUDO RESPONSE REGULATORS9*, *7* and *5*, which are clock components, show longer petioles and smaller leaves under light/dark cycle than those under continuous lighting. These S.A.R-like phenotypes cannot be explained by their hyposensitivity to red light. We demonstrated detailed analyses of this mutant to reveal the leaf-specific S.A.R regulated by circadian clock. Expression analyses of S.A.R-related genes suggested that *PRR5* functions as a repressor of S.A.R. Morphological analyses of leaves under different light condition revealed that *PRR5* is involved in the inhibition of leaf expansion in S.A.R.

Introduction

In plants, shade avoidance response (S.A.R), such as promotion of stem and/or petiole elongation and repression of leaf expansion, is one of the important adaptive responses to changes of light environment. Under shade cast by canopies (canopy-shade), the ratio of red light (R) to infrared light (FR) (R/FR) and intensity of blue light are decreased by the absorption of red and blue light by photosynthetic pigments. The former and latter are perceived by red/infrared photoreceptors: phytochromes and blue/UV receptors: cryptochromes, respectively.[1-4] In *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis), five phytochrome-encoding genes, *PHYA* to *PHYE*, and two cryptochrome genes, *CRY1* and *CRY2*, have been isolated.[1-6] *PHYB* and *CRY1* play major roles in S.A.R.[3,7,8] The ratio of R to FR is perceived as the photoequilibrium between FR-absorbing form (Pfr) and R-absorbing form (Pr) of PHYB. Pfr and Pr reversibly photointerconvert each other.[9] On the other hand, CRY1 is activated in a manner depending on intensity of blue light.[10] Pfr form of PHYB (PhyBfr) and activated CRY1 repress S.A.R.[8,11]

PhyBfr accumulates in nuclei and interacts with a group of basic helix-loop-helix (bHLH) transcriptional factors, which are called phytochrome interacting factors (PIFs).[12-14] Their functions partially and intricately overlap each other.[15] Especially, PIF3, PIF4 and PIF5, which interact with and degraded by PhyBfr, act as positive regulators of S.A.R.[16-19] They directly bind to G-box and/or E-BOX motifs located in the promoter regions of the downstream genes in phytochrome-mediated signal transduction pathway, such as other bHLH transcriptional factors (e.g., *PIL1* and *HFR1*), *YUCCA*s that participate in auxin biosynthesis, *IAA*/*AUX* which regulate auxin signal transduction and a *HD-Zip* gene, *AtHB-2*.[20-22] Particularly, *PIL1*, *AtHB-2* and *HFR1* are rapidly induced by canopy-shade stimulus, thus their expression are often used as indexes of S.A.R.[23,24]

S.A.R is regulated not only by light but also by circadian clock.[25,26] A lot of mutants of circadian clock genes display abnormalities in photomorphogenesis, such as hypocotyl elongation.[27-29] Currently, it is thought that circadian clock regulates S.A.R mainly to repress the expression of *PIF4* and *PIF5* from dusk until midnight.[26,28,30] This diurnal change in the regulation of S.A.R helps plants to respond to light environment only during light period.

A mutant of *PSEUDO RESPONSE REGULATOR9*, *7* and *5* (*PRR9*, *PRR7* and *PRR5*), which are components for circadian

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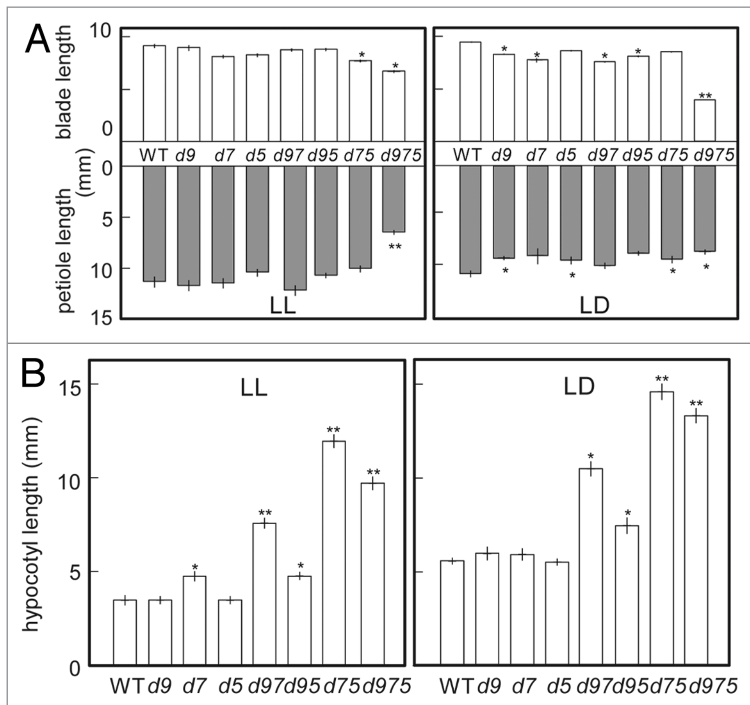

Figure 1. Length of leaf blade, petiole and hypocotyl of *prr* mutants grown under continuous light (LL, left panel) or 16 h light/8 h dark (LD, right panel). *d9*, *d7* and *d5* in these figures mean *prr9*, *prr7 and prr5* mutants, respectively. Double and triple mutants were represented by the combinations of these abbreviations. (**A**) Plants were grown under for 22 d.a.g under 40 μmol $m^{-2}s^{-1}$ PPFD, and then 3rd leaves were measured (n ≥ 6). (**B**) Seedlings were grown for 7 d.a.g under 20 μmol $m^{-2}s^{-1}$ PPFD and measured (n ≥ 12). Error bars represent SE, and single or double asterisks indicate significant difference from WT using Tukey's LSD (*p ≤ 0.05, **p ≤ 0.01).

clock, is also one of the evidences of the connection between circadian clock and light signal transduction pathway of S.A.R. *prr9*;*prr7*;*prr5* mutants show extreme hyposensitivity to red light in hypocotyl elongation and the arrhythmic expression of other major clock genes, *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *TIMIMG OF CAB EXPRESSION 1* (*TOC1*) and *GIGANTEA* (*GI*).[27] Interestingly, Niinuma et al.[31] reported that the leaves of *prr9*;*prr7*;*prr5* mutants grown under 16 h light/8 h dark cycle (LD) show extremely longer petioles and smaller leaf blades than those grown under continuous lighting (LL), while their hypocotyls are much longer than those of wild type (WT) under LD and LL. Similar phenomena to these have not been reported. Ordinarily, both hypocotyls and petioles are the stalk organs to support photosynthetic organs: leaves physically, and they have same behaviors of longitudinal growth promotion in S.A.R.[19,32,33] Those phenomena of *prr9;prr7;prr5* mutants cannot be explained only by the abnormal S.A.R caused by defective circadian clock.

In this study, we assured that the above-mentioned phenomena of *prr9*;*prr7*;*prr5* mutants were the clues to reveal the leaf-specific regulation of S.A.R, and performed detailed analyses of light responses in the leaves of *prr* mutants. Morphological analyses of leaves under different light qualities suggested that sensitivity to red light kept in *prr9;prr7;prr5* mutants is involved in leaf-specific S.A.R. Analyses of S.A.R-related gene expressions revealed that *PRR5* has a unique function to repress the amplitude of S.A.R in leaves. Furthermore, genetic analyses indicated that *PRR5* regulates S.A.R in the downstream of *PIF5*. This suggested the existence of direct regulation of S.A.R in the downstream of *PIF*s by circadian clock components. Interestingly, low R/FR treatment of *prr* mutants suggested that *PRR5* is involved in the inhibition of leaf expansion in S.A.R. Collectively, this study indicates a new point of contact between circadian clock and S.A.R and a tissue-specific function of a circadian clock component.

Result

***prr9*;*prr7*;*prr5* mutants have deficit in red light signaling pathway.** In order to analyze nature of reported S.A.R-like phenomena exhibited by the *prr9*;*prr7*;*prr5* mutants, we measured the length of leaf blade and petioles of *prr* mutants under continuous light LL or LD (**Fig. 1A**). In this study, it was noted that the T-DNA insertion lines of *prr9-10* (SALK_007551) and *prr7-11* (SALK_030430) were considered as null alleles of *PRR9* and *PRR7*, respectively, because their transcripts were not detected in the respective lines.[27] Although a truncated transcript of *PRR5* was expressed in *prr5-11* (KG24599) mutants, they showed almost the same phenotypes of a null allele of *PRR5*: *prr5-3* (SALK_064538) in light responsibility and period of rhythms in LL, as previously reported.[34] Thus, *prr5-11* line was assumed to be a null allele of *PRR5*. Plants were grown for 22 d after germination (d.a.g) under LL or LD, and then their 3rd leaves were sampled and measured. Under LL, the petiole lengths of all *prr* mutants except for *prr9*;*prr7*;*prr5* mutants were not different from those of WT. The leaf blades of *prr7*;*prr5* mutants were slightly shorter than those of WT, and the leaf blades and petioles of *prr9*;*prr7*;*prr5* mutants were much shorter than those of WT. Under LD, the leaf blades and petioles of all *prr* mutants except for *prr9*;*prr7*;*prr5* mutants were shorter than those of WT. *prr9*;*prr7*;*prr5* mutants only displayed extremely short leaf blades under LD. Interestingly, the petioles of *prr9*;*prr7*;*prr5* mutants under LD were much longer than those of them under LL, unlike the other *prr* mutants and WT (**Fig. 1A**). Although the hypocotyls of *prr9*;*prr7*, *prr9*;*prr5* and *prr7*;*prr5* mutants were longer than those of WT under both LL and LD, their leaf shapes were hardly different from those of WT (**Fig. 1A and B**). These results indicated that the behavior of hypocotyl elongation is not always the same with that of petiole elongation.

To determine which light signal transduction pathways are involved in the S.A.R-like phenomena of *prr9*;*prr7*;*prr5* mutants under LD, we measured the leaf shapes of *prr9*;*prr7*;*prr5* mutants treated with continuous red or blue light (**Fig. 2A**). Because the leaves of *prr9;prr7;prr5* mutants were much smaller than those of WT under any kind of light condition, we could not compare the effects of light quality on leaf shape using their absolute dimensions. Thus, in each line, the dimensions of leaves under red or

blue light were normalized to the average dimensions of those under white light. Under red light, *prr9*;*prr7*;*prr5* mutants displayed longer petioles and smaller leaf blades, similarly to S.A.R, than those grown under continuous white light (**Fig. 2B**). Under blue light, the leaf shapes of *prr9*;*prr7*;*prr5* mutants were not different from those of WT, except in size (**Fig. 2A and C**). These demonstrate that the leaves of *prr9*;*prr7*;*prr5* mutants have deficits in red light signal transduction pathway in photomorphogenesis as well as their hypocotyls.[27]

***PIF4* and *PIF5* mis-expression during dark period in *prr9*;*prr7*;*prr5*.** To gain insight into S.A.R-like phenomena in *prr9*;*prr7*;*prr5* mutants under LD, we analyzed the expression of the molecular markers for S.A.R: *HFR1* and *AtHB-2, PIF4* and *PIF5*. We first measured the expressions of these marker genes in *prr* mutants during dark period. Plants were grown under LD for 20 d.a.g, and then their third and fourth leaves were sampled and measured. *AtHB-2* expression was rapidly and intensely induced during dark period in the leaves of *prr9*;*prr7*;*prr5* mutants, and the same behavior was observed in the leaves of *prr7*;*prr5* mutants without some lag (**Fig. 3**). These results suggested that S.A.R might be induced in the leaves of *prr7*;*prr5* and *prr9*;*prr7*;*prr5* mutants during dark period. However, leaves of *prr7*;*prr5* mutants did not show S.A.R-like phenomena unlike their hypocotyls under LD (16 h light/8 h dark) (**Fig. 1A**). Besides the fact that leaves of *prr7*;*prr5* mutants displayed longer petioles than those of WT under 12 h light/12 h dark cycle (**Fig. S1**), it was supposed that 8 h dark was not long enough for their leaves to cause S.A.R-like phenomena. Additionally, *AtHB-2* highly expressed in *prr9*, *prr5* and *prr9*;*prr5* mutants in comparison with WT at the end of dark period (**Fig. 3**). However, *HFR1* expression in them was similar to that in WT (**Fig. S2**).

Second, to obtain further insight into what occurred in *prr9*;*prr7*;*prr5* mutants during dark period, we measured the gene expression profiles of *HFR1*, *PIF4* and *PIF5*. HFR1 expression in the leaves of *prr9*;*prr7*;*prr5* mutants was rapidly induced during dark period and remained high as compared with that in the leave of WT throughout the days despite the rapid reduction at dawn (**Fig. 4**). *PIF4* expression in the leaves of *prr9*;*prr7*;*prr5* mutants under LD was kept considerably higher than that in the leaves of WT throughout the days except at dusk, and *PIF5* expression oscillated arrhythmically and dramatically in amplitude (**Fig. 4**). Besides the arrhythmic phenotypes of *prr9*;*prr7*;*prr5* mutants as previously reported, these results suggested that mis-expression of *PIF4* and *PIF5* during dark period induces S.A.R-like phenomena under LD.[27] However, this does not explain the rapid induction of *AtHB-2* and *HFR1* in the beginning of dark period in the leaves of *prr9*;*prr7*;*prr5* mutants (**Figs. 3 and 4**). Photoactivated phytochrome B (PhyBfr), which degrades PIF4 and PIF5, is stable under dark condition.[19,35] Moreover, The dark-induced expressions of *AtHB-2* and *HFR1* were rapidly reduced by red light irradiation in the leaves of *prr9*;*prr7*;*prr5* mutants (**Fig. S3**). Thus, PhyBfr in the leaves of *prr9*;*prr7*;*prr5* mutants was unlikely to change rapidly in abundance or in conformation in the beginning of dark period.

Extreme induction of S.A.R by FR irradiation in *prr9*;*prr7*;*prr5* and *prr7*;*prr5* mutants. To gain insight into the rapid induction of the marker genes for S.A.R in the beginning of dark period in the leaves of *prr9*;*prr7*;*prr5* mutants, we measured expression levels of these genes with end-of-day FR (E.O.D-FR) treatment in loss-of-function mutants and overexpressor (ox) lines of the PRR genes. Plants were grown under LD for 20 d.a.g, and then given FR irradiation at the end of light period. Previous studies reported that *phyB* mutants and *PIF5*ox, which display constitutive S.A.R, were hardly different from WT in the expressions of these marker genes induced by low R/FR or E.O.D-FR treatment.[19,25,36] Interestingly, the expressions of *AtHB-2* and *HFR1* in *prr7*;*prr5* and *prr9*;*prr7*;*prr5* mutants were induced rapidly and approximately 2.5 to 4-fold more highly than those in WT with E.O.D-FR treatment, whereas such intense inductions were not observed in *phyB* mutants or *PIF5*ox plants (**Fig. 5A**; **Fig. S4**). It was also noted that *prr5* mutants with E.O.D-FR treatment displayed intense induction of these marker genes as compared

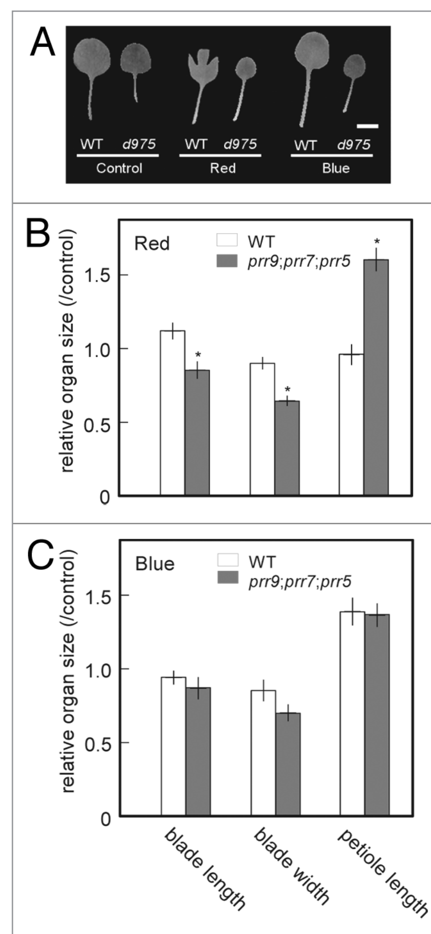

Figure 2. Dimensions of leaf blades and petioles of *prr9;prr7;prr5* mutants under red or blue light. Plants were grown under continuous white light (60 μmol $m^{-2}s^{-1}$ PPFD) for 16 d.a.g as control groups, or for 10 d.a.g under continuous white light and 6 d under continuous red or blue light (60 μmol $m^{-2}s^{-1}$ PFD). 1st and 2nd leaves were measured at 17 d.a.g. (**A**) The Images of the leaves of WT and *prr9;prr7;prr5* (*d975*) mutants under every light conditions. A scale bar indicates 5 mm. (**B**) and (**C**) In each line, each organ size was normalized to the average size of the control group. Error bars represent SE, and asterisks indicate significant difference from WT using Student's t-test ($n \geq 6$, $p \leq 0.05$).

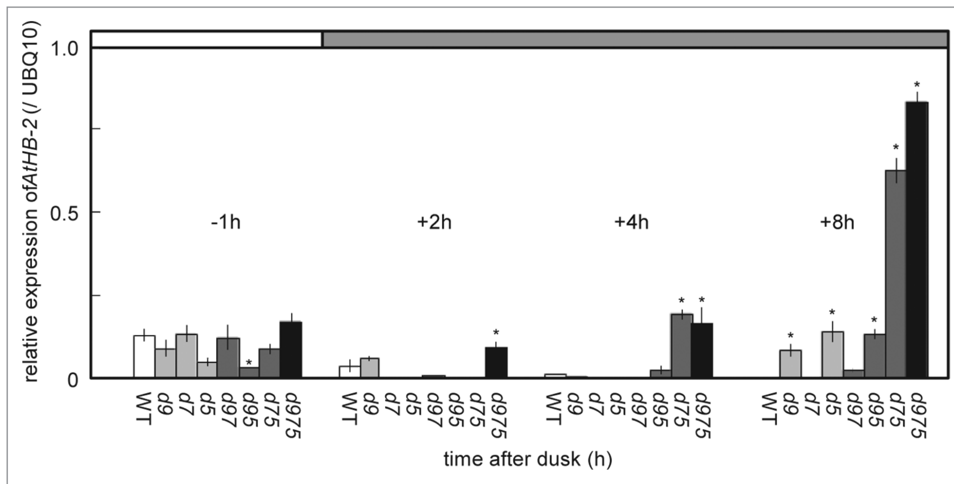

Figure 3. The expression of *AtHB-2* during dark period in *prr* mutants. Plants were grown for 22 d.a.g under LD (40 μmol $m^{-2}s^{-1}$ PPFD), and then RNA samples were prepared at specific time points from the end of light period to the end of dark period. The abbreviations of *prr* mutants are described in **Figure 1**. Error bars represent SE, and asterisks indicate significant difference from WT using Tukey's LSD ($p \leq 0.05$).

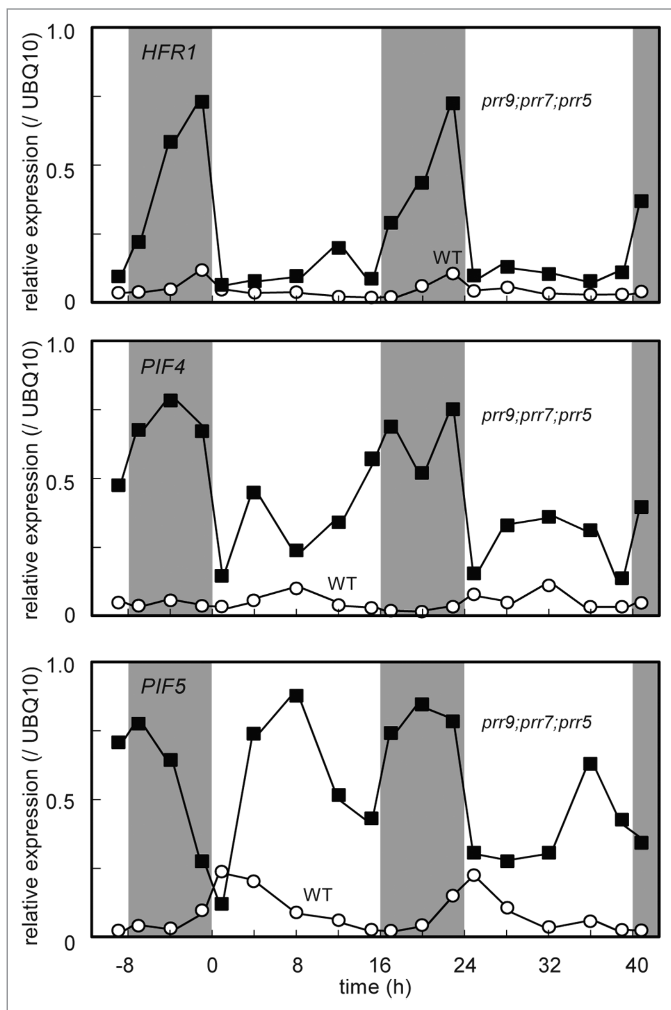

Figure 4. Diurnal expression profiles of *HFR1*, *PIF4* and *PIF5* in the leaves of **Figures 3** and **4** mutants under LD. Plants were grown under LD (40 μmol $m^{-2}s^{-1}$ PPFD) for 20 d.a.g, and then RNA samples were prepared at specific time points.

with WT and the other *prr* single mutants with E.O.D-FR treatment (**Fig. 5B**). Furthermore, remarkable suppression of the expression of these marker genes was observed only in *PRR5* overexpressors (*PRR5*ox) with E.O.D-FR treatment, whereas the other *PRR* overexpressors were hardly different from WT in the marker genes expression (**Fig. 5C**). Besides the fact that *prr5* mutants did not display conspicuous phenotypes under normal conditions, these results suggested that *PRR5* has a unique function to reduce the amplitude of induced S.A.R, unlike *PRR9* and *PRR7*.

Repression of S.A.R by *PRR5* in the downstream of *PIF5*. To further analyze this unique function of *PRR5* in the regulation of S.A.R, we analyzed the phenotypes of the double overexpressors of *PIF5*ox and *PRR5*ox (*PIF5*ox*PRR5*ox). *PIF5*ox plants displayed elongated hypocotyls and constitutive expression of *AtHB-2* and *HFR1* under LL like *phyB* mutants (**Fig. 6A and B**). In contrast, *PRR5*ox plants showed much shorter hypocotyls than those of WT. It was noteworthy that *PIF5*ox*PRR5*ox plants displayed as short hypocotyls and low-level expression of *AtHB-2 and HFR1* as *PRR5*ox plants (**Fig. 6A and B**). These results suggested that *PRR5* represses S.A.R in the downstream of *PIF5* in phytochrome-mediated light signaling pathway.

The regulation of leaf-specific S.A.R involved in *PRR5*. We empirically noticed that *prr5* mutants had larger leaves than WT and the other *prr* mutants when densely planted. It was speculated that *prr5* mutants had some defects in S.A.R in leaves. To confirm this speculation, we measured the petiole lengths and the areas of leaf blades of *prr* single mutants under high or low R/FR. In WT, *prr9* and *prr7* mutants, smaller leaf blades and longer petioles were shown under low R/FR than those under high R/FR. It was notable that *prr5* mutants under low R/FR displayed extremely elongated petioles, and that their leaf blades were as large as those under high R/FR (**Fig. 7B**). Extreme petiole elongation of *prr5* mutants under low R/FR was consistent with the intense induction of the marker genes of S.A.R in them with E.O.D-FR treatment (**Fig. 5B**). However, the normal leaf expansion in *prr5* mutants under low R/FR could not be explained by it. Besides the slight hyposensitivity to red light in hypocotyl elongation of *prr5* mutants, these results suggested that *PRR5* is involved in the leaf-specific S.A.R.[27] It was also noticed that petiole elongation is not always correlated with the reduction of the leaf blade areas in S.A.R.

Discussion

Hyposensitivity to red light in the leaves of *prr9;prr7;prr5* mutants. Previous analyses of early photomorphogenesis showed

that *PRR9*, *PRR7* and *PRR5* act as positive regulators of red light signaling pathway and that *prr9;prr7;prr5* mutants are extremely hyposensitive to red light in spite of their normal sensitivity to blue light.[30,37-39] Consistent with these findings, the leaves of *prr9*;*prr7*;*prr5* mutants are as hyposensitive as their hypocotyls to red light in spite of their normal sensitivity to blue light (**Fig. 2**). *phyB* mutants and *PIF5*ox plants also show extreme hyposensitivity to red light and normal sensitivities to blue light in hypocotyl elongation.[17] However, in this study, their leaves showed S.A.R under both LL and LD, and dramatic change in leaf shape dependent on light/dark cycle was not observed in them, unlike in *prr9*;*prr7*;*prr5* mutants (data not shown). This suggested that the sensitivity to red light is not lost completely in the leaves of *prr9*;*prr7*;*prr5* mutants. This is reinforced by the result that the dark-induced expressions of the marker genes for S.A.R were rapidly reduced by red light irradiation (**Fig. S3**). Considering the result that *prr9*;*prr7*;*prr5* mutants showed elongated hypocotyls as well as *phyB* mutants and *PIF5*ox plants under LL (**Fig. 6A**), the red light sensitivity kept in *prr9*;*prr7*;*prr5* mutants seems to be involved in leaf-specific S.A.R.

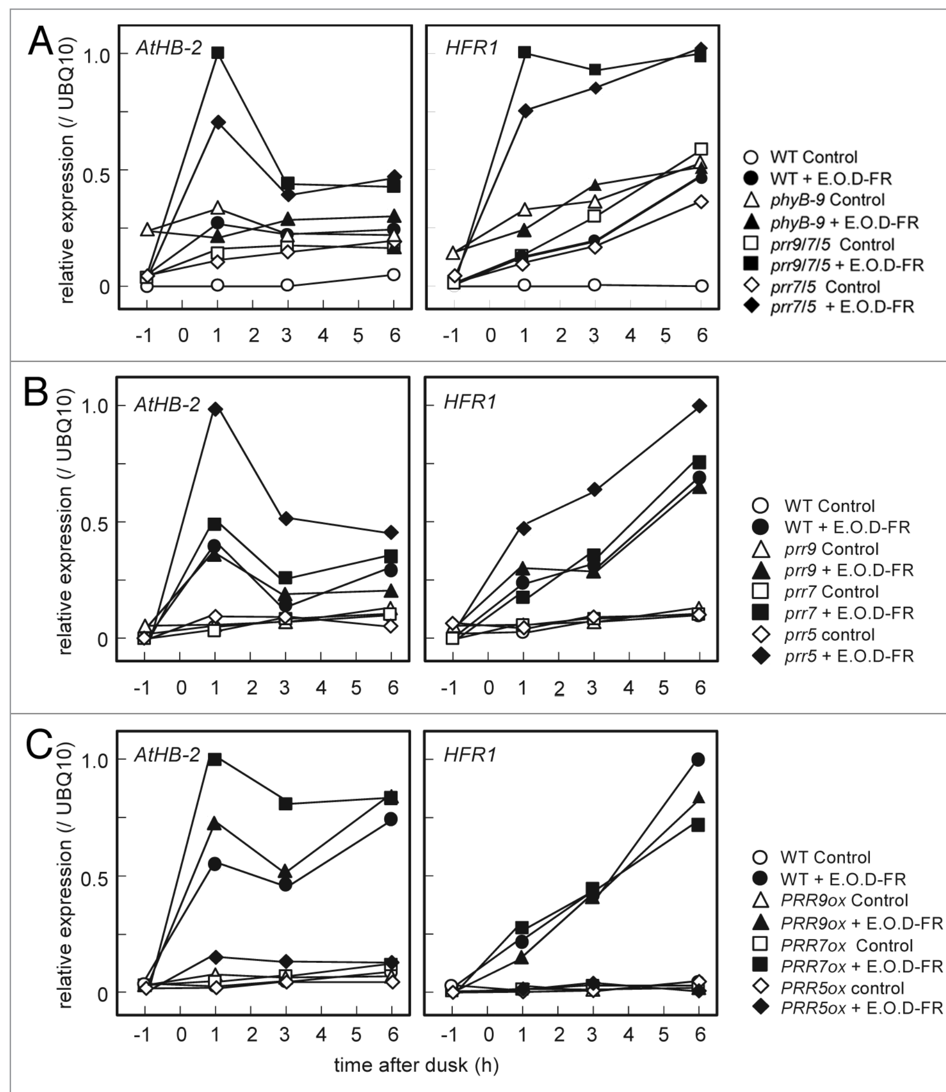

Figure 5. Effect of E.O.D-FR on the expression of *AtHB-2* and *HFR1* in the leaves of loss-of-function mutants and overexpressor lines of *PRR* genes. Plants were grown for 20 d.a.g under LD (40 μmol $m^{-2}s^{-1}$ PPFD), and then some of them were irradiated with PFD of FR for 15 min (closed symbols) at the end of light period, and the others were not (open symbols). RNA samples were prepared at specific time points. (**A**) Extreme induction of these marker genes by E.O.D-FR in the leaves of *prr7;prr5* and *prr9;prr7;prr5* mutants. (**B**) Intense induction of the marker genes by E.O.D-FR in the leaves of *prr5* mutants. (**C**) Remarkable suppression of the induction of the marker genes by E.O.D-FR in the leaves of *PRR5*ox.

A unique function of *PRR5* to reduce the amplitude of S.A.R. According to previous studies, degradation of PIF4 and PIF5 by PhyBfr partially represses S.A.R during light period.[19,28] In contrast, the expression of *PIF4* and *PIF5* are upregulated during light period and downregulated during dark period by light and circadian clock.[30] In this study, the mis-expression of *PIF4* and *PIF5* during dark period was observed in the leaves of *prr9;prr7;prr5* mutants (**Fig. 4**), and this seems to be the cause of S.A.S-like phenotypes of the leaves of *prr9;prr7;prr5* mutants under LD. This is consistent with the arrhythmicity of *prr9;prr7;prr5* mutants reported previously.[21,27] However, this supposition cannot explain the rapid induction of the marker genes for S.A.R in the beginning of dark period in the leaves *prr9*;*prr7*;*prr5* mutants (**Fig. 4**; **Fig. S2**). This is because *PIF5*ox plants, in which abundance of PIF5 increases rapidly in the beginning of dark period, did not show such rapid induction of the marker genes (**Fig. S4**).[19,28] Furthermore, we found that, with E.O.D-FR treatment, the expressions of the marker genes were intensely induced in the leaves of *prr5* mutants and remarkably suppressed in those of *PRR5*ox plants (**Fig. 5B and C**). This suggested that *PRR5* has a unique function to reduce the amplitude of S.A.R, unlike *PRR9* or *PRR7*. This newly discovered function of *PRR5* can explain that, in the leaves of *prr9;prr7;prr5* mutants, the absence of *PRR5* enhanced the influence of the mis-expressed *PIF4* and *PIF5* in the beginning of dark period. The same seems to be true for the conspicuous induction of the marker genes by E.O.D-FR treatment in the leaves of *prr7*;*prr5* and *prr9*;*prr7*;*prr5* mutants (**Fig. 5A**).

Molecular function of *PRR5* in phytochrome signaling pathway. It has been proposed that *PRR5* together with *PRR9* and *PRR7* acts as a circadian clock element, and participates in

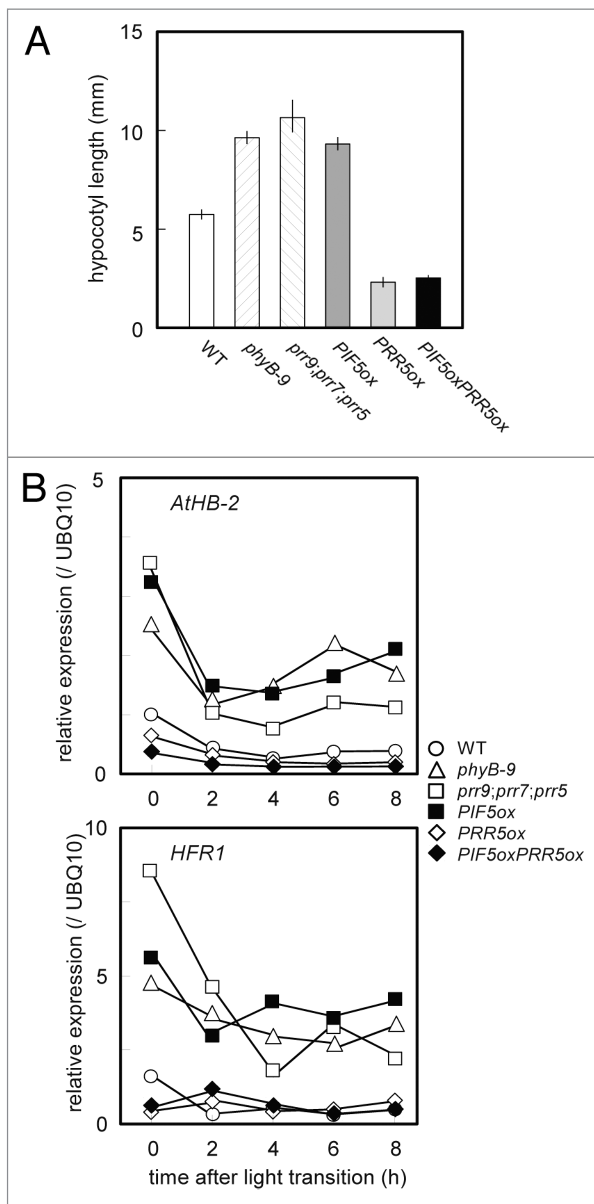

Figure 6. Repression of shade avoidance response by *PRR5*. (**A**) Hypocotyl length of *phyB*, *prr9;prr7;prr5* mutants, *PIF5ox*, *PRR5ox* and *PIF5oxPRR5ox*. Seedlings were grown under LL (20 μmol $m^{-2}s^{-1}$ PPFD) for 10 d.a.g, and then measured (n ≥ 12). Error bars represent SE. (**B**) The expression profiles of *AtHB-2* and *HFR1* in these seedlings under LL. The seedlings were grown under the same condition of (A) for 12 d.a.g, and then sampled at specific time points after 12 h of dark treatment. RNA samples were extracted from whole above ground parts of the seedlings.

phytochrome-mediated light signal transduction pathways to regulate periodic expression of PIFs directly or indirectly.[17,21,28] According to recent studies, PRR5 directly represses the expression of *LHY* and *CCA1*, which repress *TOC1* transcription, and PRR5 and TOC1 oligomerize with their PRR domain to enhance the stability and nuclear localization of TOC1.[40,41] Moreover, overexpressors of *TOC1* (*TOC1*ox) show extreme hypersensitivity to red light in hypocotyl elongation.[37,38] On the basis of these findings, the intense repression of S.A.R in *PRR5*ox seems to be attributed to enhanced accumulation and nuclear localization of TOC1. Contrary to this supposition, in hypocotyl elongation, overexpressors of PRR5 without PRR domain, which cannot interact with TOC1, show extreme hypersensitivity to red light, and *prr5 TOC1*ox plants are not different from *TOC1*ox in sensitivity to red light.[37,42] From the above, *PRR5* is suggested to reduce the amplitude of S.A.R independently of *TOC1*. Furthermore, this study showed that the hypocotyl elongation and expression of *AtHB-2* and *HFR1* in *PIF5*ox*PRR5*ox plants were intensely repressed as well as those in *PRR5*ox plants (**Fig. 6A and B**). This result suggests that *PRR5* suppresses S.A.R in the downstream of *PIF5* directly or indirectly. Recently, Nakamichi et al. reported that PRR5, as well as PRR9 and PRR7, directly binds to the upstream regions of *HFR1* and a C2C2-CO-like transcriptional factor: *B-BOX DOMAIN PROTEIN 24* (*BBX24*), which was reported as an enhancer for S.A.R, and generally acts as a transcriptional repressor.[43,44] This can explain the repression of *HFR1* and the inhibition of hypocotyl elongation in *PRR5ox* and *PIF5*ox*PRR5*ox plants. However, the mechanism for the repression of *AtHB-2* in them and the functional uniqueness of *PRR5* in the suppression of S.A.R still remain unclear. Taken together, these findings suggest that *PRR5* regulates S.A.R not only by control of periodic expression of *PIF*s as a clock component but also by direct regulation of the downstream of *PIF*s in phytochrome-mediated light signaling pathway.

***PRR5*-regulated leaf-specific S.A.R.** Although both leaf blades and petioles are components of eudicot leaves, their morphogenic behaviors in S.A.R differ largely.[19,45] According to previous anatomical analyses of leaves in several mutants, leaf blades and petioles can be regarded as different organs.[46] In facts, approximately half of the genes induced by E.O.D-FR treatment in leaves are petiole-specific.[24] In this study, we found that *prr5* mutants showed normal leaf expansion under low R/FR in spite of their extreme petiole elongation, unlike *prr9* and *prr7* mutants (**Fig.** 7). The extreme petiole elongation in *prr5* mutants under low R/FR can be explained by the above-mentioned function of *PRR5* as a negative regulator of S.A.R. On the other hand, the normal leaf expansion in *prr5* mutants under low R/FR suggests that *PRR5* is involved in the leaf-specific S.A.R. It was previously reported that phytochrome in leaf blades but not that in petioles regulates S.A.R in petioles.[24] On the basis of these findings, canopy-shade signal seems to be normally transduced from leaf blades to petioles in *prr5* mutants. Further analyses of *prr5* mutants will reveal the mechanism of inter-organic growth regulation in S.A.R.

Roles of *PRR5* in diurnal regulation of phytochrome-meditated S.A.R. According to previous reports, the expression of *PRR5* peaks between middle and end of light period, and the abundance of PRR5 peaks with some lag and is reduced rapidly in darkness by ZEITLUPE (ZTL).[40,47] ZTL consists of a LOV domain, a F-box and six Kelch repeats, and interacts with other proteins under influence of light.[48-50] The degradation of PRR5 by ZTL is also repressed by blue light.[44] On the basis of these findings, it is speculated that *PRR5* represses S.A.R from late light period to early dark period. This is consistent with the repression of S.A.R at dusk reported previously.[25] Interestingly,

it was reported that, in early photomorphogenesis, loss of function mutants of *ZTL* (*ztl*) is hyposensitive to red light in spite of their normal sensitivity to blue light.[49] These phenomena can be explained that accumulated PRR5 due to absence of ZTL represses phytochrome-meditated S.A.R. Taken together, *PRR5* seems to gate phytochrome-meditated S.A.R around dusk and to be one of the cross-talk points between red and blue light signal transduction pathway. The details of *PRR5* as a regulator of S.A.R will be revealed by further analyses using artificial induction of *PRR5* in various mutants of clock and photomorphogenetic genes.

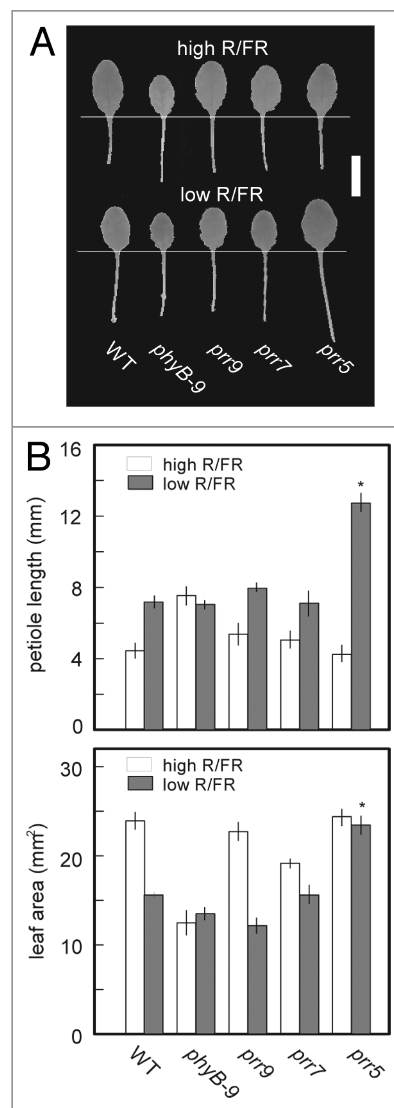

Figure 7. Extreme petiole elongation and normal leaf expansion under low R/FR in the leaves of *prr5* mutants. Plants were grown under high R/FR (LD, 40 μmol $m^{-2}s^{-1}$ PPFD) for 13 d.a.g, and then transferred to continuous high or low R/FR for 7 d. Their 3rd leaves were measured (n = 6). (**A**) Representative phenotypes of 3rd leaves in WT, *phyB-9* and *prr* mutants grown under high (upper side) or low (lower side) R/FR. A scale bar indicates 5 mm. (**B**) Petiole length and leaf area of 3rd leaves. Error bars represent SE. Asterisks indicate significant difference from WT using Tukey's LSD ($p \leq 0.05$).

Materials and Methods

Plant materials and growth conditions. Arabidopsis accession Columbia-0 (Col-0) was used as WT in this study. *prr9-10* (SALK_007551), *prr7-11* (SALK_030430), *prr5-11* (KG24599; from the KAZUSA DNA Research Institute) and their hybrid lines were described previously.[27] Overexpressors of *PRR9*, *PRR7* and *PRR5* were kind gifts from Dr. Yamashino.[38,47] *PIF5*ox tagged with GFP was a kind gift from Dr. Lorrain.[19] *phyB-9* was described previously.[51] *PIF5*ox*PRR5*ox plants were obtained by crossing. *PIF5ox* was genotyped using GFP fluorescence in root, and *PRR5ox* was genotyped based on hypocotyl length and the expression of *PRR5* detected by RT-PCR. For morphometry and gene expression profiling, seeds were sown on rock wool, and the seedlings were watered with 0.5 gL^{-1} Hyponex solution (Hyponex, Co. Ltd.). For selection of crossed lines, seeds were sown on Murashige and Skoog (MS) medium[52] with 2% sucrose, solidified with 0.5% Gelann Gum. After 2 d of darkness at 4°C, seedlings were transferred under several light conditions at 23°C in closed growth chambers (LH-80CCFL-DT, NKsystem Co. Ltd.).

Light source and E.O.D-FR treatment. White light was provided by white cold cathode fluorescent lamp (CCFL-1; NKsystem Co. Ltd.). Red, blue and infrared lights were provided by light-emitting diodes (LEDs) at peak wavelengths of 660 nm (LED-mR; Eyela Co. Ltd.), 470 nm (LED-mB; Eyela Co. Ltd.) and 735 nm (LED-mFR; Eyela Co. Ltd.), respectively. High R/FR light means white light (R/FR = 4.2), and Low R/FR light (R/FR = 0.2) was provided by the white light with supplementary FR LEDs. For E.O.D-FR treatment, plants were irradiated with 20 μmol $m^{-2}s^{-1}$ PFD of FR for 15 min in the end of light period. For sampling under dark condition, irradiation with green LEDs at peak wavelength of 525 nm (OSPG5161P; Optosupply Co. Ltd.) was used as a working light.

Measurements of leaves and hypocotyls. For leaf measurement, leaves were excised at the basal portion of petiole and placed on white papers with white tape; then images of the leaves were scanned with a scanner. For hypocotyl measurement, seedlings were directly placed on a scanner and imaged. The lengths of hypocotyls, leaf blades and petioles were determined using the software Image J (http://rsb.info.nih.gov/ij/, NIH).

Gene expression profiling. Third and fourth leaves of 20 d.a.g old plants were excised at the basal portion of petiole and immediately frozen in liquid nitrogen at specific time points. RNA in the frozen samples was extracted using Plant RNA Isolation Reagent (Invitrogen Co. Ltd.), and reverse-transcribed using the PrimeScript Reverse Transcriptase (TAKARA, Co. Ltd.). Quantitative PCR was performed with the 7500 Real-Time PCR System (Applied Biosystems). Each reaction was performed in triplicate using a primer concentration of 0.3 μM. The sequences of the primer sets used in this study are given in **Table S1**.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be downloaded here: www.landesbioscience.com/journals/psb/article/23534/

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