Aberrant Expression of the *Arabidopsis* Circadian-Regulated *APRR5* Gene Belonging to the *APRR1/TOC1* Quintet Results in Early Flowering and Hypersensitiveness to Light in Early Photomorphogenesis

Eriko Sato, Norihito Nakamichi, Takafumi Yamashino and Takeshi Mizuno

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-8601 Japan

In *Arabidopsis thaliana*, the transcripts of the *APRR1/TOC1* family genes each start accumulating after dawn rhythmically and one after another at intervals in the order of *APRR9→APRR7→APRR5→APRR3→APRR1/TOC1* under continuous light. Except for the well-characterized *APRR1/TOC1*, however, no evidence has been provided that other *APRR1/TOC1* family genes are indeed implicated in the mechanisms underlying circadian rhythms. We here attempted to provide such evidence by characterizing transgenic plants that constitutively express the *APRR5* gene. The resulting *APRR5*-overexpressing (*APRR5*-ox) plants showed intriguing properties with regard to not only circadian rhythms, but also control of flowering time and light response. First, the aberrant expression of *APRR5* in such transgenic plants resulted in a characteristic phenotype with regard to transcriptional events, in which free-running rhythms were considerably altered for certain circadian-regulated genes, including *CCA1, LHY, APRR1/TOC1*, other *APRR1/TOC1* members, *GI* and *CAB2*, although each rhythm was clearly sustained even after plants were transferred to continuous light. With regard to biological events, *APRR5*-ox plants flowered much earlier than wild-type plants, more or less, in a manner independent of photoperiodicity (or under short-day conditions). Furthermore, *APRR5*-ox plants showed an SRL (short-hypocotyl under red light) phenotype that is indicative of hypersensitiveness to red light in early photomorphogenesis. Both *APRR1*-ox and *APRR9*-ox plants also showed the same phenotype. Therefore, *APRR5* (together with *APRR1/TOC1* and *APRR9*) must be taken into consideration for a better understanding of the molecular links between circadian rhythms, control of flowering time through the photoperiodic long-day pathway, and also light signaling-controlled plant development.

**Keywords:** *Arabidopsis* — Circadian rhythm — Flowering time — Light signaling.

**Introduction**

Circadian rhythms are driven by an endogenous biolog-

1Corresponding author: E-mail, tmizuno@agr.nagoya-u.ac.jp; Fax, +81-52-789-4091.
the circadian clock (Wang and Tobin 1998, Schaffer et al. 1998, Green and Tobin 1999). So far, these homologous proteins are the best candidates for such clock components (Alabadi et al. 2002, Mizoguchi et al. 2002). Another gene, TIMING OF CAB EXPRESSION 1 (TOC1), is also believed to encode a component of the central oscillator, and a mutation of the TOC1 gene affects the periods of many circadian rhythms including expression of the CAB2 gene (encoding light-harvesting chlorophyll-a/b-binding protein) (Millar et al. 1995, Somers et al. 1998b). Furthermore, reciprocal and intimate interactions were observed between the functions of CCA1/LHY and TOC1 (Alabadi et al. 2001, Makino et al. 2002, Matsushika et al. 2002b, Mizoguchi et al. 2002).

With regard to biological consequences of the clock functions (i.e. circadian-regulated output pathways), a number of circadian-regulated genes were identified through systematic analyses by means of Arabidopsis DNA microarrays (Harmer et al. 2000, Schaffer et al. 2001). CAB2 is one of the hallmarks of such downstream target genes (Millar et al. 1995). Nevertheless, little is known about such output pathways, through which a wide range of biological events and gene expressions are regulated in a circadian clock-dependent manner. However, it has been relatively well documented that, through such an output pathway, the circadian clock regulates the photoperiodic induction of flowering, in that a circadian-regulated downstream gene, CO (CONSTANS), appears to play a crucial role (Onouchi et al. 2000, Suarez-Lopez et al. 2001). Another gene, GI (GIGANTEA), is also intriguing, because this gene was proposed to be implicated both in a light signal input pathway and an output pathway leading to the photoperiodic control of flowering (Fowler et al. 1999, Park et al. 1999, Huq et al. 2000a). In general, certain mutations (or aberrant overexpressions) in some of these circadian-associated genes confer pleiotropic phenotypes with regard to the controls of flowering time and photomorphogenesis, as well as circadian rhythms (for reviews, see Barak et al. 2000, McClung 2000, Samach and Coupland 2000, Carre 2001, Putterill 2001, Somers 2001). Thus, clarification of the molecular mechanisms underlying circadian rhythms should provide us with the basis for a better understanding of the fundamental biological processes of plants, including the photoperiodic induction of flowering and the light-regulated plant development.

In this context, we have been characterizing a novel family of genes encoding Arabidopsis pseudo-response regulators (designated as APRR1, APRR3, APRR5, APRR7, and APRR9) (Makino et al. 2000, Makino et al. 2001, Makino et al. 2002, Matsushika et al. 2000, Matsushika et al. 2002a, Matsushika et al. 2002b, Murakami-Kojima et al. 2002). These APRR products are nuclear-localized proteins with a common motif similar to the receiver of response regulators that are implicated in widespread two-component signal transduction systems (Mizuno 1998). Furthermore, they also share an additional (CCT) motif with CO (Makino et al. 2000, Strayer et al. 2000). Interestingly, APRR1 turned out to be identical to TOC1 (Matsushika et al. 2000, Strayer et al. 2000). More interestingly, not only APRR1/TOC1, but also other APRRs are all subjected to circadian rhythms at the level of transcription, in such a manner that each APRR transcript starts accumulating after dawn sequentially at intervals in the order of APRR9→APRR7→APRR5→APRR3→APRR1/TOC1 (Matsushika et al. 2000), in which the wave of APRR9 first appears immediately after dawn. Also, the expression of APRR9 is rapidly and transiently induced, when dark-grown etiolated seedlings are exposed to red light (Makino et al. 2001). Based on these findings, we previously speculated that such sequential and rhythmic events, termed “circadian waves of the APRR1/TOC1 quintet”, might be the basis of a biological clock. Nevertheless, no evidence has been provided that other quintet members, except for APRR1/TOC1, are indeed relevant to the mechanisms underlying circadian rhythms. To clarify the biological functions of these intriguing pseudo-response regulators, we previously characterized certain transgenic plants (APRR1-ox and APRR9-ox) that constitutively express APRR1/TOC1 and APRR9, respectively (Makino et al. 2002, Matsushika et al. 2002a, Matsushika et al. 2002b). In this study, we further attempted to characterize transgenic plants that aberrantly (or constitutively) express the APRR5 gene in a manner independent of circadian rhythms. Evidence will be presented that not only APRR1/TOC1, but also APRR5 must be taken into consideration for a better understanding of the molecular links between circadian rhythms, control of flowering time through the photoperiodic long-day pathway, and also light signaling.
Isolation of plants carrying a 35S::APRR5 transgene

According to the conventional Agrobacterium-mediated DNA delivery method, we isolated 10 independent T2 transgenic lines (or seeds), each presumably carrying a monogenic 35S::APRR5 transgene, in which the entire coding region of APRR5-cDNA was placed under control of the cauliflower mosaic virus (CaMV) 35S promoter so as to be aberrantly (or constitutively) expressed in plants. These transgenic plants, together with wild-type plants (Columbia, Col.), were grown under 16 h light/8 h dark conditions for 12 d, and then RNA samples were prepared from leaves at both CT2 (circadian time 2 h corresponding to morning) and CT8 (afternoon). They were analyzed by Northern blot hybridization with a probe specific for the APRR5-coding sequence (Fig. 1). In wild-type plants, the expression of APRR5 was at its basal level in the morning, and the transcript peaked in the afternoon (lanes denoted by Col.), as previously reported (Makino et al. 2000). Under these conditions, each of these transgenic lines showed, more or less, a constitutive level of the APRR5 transcript, regardless of the timing (morning and afternoon) (Fig. 1). Among these transgenic lines, L11, L111, and L210 were selected, and then their homozygous T3 seeds carrying the 35S::APRR5 transgene were isolated. They were designated hereafter as APRR5-over-expressing (APRR5-ox) plants, for clarity of the text, and then they were further characterized with special reference to circadian rhythms (L210 were mainly used in this study, unless otherwise noted).

Rhythms of certain circadian-controlled genes in APRR5-ox plants

A well-known hallmark of circadian-regulated events in Arabidopsis is the rhythmic expression of CAB2 under continu-
Circadian rhythms in Arabidopsis

ous light, as mentioned above. The CAB2 gene is presumably the most downstream target of a circadian output pathway (Millar et al. 1995). To gain a first insight into the properties of APRR5-ox plants in terms of circadian rhythms, such a hallmark rhythmic expression of CAB2 was examined, after they had been grown under continuous light (LL conditions) (Fig. 2A). Both wild-type (Col.) and APRR5-ox plants were grown for 22 d under 12 h light/12 h dark (LD conditions), and then they were transferred to continuous white light. RNA samples were prepared from leaves with primary inflorescences at appropriate intervals (3 h), and then Northern blot hybridization analyses were carried out. In wild-type plants, the expression of CAB2 showed a robust free-running rhythm with a peak before noon, as has been well documented (Piechulla 1999). Such rhythmic expression of CAB2 was clearly seen in APRR5-ox plants. However, the circadian profile was considerably different from that seen in wild-type plants. In particular, the amplitude of rhythmic peaks of CAB2 was considerably reduced in APRR5-ox plants, as compared with in the case of wild-type plants. This event became evident particularly for the sustained 2nd peak. As another example of circadian-controlled gene, the expression of GI in APRR5-ox plants was also examined (Fig. 2B). In wild-type plants, the expression of GI showed a clear free-running rhythm with a peak at the subjective afternoon, as has been reported (Fowler et al. 1999, Park et al. 1999). In APRR5-ox plants, the amplitude of free-running rhythm of GI was markedly reduced to its trough level, the event of which was more dramatic than that seen for CAB2.

As mentioned above, several genes have been proposed to encode proteins that function within, or close to, the central oscillator(s). Among them, CCA1 and LHY are very intriguing, because these homologous proteins are the best candidates for central oscillator components (Alabadi et al. 2002, Mizoguchi et al. 2002). It is thus critical to examine their circadian profiles in APRR5-ox plants. Northern blot hybridization analyses were carried out, as described above for CAB2 and GI. The results showed that the circadian amplitudes of both CCA1 and LHY were also significantly reduced in APRR5-ox plants, as compared with in wild-type plants (Fig. 3). In wild-type plants, both CCA1 and LHY showed a typical circadian rhythm with a peak at the subjective dawn, as anticipated (Wang and Tobin 1998, Schaffer et al. 1998). In APRR5-ox plants, such rhythms of CCA1 and LHY were not abolished, but the rhythmic amplitudes of both the CCA1 and LHY transcripts were markedly

Fig. 3 Northern blot hybridization analyses of the transcripts of CCA1 and LHY in APRR5-ox plants. Both wild-type (Col.) and APRR5-ox plants (L210) were grown under LD conditions of 12 h light/12 h dark for 26 d, and then they were transferred to continuous light (LL conditions). RNA samples were prepared from leaves with primary inflorescences at the times with appropriate intervals (3 h), as schematically shown at the bottom (shaded boxes indicate subjective night). They were analyzed with a probe specific for the CCA1 coding sequence (upper panels). Similarly, Northern blot hybridization was carried out with a probe specific for the LHY coding sequence (lower panels). Other details are the same as those given in the legend to Fig. 1.
reduced in APRR5-ox. In particular, the free-running rhythm of LHY was more severely affected than that of CCA1 (Fig. 3).

In any case (CAB2, GI, CCA1 and LHY), it should be emphasized that their free-running rhythms have not completely been abolished in APRR5-ox plants. However, the common event, observed in APRR5-ox, was a significant and rapid reduction of their amplitudes of rhythms under continuous light, although the extents of such effects varied from one instance to another.
TOC1 quintet genes were expressed in a sequential and rhythmic manner in the characteristic order of APRR9→APRR7→APRR5→APRR3→APRR1/TOC1, as has been repeatedly demonstrated (see upper and color-coordinated profiles, together with the corresponding raw data, in Fig. 4A). This is the event that has been referred to as “circadian waves of the APRR1/TOC1 quintet”. In APRR5-ox plants, however, a higher level of the APRR5 transcript was detected at any given time, as expected (see lower and color-coordinated profiles in Fig. 4A). In contrast, the rhythmic expressions of other members were markedly altered in APRR5-ox plants under continuous light, in a way that their rhythms became dampened rapidly. However, it may be worth noting that the level of the APRR9 transcript was reduced to its trough-level, while the APRR1 transcript increased to its peak-level. Interestingly, the profile of the APRR7 transcript was also altered in a manner similar to that of the APRR9 transcript, whereas the profile of the APRR3 transcript was altered in a manner similar to that of the APRR1. As the result, the free-running circadian waves were almost completely abolished in APRR5-ox plants at the second cycle, after transfer to LL conditions.

Aberrant expression of APRR5 results in the phenotype of early flowering

The most critical issue with regard to APRR5-ox plants, which should be addressed here, is whether or not they show any biological phenotype. To examine this, two homozygous APRR5-ox lines (T3 seeds of L11 and L210) were germinated, and plants were grown under either long-day (16 h light/8 h dark) or short-day (10 h light/14 h dark) conditions. In each case, they grew well and flowered eventually. The structures (or morphologies) of adult plants were essentially the same as those of wild-type plants, although the apical parts of APRR5-ox plants were slightly smaller in average size than that of wild-type plants (see Fig. 5). In any event, we noticed that these APRR5-ox plants flowered slightly earlier under the long-day conditions, and also very early under the short-day conditions, as compared with wild-type plants, as demonstrated below (Fig. 5). The upper picture shows representatives of 23-day-old APRR5-ox plants, together with wild-type...
plants, grown under the long-day conditions. The picture (lower left) shows a representative 39-day-old APRR5-ox plant with flowers, together with a wild-type plant of the same age, and another (lower right) shows a representative 57-day-old wild-type plant with a primary inflorescence, all of which were grown under the short-day conditions. These observations suggested that APRR5-ox plants showed a characteristic phenotype of early flowering. To further confirm this idea, we statistically monitored the flowering time of APRR5-ox plants (L11 and L210), in comparison with that of wild-type plants, by counting leaves upon the onset of flowering (Table 1). The results clearly showed that APRR5-ox plants exhibited the characteristic phenotype of early flowering, which was more or less independent of photoperiodicity. This finding is compatible with the idea that APRR5 plays a role in the mechanism, directly or indirectly, by which the photoperiodic flowering pathway is modulated.

**APRR5-ox plants were hypersensitive in red right-mediated de-etiolation process**

It has been suggested that some circadian-associated genes are implicated not only in control of flowering time, but also photomorphogenesis in light with a given spectrum (e.g. phyB signaling for red light) (for a review, see Quail 2002). Such circadian-associated genes include *ELF3* and *GI*, as mentioned earlier (see Introduction). These facts prompted us to examine APRR5-ox plants with special reference to such photosensory signal transduction. A well-established and visible hallmark of photosensory signal transduction is the de-etiolation of seedlings under light with a certain spectrum (e.g. continuous red light) (Huq and Quail 2002). When both wild-type and APRR5-ox plants were germinated in the dark, the hypocotyl-lengths of their etiolated seedlings with closed and yellow cotyledons were relatively long, similar to each other (Fig. 6A, bottom panel). However, when they were germinated and grown under continuous red light (cR) (fluence rate, 4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), APRR5-ox plants displayed very short hypocotyls with fully expanded green cotyledons (Fig. 6A, top panel). Although wild-type plants showed a de-etiolated morphology, the hypocotyl-lengths of APRR5-ox seedlings were strikingly shorter than those of wild-type seedlings. This event was observed over a broad range of cR fluence rates (Fig. 6, 0.4 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 0.04 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)). These events were statistically confirmed by examining fluence-rate response curves of hypocotyl lengths (Fig. 6B). This is indicative of the photobiological event, referred to as “hypersensitivity to red light in early photomorphogenesis”, which is presumably mediated through the phytochrome (phy)-dependent signaling pathways (Quail 2002).

We previously isolated and characterized certain APRR1-ox and APRR9-ox plants, respectively (Makino et al. 2002, Matsushika et al. 2002b). Thus, APRR1-ox and APRR9-ox plants were also characterized in terms of such sensitivity to cR in early photomorphogenesis (Fig. 6A). The results showed that APRR1-ox plants were also hypersensitive to cR. It would be worth mentioning that APRR1-ox plants were even more sensitive to cR than APRR5-ox (see panel of 0.04 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), also see Fig. 6B). APRR9-ox plants were also considerably hypersensitive to cR, although the magnitude of sensitivity was lower that that seen for APRR5-ox and APRR1-ox. For these experiments, we examined each particular transgenic line (L210 for APRR5-ox, L3 for APRR1-ox, and L6 for APRR9-ox). Essentially the same events were reproducibly confirmed for each alternative transgenic line (L11 for APRR5-ox, L5 for

### Table 1 The phenotype of APRR5-ox plants with regard to the flowering time

<table>
<thead>
<tr>
<th>野型（Col.）</th>
<th>APRR5-ox（L11）</th>
<th>APRR5-ox（L210）</th>
</tr>
</thead>
<tbody>
<tr>
<td>光周期（16 h光）</td>
<td></td>
<td></td>
</tr>
<tr>
<td>可见花序出现日数</td>
<td>22.0±0.6</td>
<td>18.6±1.0</td>
</tr>
<tr>
<td>每株花序的叶数</td>
<td>7.4±0.6</td>
<td>4.8±0.5</td>
</tr>
<tr>
<td>每株植物的总植株数</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>短日照（10 h光）</td>
<td></td>
<td></td>
</tr>
<tr>
<td>可见花序出现日数</td>
<td>53.1±2.4</td>
<td>36.5±1.9</td>
</tr>
<tr>
<td>每株花序的叶数</td>
<td>36.5±2.2</td>
<td>13.1±0.6</td>
</tr>
<tr>
<td>每株植物的总植株数</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

---

The plants were grown as follows. Seeds were imbibed directly on “rock-fiber” (Nittobo, Tokyo, Japan), soaked in a solution of 1,000-times diluted HYPONEX (N : P : K = 5 : 10 : 5) (HYPONEX-JAPAN, Osaka, Japan), and then they were cold-treated at 4°C for 5 d in the dark before germination under light. They were transferred for germination under light, and then were grown in chambers (22°C), either under conditions of long days (16 h light) or short days (10 h light). Note that at every 14 d, a fresh solution of the diluted HYPONEX was supplied.

Days to visible inflorescence was defined as the time at which a given plant possessed the flower primordia visible to the unaided eye.

For number of leaves at flowering, the leaf count was taken on the day the flower primordia were first observed on a given plant.
APRR1-ox, and L2 for APRR9-ox). Thus, we assume that the aberrant expressions of certain APRR1/TOC1 quintet members (APRR1/TOC1, APRR5, APRR9) commonly resulted in the phenotype of hypersensitivity to cR in early photomorphogenesis, thereby giving rise to short hypocotyls when they were germinated under cR.

Such photomorphological differences among wild-type, APRR5-ox, APRR1-ox, and APRR9-ox de-etiolated seedlings were less evident when they were grown under continuous far red light (1 μmol m$^{-2}$ s$^{-1}$) (Fig. 7). It may be noted that APRR1-ox seedlings seemed to be slightly hypersensitive to far red light, as compared with the wild-type seedlings. We also examined such photomorphogenesis of APRR5-ox, APRR1-ox, and APRR9-ox seedlings under blue light (10 μmol m$^{-2}$ s$^{-1}$) (Fig. 7). APRR5-ox seedlings were considerably hypersensitive to blue light. APRR5-ox seedlings also seemed to be slightly hypersensitive to blue light. Clarification of these observations must await more intensive analyses. At least, however, it was clearly demonstrated for the first time that APRR5-ox plants are highly sensitive to cR, as far as the early photomorphogenesis of young seedlings is concerned, regardless of whether it is specific to red light or not.

**Discussion**

Clarification of the molecular mechanisms underlying plant biological clocks is a paradigm of current *Arabidopsis* biology, as mentioned above. In this respect, the circadian waves of the APRR1/TOC1 quintet, which are characteristic events in gene expression, are intriguing. The finding of this circadian-related event previously led us to speculate that such rhythmic events might be the basis of a biological clock. To gain insight into this idea, we have previously characterized APRR1/TOC1-ox and APRR9-ox plants, respectively (Makino et al. 2002, Matsushika et al. 2002a, Matsushika et al. 2002b). Indeed, aberrant expression of *APRR1* in APRR1-ox plants resulted in a dramatic alteration of free-running circadian rhythms of certain circadian-controlled genes, including *CCA1*, *LHY*, *GI*, and *CAB2*, as well as the APRR1/TOC1 quintet members. Also, aberrant expression of *APRR9* in APRR9-ox plants resulted in an intriguing phenotype with regard to circadian-related events, in which short-period rhythms were commonly observed for certain circadian-controlled genes that included those affected in APRR1-ox plants. These results are well compatible with the idea that APRR9 plays a role together with APRR1/TOC1 in a mechanism underlying circadian rhythms. By characterizing APRR5-ox plants, we have provided further

![Fig. 6 Red light response of APRR5-ox plants in early photomorphogenesis. (A) Photographs of seedlings that were grown under continuous red light with a varied range of fluence rates (4 μmol m$^{-2}$ s$^{-1}$ to 0.04 μmol m$^{-2}$ s$^{-1}$) or in the dark. Col., wild-type; 5ox., APRR5-ox; 1ox., APRR1-ox; 9ox., APRR9-ox. Seeds were sown on gellanum (0.3%) plates containing MS salts without sucrose, and they were then kept at 4°C for 48 h in the dark. After seeds were exposed to white light for 3 h in order to enhance germination, they were kept at 22°C for 21 h again in the dark. Plants were grown for 72 h under cR with a varied range of fluence rates or in the dark, as indicated. For each experiment, ten plants that germinated normally were examined, and representatives (two each) were photographed. Note that APRR9-ox plants were grown under the fluence rate of 6 μmol m$^{-2}$ s$^{-1}$, instead of 4 μmol m$^{-2}$ s$^{-1}$. (B) Fluence-rate response curves of hypocotyl lengths are shown (means for ten plants with standard deviations). The experiments were carried out as described above.](image-url)
plants, was the phenotypic alteration of flowering time. APRR5-ox plants flowered much earlier than wild-type plants, more or less, in a manner independent of photoperiodicity (or under short-day conditions). This is not surprising for us, because we have already learnt that APRR9-ox plants also show a similar phenotype of early flowering (Matsushika et al. 2002a). These findings are important in the sense that solid evidence for a role of the plant circadian clocks in flowering has already been obtained with the isolation of Arabidopsis mutants with both altered circadian rhythms and flowering time. These genes include not only certain photoreceptor genes (PHYA/B and CRY1/2) (Somers et al. 1998a), but also ELF3 (McWatters et al. 2000, Covington et al. 2001, Hicks et al. 2001, Liu et al. 2001), ZTL and its homologs (FKF1 and LKP2) (Somers et al. 2000, Nelson et al. 2000, Schultz et al. 2001), CCA1 (Green and Tobin 1999), LHY (Schaffer et al. 1998), TOC1 (Somers et al. 1998b), GI (Fowler et al. 1999, Park et al. 1999, Huq et al. 2000a) (for more references, see Mouradov et al. 2002). These recent intensive studies on the regulation of flowering time have revealed the framework of a clock-controlled and photoperiodic long-day pathway, which is integrated through CO (a major downstream player, see Onouchi et al. 2000, Suarez-Lopez et al. 2001) into the mechanism by which certain genes for floral pathway integrators are properly regulated so as to promote flowering (for recent reviews, see Samach and Coupland 2000, McClung 2000, Putterill 2001, Mouradov et al. 2002). Nevertheless, little is known about molecular interactions among those components listed above. It is also reasonably assumed that as yet unknown factors must also be integrated into the scenario described above. In this context, the results of our present and previous studies suggested that the circadian-controlled APRR5 and APRR9 proteins must also be added as new members to a flowering-club of the photoperiodic long-day pathway. In any case, the cellular and molecular mechanisms by which APRR5 and APRR9 exert their regulatory roles in flowering also remain to be fully elucidated.

Another intriguing biological event, observed here for APRR5-ox plants, is its apparent hypersensitivity to red light in early photomorphogenesis. When APRR5-ox plants were germinated and grown under cR, they displayed very short hypocotyls with fully expanded green cotyledons. Although wild-type plants also showed a de-etiolated morphology under such conditions, the hypocotyl lengths of APRR5-ox seedlings were strikingly shorter than those of wild-type seedlings. It is unlikely that this event is due to a general defect of growth in APRR5-ox plants, because cotyledons of APRR5-ox plants have expanded even more vigorously than those of wild-type plants. Another important finding with this regard is that essentially the same event was seen for APRR1-ox and APRR9-ox plants. These observed biological events appear to be relevant to phyto-dependent photosensory signal transduction.

To adapt to constantly changing quality and quantity of environmental light signals, plants have a number of distinc-

---

**Fig. 7** Light responses of APRR5-ox plants in early photomorphogenesis. Seeds (Col., wild-type; 5ox., APRR5-ox; 1ox., APRR1-ox; 9ox., APRR9-ox) were sown on gellanum (0.3%) plates containing MS salts without sucrose, and they were then kept at 4°C for 48 h in the dark. After seeds were exposed to white light for 3 h in order to enhance germination, they were kept at 22°C for 21 h again in the dark. Plants were grown for 72 h in the dark, under continuous far red light (1 μmol m⁻² s⁻¹) and continuous blue light (10 μmol m⁻² s⁻¹). Lengths of hypocotyls were measured for seedlings that germinated normally (n > 10).
tive informational photoreceptors (for reviews, see Chory 1997, Cashmore et al. 1999, Briggs and Christie 2002). Among them, phys are responsible for sensing the red/far red region of the light spectrum (for reviews, see Deng and Quail 1999, Hudson 2000, Quail 2002). There is a small gene (PHYA-PHYE) family in Arabidopsis, among which the functions of phyA and phyB have been characterized intensively (Chory 1997). A number of approaches have been used to investigate such phy-dependent signaling pathways, including biochemical approaches, yeast two-hybrid screening coupled with reverse genetic approaches, as well as forward genetic approaches (for reviews, see Quail 2000, Quail 2002). They revealed a number of components that are implicated directly or indirectly in phy-dependent signaling pathways. Among them, here we focus our attention mainly on those revealed by molecular genetic approaches, and those implicated in photosensitivity to red light, because they appear to be more closely relevant to the findings of this study for APRR5-ox plants. These genes of components that are implicated directly or indirectly in phy-dependent signaling pathways, in which APRR5-ox plants. These suggest that there might be a complicated linkage between the photoperiodic control of flowering and the phy-dependent signaling pathways, in which APRR5 might be implicated together with ELF3 and GI. In this connection, another important finding of this study is that APRR1/TOC1-ox and APRR9-ox plants also showed the SRL phenotype. Together with the fact that the APRR9 transcription is rapidly induced by red light, our results suggested that the APRR1/TOC1 quintet members might also play coordinate roles in a phy-dependent photosensory signaling pathway, which is crucially involved in light-regulated plant development. However, it should be noted that the scenario might not be so simple as mentioned above, because APRR1-ox seedlings appear to be hypersensitive also to blue light in early photomorphogenesis, as judged by hypocotyl-elongation (see Fig. 7). In any case, clarification of these hypothetical views must also await further examinations. For example, the well-known circadian-regulated elongation of hypocotyls remains to be examined for APRR5-ox and APRR9-ox plants.

In our previous studies, it was naively thought that the APRR1/TOC1 quintet members might play roles mainly in circadian rhythms. The results of further studies on APRR1-ox, APRR9-ox, and APRR5-ox provided us with new insight into the cellular and biological functions of the APRR1/TOC1 quintet members. As discussed above, these circadian-associated components appear to play even more sophisticated roles than we originally thought. In short, the results of this study indicate for the first time that not only APRR1/TOC1, but also APRR5 (and APRR9) must be taken into consideration for a better understanding of the molecular links between circadian rhythms, control of flowering time through the photoperiodic long-day pathway, and also photosensory signal transduction. To clarify these issues, characterization of each null mutant for APRR5 and APRR9 is very critical. These lines of experiments are underway.

Materials and Methods

Plant growth conditions and related materials

Arabidopsis thaliana (Columbia ecotype, Col.) was mainly used as wild-type plants. Seeds were imbibed and cold treated at 4°C for 3 d in the dark before germination under light, and then plants were grown at 22°C. Note that the imbibed seeds were exposed to white light for 30 min before incubation in the dark. Plants were grown in a chamber with light from fluorescent lights (150–200 μmol m⁻² s⁻¹) at 22°C on soil and/or on agar-plates containing MS salts and 2% sucrose, as described previously. Light/dark conditions used were either 16 h light/8 h dark (LD, long-day conditions), 12 h light/12 h dark, or 10 h light/14 h dark (SD, short-day conditions), as specifically noted for each experiment in the text. Otherwise, the conditions were given in detail in the text (see also Table 1).

Preparing RNA and Northern blotting

Total RNA was isolated from appropriate organs (mainly leaves) of Arabidopsis by the aurintricarboxylic acid (ATA) method. For Northern blot hybridization, RNA was separated in agarose gels (1%) containing 0.67 M formaldehyde, then transferred to Hybond-N+ membranes. The fixed membranes were hybridized with 32P-labeled DNA fragments in 6× standard saline phosphate and EDTA (1× SSPE = 0.18 M NaCl, 10 mM phosphate buffer, 1 mM EDTA, pH 7.4), 5×
References

cal role for CCA1 and LHY in maintaining circadian rhythmicity in Arabi-

Alabadi, D., Oyama, T., Yanovsky, M.J., Harmon, E.G., Mas, P. and Kay, S.A. (2001) Reciprocal regulation between TOCI and LHY/CCA1 within the Arabi-


gene transfer by infiltration of adult Arabidopsis thaliana plants. C. R. Acad.

Carre, I.A. (2001) Day-length perception and the photoperiodic regulation of


1225–1234.


757–761.

Hicks, K.A., Albertson, T.M. and Wagner, D.R. (2001) CCA1 and LHY play a
role for CCA1 and LHY in maintaining circadian rhythmicity in

Huq, E. and Quail, P.H. (2002) PIF4, a phytochrome-interacting bHLH factor,
encodes a novel protein that regulates circadian clock function and flowering

Huq, E., Pepperman, J.M. and Quail, P.H. (2000a) GIGANTEA is a nuclear pro-
tein implicated in phytochrome signaling because of a T DNA insertion in the
promoter of PIF3, a gene encoding a phytochrome-interacting

Harmer, S.L., Hogeness, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T,

USA 96: 4176–4179.

Halliday, K.J., Hudson, M., Ni, M., Qin, M.-M. and Quail, P.H. (1999) PIF1: An
Arabidopsis mutant perturbed in phytochrome signalling because of a T DNA
insertion in the promoter of PIF3, a gene encoding a phytochrome-interacting

Harmer, S.L., Hogeness, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T,

USA 96: 4176–4179.

Arabidopsis mutant perturbed in phytochrome signaling because of a T DNA
insertion in the promoter of PIF3, a gene encoding a phytochrome-interacting

Harmer, S.L., Hogeness, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T,

USA 96: 4176–4179.

Huq, E., Pepperman, J.M. and Quail, P.H. (2000a) GIGANTEA is a nuclear pro-
tein implicated in phytochrome signaling because of a T DNA insertion in the
promoter of PIF3, a gene encoding a phytochrome-interacting

Harmer, S.L., Hogeness, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T,

USA 96: 4176–4179.

cis-regulatory element sufficient to confer dark-inducible and light down-
regulated expression to a minimal promoter in pea. J. Biol. Chem. 275:
19723–19727.

Kojima, S., Banno, H., Yoshioka, Y., Oka, A., Machida, C. and Machida, Y.
(1997) Coordination of plant metabolism and development by the circadian

ELF3 encodes a circadian clock-regulated nuclear protein that functions in an


(Received August 19, 2002; Accepted September 9, 2002)