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The APRR1/TOC1 Quintet Implicated in Circadian Rhythms of *Arabidopsis thaliana*: I. Characterization with APRR1-Overexpressing Plants

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Several Arabidopsis genes have been proposed to encode potential clock-associated components, including the Myb-related CCA1 and LHY transcription factors and a member of the novel family of pseudo response regulators (APRR1/TOC1). We previously showed that mRNAs of the APRR1/TOC1 family of genes start accumulating after dawn rhythmically and sequentially at approximately 2 h intervals in the order: *APRR9*→*APRR7*→*APRR5*→*APRR3*→*APRR1/TOC1*. Here we constructed APRR1-overexpressing (APRR1-ox) plants, and examined certain circadian profiles for *APRRs*, *CCA1*, *LHY*, *GI*, *CCR2*, and *CAB2*. The free-running circadian rhythms of the APRR1/TOC1 family of genes, including *APRR1*, were dampened in APRR1-ox plants. In particular, the light-inducible expression of *APRR9* was severely repressed in APRR1-ox plants, suggesting that there is a negative APRR1→APRR9 regulation. The free-running robust rhythm of *CAB2* was also dampened in APRR1-ox. The circadian profiles of potential clock-associated genes, *CCA1*, *LHY*, *GI*, and *CCR2* were all markedly altered in APRR1-ox, each in characteristic fashion. To gain further insight into the molecular function of APRR1, we then identified a novel Myc-related bHLH transcription factor, which physically associated with APRR1. This protein (named PIL1) is similar in its amino acid sequence to PIF3, which has been identified as a phytochrome-interacting transcription factor. These results are discussed in relation to the current idea that APRR1 (TOC1) plays a role within, or close to, the Arabidopsis central oscillator.

Key words: Arabidopsis — Circadian rhythm — Clock components — Transgenic plants.

Introduction

During the past few years, there has been a tremendous increase in our understanding of the molecular bases of the biological clocks in a wide range of organisms, including *Neurospora*, *Drosophila* and *Synechococcus* (Dunlap 1999). In higher plants, such a clock-dependent circadian rhythm is a very old issue in physiology (Garner and Allard 1920), and a newly emerging paradigm of molecular biology (for reviews see Anderson and Kay 1996, Thomas and Vince-Prue 1997, Kreps and Kay 1997, Koornneef et al. 1998, Piechulla 1999, Staiger and Heintzen 1999, Murtas and Millar 2000, Samach and Coupland 2000, Barak et al. 2000, Carre 2001). A number of circadian-regulated genes have been identified through extensive analyses with DNA microarrays of Arabidopsis (Harmer et al. 2000, Schaffer et al. 2001). In general, a biological clock is presumed to consist of input pathways, central oscillators, and output pathways (Dunlap 1999). A central oscillator is thought to autonomously generate a rhythm through transcription/translation positive/negative feedback loops. In the model higher plant, *Arabidopsis thaliana*, biological and genetic studies have begun to shed light on the molecular bases of such clocks in higher plants (see the reviews cited above).

With regard to the input pathways responsible for the entrainment of plant circadian clocks, light is obviously an important factor. Members of both the phytochrome (PHY) and cryptochrome (CRY) families of Arabidopsis photoreceptors have been shown to act as part of the input pathways (Somers et al. 1998a). In white light, however, a *phyA phyB cry1 cry2* quadruple mutant retains robust circadian rhythmicity of leaf movement, suggesting that an, as yet unidentified, photoreceptor(s) can also function in the input pathways (for a review see Barak et al. 2000). Through intensive genetic approaches, several other Arabidopsis genes have also been uncovered as ones encoding additional components of the input pathways. They include the *ELF3*, *GI*, *ZTL*, and *FKF1* genes. Mutations in *ELF3* (*EARLY FLOWERING 3*) result in arhythmicity in light but not in the dark (Hicks et al. 1996, Zagotta et al. 1996, Reed et al. 2000). The *ELF3* gene product was recently identified and extensively characterized (Hicks et al. 2001, Liu et al. 2001, Covington et al. 2001). Mutations in *GI* (*GIGANTEA*) cause altered periods and reduced amplitudes of expression of certain circadian-associated genes, including *CCA1* and *LHY* (Fowler et al. 1999, Park et al. 1999, Huq et al. 2000, and see below for CCA1 and LHY). The *ZTL* (*ZEITLUPE*) and *FKF1* (flavin-binding, kelch repeat, F-box) genes both encode a protein that contains a common LOV (light, oxygen, voltage) domain, together with an F-box and kelch-repeats (Nelson et al. 2000, Somers et al. 2000). Although all these components are assumed to play roles in controlling input from light signals to the clock, their molecular functions are not yet fully understood.

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With regard to the Arabidopsis circadian oscillator(s), several genes have proposed to encode potential clock-associated components, although none of them have yet been fully established as a bona fide clock component. Best characterized are the Myb-related *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) genes, each of which encodes a putative transcription factor similar to each other (Wang et al. 1997, Green and Tobin 1999, Schaffer et al. 1998). Both *CCA1* and *LHY* show robust and free-running circadian oscillations at both the transcript and protein levels. Overexpression of *CCA1* in plants stops overt rhythmicity, including the expression of genes that are under control of the circadian rhythm (Wang and Tobin 1998). The examples of such circadian-controlled genes are those encoding light-harvesting chlorophyll-*a/b*-binding proteins (e.g. *CAB2*) and a catalase (*CAT3*) (for a review see Piechulla 1999). Another gene, *TIMING OF CAB 1* (*TOC1*), is also intriguing, because a semi-dominant (*toc1-1*) mutant exhibits shortened periods of several rhythmic markers, including the *CAB2* gene, and also of the leaf movement and stomatal conductance rhythms (Somers et al. 1998b). Since this single mutation interferes with a wide range of clock-controlled output processes, the TOC1 protein was also proposed to function close to, or as part of, the oscillator itself. The *TOC1* gene was recently isolated and found to encode a nuclear protein with a motif similar to that found in the CONSTANS family of transcriptional activators (Robert et al. 1998, Strayer et al. 2000). Besides these candidate components of oscillators, a pair of putative RNA-binding proteins (CCR1/CCR2) was characterized as sub- (or slave) oscillator components (Kreps and Simon 1997, Heintzen et al. 1997).

Recent reverse-genomic studies have provided further insights into such Arabidopsis circadian clocks. Thanks to completion of the entire Arabidopsis genome sequence (The Arabidopsis Genome Initiative 2000), we previously characterized a novel family of genes, encoding Arabidopsis pseudo response regulators (designated as *APRR1*, *APRR3*, *APRR5*, *APRR7*, and *APRR9*) (Makino et al. 2000, Matsushika et al. 2000, Makino et al. 2001). Each of these APRR products contains a domain similar to the receiver of response regulators that are implicated in widespread signal transduction systems, generally referred to as "histidine (His)-to-aspartate (Asp) phosphorelay" (Imamura et al. 1999, Makino et al. 2000). However, they are atypical in that the conserved aspartate residue that normally undergoes phosphorylation in other authentic response regulators is replaced with a glutamate residue. Importantly, *APRR1* turned out to be identical to *TOC1* (Matsushika et al. 2000, Strayer et al. 2000). More importantly, not only *APRR1/TOC1* but also other *APRRs* are all subjected to circadian rhythms at the transcription level. Furthermore, each transcript of *APRRs* starts accumulating after dawn rhythmically and sequentially at approximately 2 h intervals in the order: *APRR9*→*APRR7*→*APRR5*→*APRR3*→*APRR1/TOC1* (Matsushika et al. 2000). The wave of *APRR9*-mRNA is first boosted immediately after dawn. Consistent with this last fact, the expression of *APRR9* is rapidly and transiently induced when dark-grown etiolated seedlings are exposed to a pulse of red light (or white 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 a basis of the biological clock of Arabidopsis.

To gain further insight into such circadian-related properties of *APRRs*, here we constructed APRR1-overexpressing (APRR1-ox) transgenic plants, and then we characterized them to some extent, with special reference to certain circadian-related gene expression in Arabidopsis. Furthermore, we identified a novel protein that physically associates with APRR1. This protein is a putative transcription factor that resembles PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Ni et al. 1998, Martinez-Garcia et al. 2000). This novel protein was designated PIL1 (PIF3-LIKE 1), and its possible function was discussed in relation to other findings of this study.

Results

Constructing plants carrying a 35S::APPR1 transgene

According to a conventional *Agrobacterium*-mediated DNA delivery method, we established independent homozygous transgenic lines (T3 seeds) carrying a *35S::APRR1* transgene, in which the coding region of *APRR1*-cDNA was placed under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter so as to be constitutively expressed in plants (tentatively referred to as APRR1-overexpressing plants, APRR1-ox). Two representatives of these putative APRR1-ox transgenic lines (L3 and L5) were characterized. Fig. 1A shows typical seedlings. As compared with wild-type plants (Columbia, Col.), APRR1-ox seedlings exhibited alterations in their petiole lengths. They showed significantly shorter petioles than wild-type plants (upper panel, plants were grown for 16 d under 8 h light/16 h dark). Essentially the same phenotypes were seen for APRR1-ox plants grown under 16 h light/8 h dark (lower panels, plants were grown for 22 d). These morphological alterations appear to be due to developmental regulation rather than a general growth defect, because both the numbers and sizes of leaves, except for petiole lengths, were not significantly different between these transgenic and wild-type plants. The numbers and sizes of roots were also indistinguishable between them (see upper panel). For other transgenic lines, however, the severity of phenotypes varied from one line to another (data not shown). Therefore, clarification of phenotypes of APRR1-ox plants must await more statistic analyses, particularly with regard to circadian-regulated biological events, such as stomatal opening and flowering. Meanwhile, we wanted to address more specific issues for these putative APRR1-ox plants, with special reference to certain circadian-regulated genes, including the APRR1/TOC1 family of genes themselves.

These transgenic plants (L3 and L5), together with-wild type plants (Col.), were grown under 16 h light/8 h dark for

20 d, and RNA samples were prepared at both dawn and dusk. They were analyzed by Northern hybridization with a probe specific for the *APRR1* coding sequence (Fig. 1B). In wild-type plants expression of *APRR1* peaked at dusk, and it was minimal at dawn, as expected (Makino et al. 2000). Under these conditions, two types of *APRR1*-transcript were detected for APRR1-ox transgenic plants. Judging from their sizes, it was assumed that the longer (or slow migrating) and minor transcript was derived from the endogenous *APRR1* gene, and the shorter and major one from the transgene, because the transgene was designed so as to lack the 3′ non-coding sequence. Based on this assumption, the results suggested that the transgenic plants express a high and constitutive level of the transgenic *APRR1*-transcript, at both dawn and dusk.

We then raised a polyclonal antiserum that is cross-reactive with the C-terminal portion of the APRR1 protein (i.e. its CONSTANS-like domain). The transgenic plants (L3 and L5), together with wild-type plants (Col.), were grown under continuous light for 30 d. Cellular proteins were extracted from whole plants, and then they were analyzed by Western blotting with the anti-APRR1 antiserum (Fig. 1C). A major protein band was detected for one of the transgenic lines (L3), and a considerable amount of the same cross-reactive band was also seen for another (L5). The corresponding band was hardly detected for wild-type plants. Note that the calculated molecular weight of APRR1 is 69,195, which is coincident with the apparent molecular mass (72 kDa), estimated for the immunodetected band on the gel. We thus found that the constructed *35S::APRR1* transgenic plants show the anticipated nature in that they express a larger amount of *APRR1* at the levels of both mRNA and protein, as compared with in wild-type plants. These transgenic plants were thus referred to as APRR1-overexpressing (APRR1-ox) plants. In the following studies, the homozygous L3 line of APRR1-ox plants (T3 seeds) was mainly used.

Overexpression and circadian rhythm of APRR1 in APRR1-ox plants

It was first necessary to examine APRR1-ox plants more extensively, particularly, with regard to the circadian rhythm of *APRR1* itself. To this end, APRR1-ox plants (L3) were grown for 22 d under 16 h light/8 h dark (LD conditions). From these plants, RNA samples were prepared at appropriate intervals. Northern hybridization analyses with a probe specific for the *APRR1* coding sequence were carried out (Fig. 2A). For wild-type plants, rhythmic expression of *APRR1* was clearly seen with a peak at evening (see also lower panel). For APRR1-ox plants, however, a higher and constitutive level of the total APRR1-transcript was detected for all RNA samples prepared at any given time. An endogene-specific probe, corresponding

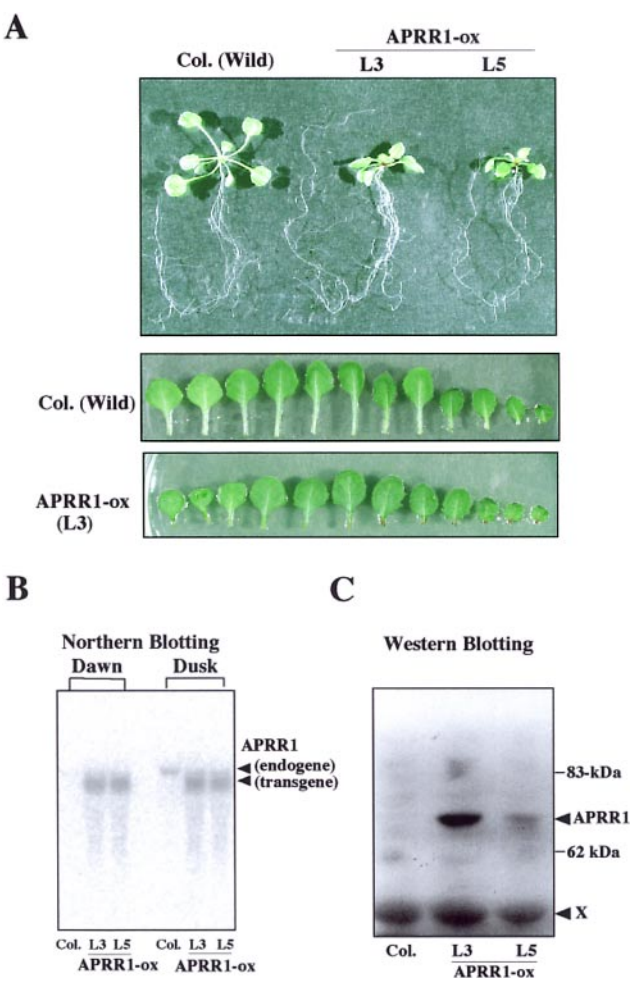

Fig. 1 Isolation and characterization of APRR1-ox transgenic plants. (A) Two lines of APRR-ox plants (homozygous T3 seeds, L3 and L5), together with wild-type plants (Columbia, Col.), were grown on agar-plates under short-day (8 h light/16 h dark) and long-day (16 h light/8 h dark) conditions. After plants were grown for 16 d under the short-day conditions, pictures were taken for each representative, as indicated (upper panel). On the other hand, after plants were grown for 25 d under the long-day conditions, pictures were taken for each representative, with special reference to their leaves (lower two panels). (B) The *APRR1*-transcript was examined for APRR1-ox plants by Northern hybridization with a probe specific for the *APRR1* coding sequence. Both wild-type (Col) and APRR1-ox plants (L3 and L5) were grown under conditions of 16 h light/8 h dark for 20 d, and then RNA samples were prepared at the times indicated (dawn and dusk). They were analyzed by Northern hybridization, followed by exposure on a phosphoimage analyzer (BAS-2500, FujiXerox, Tokyo, Japan). The positions of *APRR1* from both the endogene and transgene are indicated. (C) The APRR1 protein was examined for APRR1-ox plants by Western blotting with an antiserum, which is cross-reactive with the C-terminal portion of APRR1. Both wild-type (Col.) and APRR1-ox plants (L3 and L5) were grown under continuous light for 30 d, and then cellular proteins were isolated from whole plants. These protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoreactive bands were detected by the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The position of APRR1 is indicated, and X denotes a non-specific unknown band.

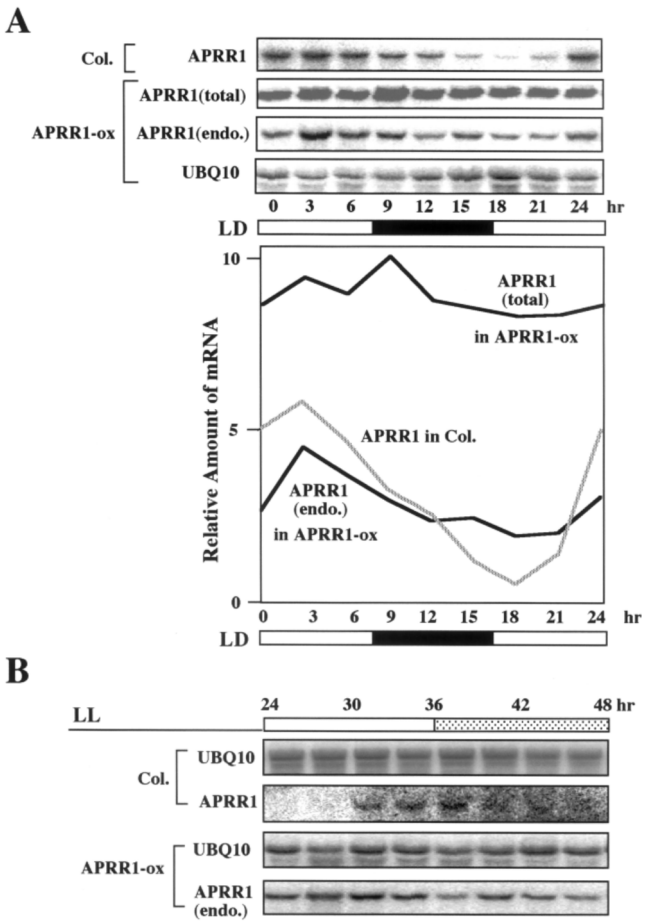

Fig. 2 Northern hybridization analyses of the *APRR1*-transcript for APRR1-ox plants. (A) Both wild-type (Col.) and APRR1-ox plants (L3) were grown under LD conditions of 16 h light/8 h dark for 22 d, as schematically shown (the filled bars indicate night). RNA samples were prepared from leaves at the appropriate intervals indicated. They were analyzed with a probe specific for the *APRR1* coding sequence to detect the total *APRR1*-transcript (i.e. transcripts from both the endogene and transgene) (upper panel). They were also analyzed with a probe specific for the 3′ non-coding region of *APRR1*, in order to detect the endogenous APRR1-transcript (labeled by endo.). The *UBQ10*-transcript was also detected as an internal and loading reference. Note that not all the reference data of *UBQ10* are shown for clarity of the figures (however, essentially the same non-fluctuated profiles were obtained for others). We then analyzed these raw data by a phosphoimage analyzer (BAS-2500, FujiXerox, Tokyo, Japan) (lower panel). The measured intensities of each band were normalized (by dividing with the *UBO10*-value). Based on these values, the relative amounts of mRNA (or transcript) were calculated and expressed as arbitrarily units. (B) Both wild-type (Col.) and APRR1-ox plants (L3) were grown under LD conditions of 12 h light/12 h dark for 20 d, and then they were transferred to continuous light (LL), as schematically shown (the shaded bar indicates subjective night). After 24 h, RNA samples were prepared from leaves at the appropriate intervals indicated. They were analyzed with a probe specific for the 3′ no-coding sequence of *APRR1* to detect the endogenous *APRR1*-transcript (labeled by endo.). The *UBQ10*-transcript was also shown as an internal and loading reference.

to the 3′ non-coding sequence of *APRR1*, was then used to detect the endogenous *APRR1*-transcript in APRR1-ox plants (labeled by endo. in Fig. 2A). As far as the light/dark (LD) conditions were concerned, the results showed that the endogenous *APRR1*-transcript was expressed rhythmically in APRR1-ox plants. However, it should be emphasized that its apparent robustness was considerably less evident, as compared with the case of wild-type plants. In any case, it was concluded that APRR1-ox plants, used in this study, overproduce the total *APRR1*-transcript constitutively.

Next, free-running expression of the endogenous *APRR1* in APRR1-ox was examined after plants were grown under continuous light (LL conditions) (Fig. 2B). Wild-type and APRR1-ox plants (L3) were grown for 20 d under 12 h light/12 h dark, and then they were transferred to LL conditions. RNA samples were prepared from these plants at appropriate intervals, and then Northern hybridization analyses were carried out with a probe specific for the 3′ non-coding sequence of *APRR1* (Fig. 2B). For wild-type plants, free-running rhythmic expression of *APRR1* was seen with a peak at subjective evening, as reported previously (Makino et al. 2000). For APRR1-ox plants, however, a constitutive level of the endogenous *APRR1*-transcript was detected for all RNA samples. Note also that essentially the same results were obtained for other RNA samples prepared during the next day (i.e. 48 h to 72 h) (data not shown). These results suggested that overexpression of *APRR1* results in loss of rhythmicity of the endogenous *APRR1*, in a manner that its robust rhythm is abolished.

Free-running circadian rhythms of the APRR1/TOC1 family of genes are dampened in APRR1-ox plants

We then examined possible effects of APRR1-ox on expressions of other members of the ARPP1/TOC1 family of genes, namely, *APRR9*, *APRR7*, *APRR5*, and *APRR3* (Fig. 3). The conditions used were essentially the same as those described above, except for the photoperiod conditions used (16 h light/8 h dark). After APRR1-ox plants were grown for 22 d, in one line of experiments the photoperiod conditions were maintained (LD conditions), whereas in the other line of experiments plants were transferred to continuous light (LL conditions). From the leaves of these plants, RNA samples were prepared, followed by Northern hybridization analyses, using the probes specific for *APRR9*, *APRR7*, *APRR5*, *APRR3*, and *UBQ10* (loading reference) (Fig. 3A), and then the hybridized bands were quantified (Fig. 3B). It should be first emphasized that the *APRR9*-transcript was hardly detected in RNA samples prepared at any given time, in either LD or LL conditions. By contrast, rhythmic expressions were observed for *APRR7*, *APRR5*, and *APRR3* in APRR1-ox plants grown under LD conditions. Under LL conditions, however, such sequential waves of these transcripts were no longer evident. In other words, each expression of *ARPP7*, *APRR5*, and *APRR3* became arhythmic, or, their rhythmicities were dampened (Fig. 3A and 3B).

In APRR1-ox plants, it was thus suggested that the

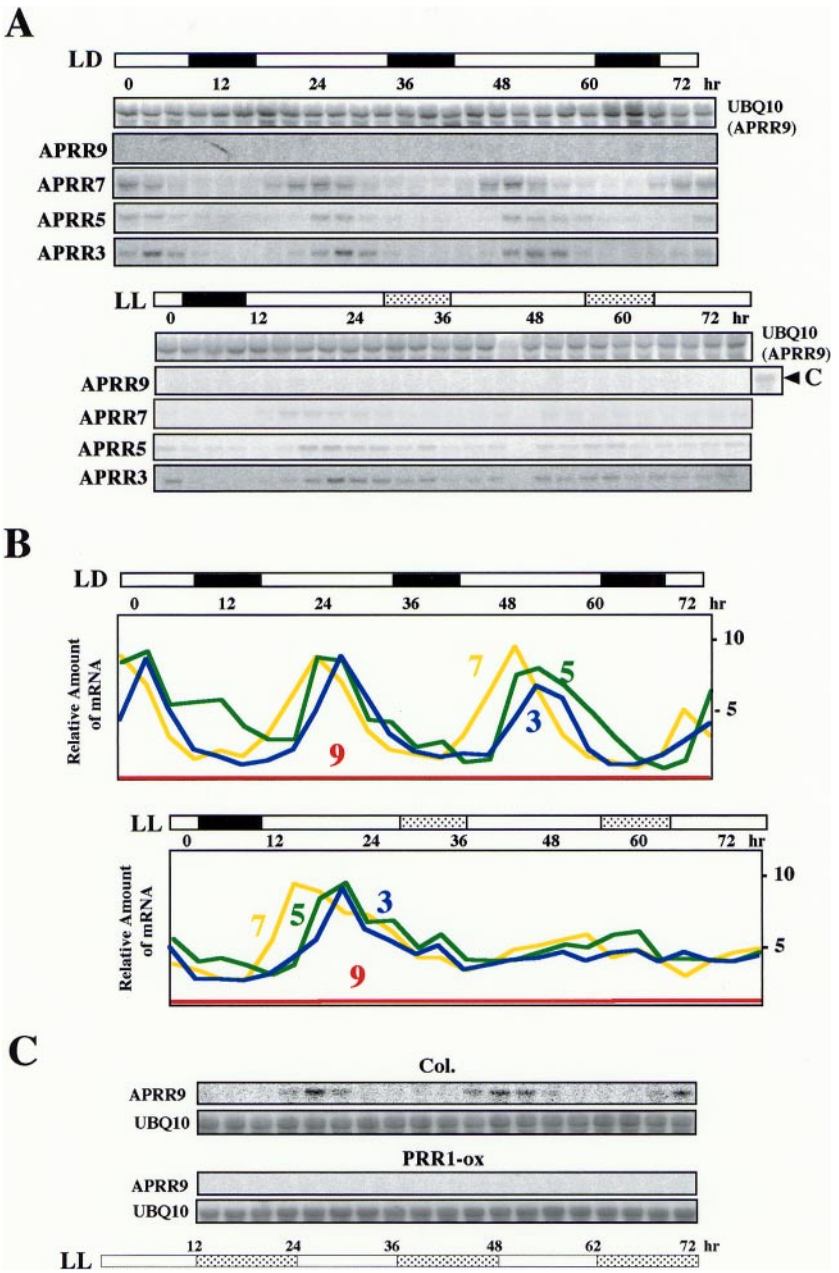

Fig. 3 Northern hybridization analyses of the *APRR*-transcripts for APRR1-ox plants. (A and B) Plants were grown under LD conditions of 16 h light/8 h dark for 22 d. They were further grown under either LD (16 h light/8 h dark) or continuous light (LL), as schematically shown (the filled and shaded bars indicate objective and subjective night, respectively). RNA samples were prepared from leaves at the intervals indicated. They were analyzed by Northern hybridization with each specific probe, as indicated (*APRR9*, *APRR7*, *APRR5*, *APRR3*, and *UBQ10*). Among these data, an appropriate and positive reference was loaded on the most right hand side of the panel for *APRR9* (see LL conditions), in order to make sure the position of the *APRR9*-transcript (labeled by C). Note also that the *UBQ10*-transcript was analyzed as an internal and loading reference in these experiments, but not all the reference data are shown for clarity of the figures (however, essentially the same non-fluctuated profiles *UBQ10* were obtained for others). These raw data (upper panels) were each quantified (by normalizing with the UBQ10-value). These quantified data are shown schematically as the relative amounts of mRNA (lower panels), in which the maximum level of each transcript was taken as 10 arbitrarily, in order to clarify the profiles. In this sense, it should be noted that one cannot directly compare the amplitudes relative to each other. In any case, the color-coordinated lines, each of which is denoted by the corresponding APRR-number, show the expression profiles for *APRR3*, *APRR5*, and *APRR7*. The red straight lines indicate the expression pattern of *APRR9*. Note that this particular profile of *APRR9* is intended to be schematic, because the *APRR9*-transcript was hardly detected. (C) The expression of *APRR9* was further examined in APRR1-ox plants grown under different growth conditions. Wild type (Col.) and APRR1-ox plants were grown for 20 d under 12 h light/12 h dark, and then they were transferred to continuous light (LL). RNA samples were prepared from leaves at the intervals indicated, as schematically shown. They were analyzed with a probe specific for the *APRR9* coding region by Northern hybridization.

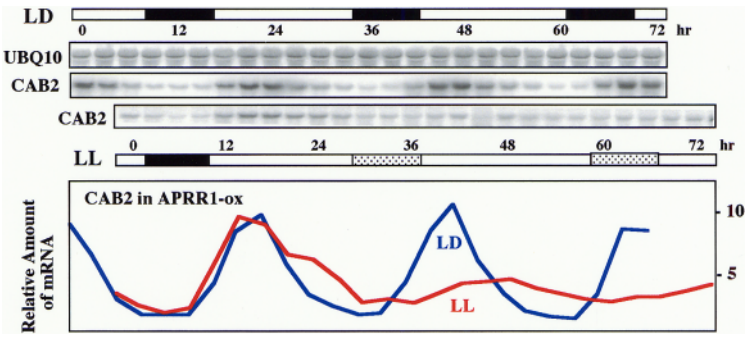

Fig. 4 The free-running circadian rhythm of *CAB2* is dampened in APRR1-ox plants. APRR1-ox plants were grown under LD conditions of 16 h light/8 h dark for 20 d. They were further grown under either LD or continuous light (LL), as schematically shown (the filled and shaded bars indicate objective and subjective night, respectively). RNA samples were prepared from leaves at the intervals indicated, and then they were analyzed by Northern hybridization with a probe specific for the *CAB2* coding sequence. These raw data are shown (upper panels). The *UBQ10*-transcript was also analyzed as an internal and loading reference. The quantified data are shown schematically (lower panel). For other experimental details, see the legends to Fig. 2 and Fig. 3. The free-running circadian rhythm of *CAB2* in wild-type plants (Col.) was not examined, because it has been well established previously (see the text).

expression of *APRR9* is severely repressed under both LD and LL conditions. Since this observation is particularly crucial, we further examined this event in APRR1-ox plants grown under different conditions (Fig. 3C). Plants were grown for 20 d under conditions of 12 h light/12 h dark (LD conditions), and then plants were transferred to continuous light (LL conditions). RNA samples were prepared from the leaves of these plants at intervals, followed by Northern hybridization analyses. In wild-type plants, rhythmic and free-running expression of *APRR9* with a peak at dawn was observed, as expected (Makino et al. 2000). In APRR1-ox plants, however, the *APRR9*-transcript was hardly detected at any time. This result is consistent with that seen in Fig. 3A. Taken together, for APRR1-ox plants, it was demonstrated that the expression of *APRR9* is severely repressed under both LD and LL conditions, whereas the free-running and rhythmic waves of others (*APRR7*, *APRR5*, and *APRR3*) are dampened, particularly, under LL conditions. From these results, one can at least envisage that there are intimate linkages between the expression of *APRR1* and those of other members of the APRR1/TOC1 family of genes (*APRR9*, *APRR7*, *APRR5*, and *APRR3*).

Circadian rhythm of CAB2 is dampened in APRR1-ox plants

A well-known hallmark of clock-regulated events in Arabidopsis is the free-running and rhythmic expression of *CAB2*, which is presumably the most downstream target of circadian output pathways. This crucial circadian-regulated event was examined in APRR1-ox plants (Fig. 4), after plants were grown under the same conditions as those described above (see Fig. 3A). In APRR1-ox plants grown under 16 h light/8 h dark, expression of *CAB2* showed robust rhythmicity with a peak around noon (Fig. 4), as has been reported for wild-type plants (Piechulla 1999). This rhythmic profile was rapidly dampened when APRR1-ox plants were transferred to continuous light (LL) (note that the anticipated 2nd and 3rd peaks disappeared). Thus, the circadian rhythm of the circadian-regulated *CAB2* gene was dampened in APRR1-ox plants, suggesting that overexpression of *APRR1* results in a defect of the free-running clock function in Arabidopsis.

Circadian rhythms of several clock-associated genes are also altered in APRR1-ox plants

As mentioned in the Introduction, several genes have been proposed to encode such proteins that function within, or close to, the central oscillator(s). They include *CCA1*/*LHY* encoding putative clock components, *GI* encoding a modulator of flowering time, and *CCR2* encoding a component of a sub- (or slave) oscillator. It should be noted that it is well known that the accumulations of the respective transcripts themselves are also oscillated under free-running conditions (for a review see Barak et al. 2000). These events are assumed to be crucial for their circadian-associated functions. Thus, it is of interest to examine such oscillated profiles of these circadian-associated genes in APRR1-ox plant. The results are shown in Fig. 5.

In wild-type plants, both *CCA1* and *LHY* showed each typical circadian rhythm with a peak at subjective dawn under LL conditions, as anticipated (Wang et al. 1997, Schaffer et al. 1998). In APRR1-ox plants, however, the amplitude of the peak of *CCA1* decreased significantly. Furthermore, the *CCA1*-transcript peaked at a significantly delayed timing in APRR1-ox plants (Fig. 5A). In other words, in this particular experiment with APRR1-ox plants under LL conditions, the 1st peak of *CCA1* appeared about 5 h later than that under LD conditions, and the re-appearance of 2nd peak was delayed further. The estimated period between the 1st and 2nd peaks of *CCA1* in APRR1-ox plants was approximately 30 h, considerably longer than anticipated. A similar event was observed for *LHY* in APRR1-ox plants under LL conditions (Fig. 5B).

Another intriguing event was seen in the case of *GI* (Fig. 5C). In wild-type plants, the *GI*-transcript peaked rhythmically

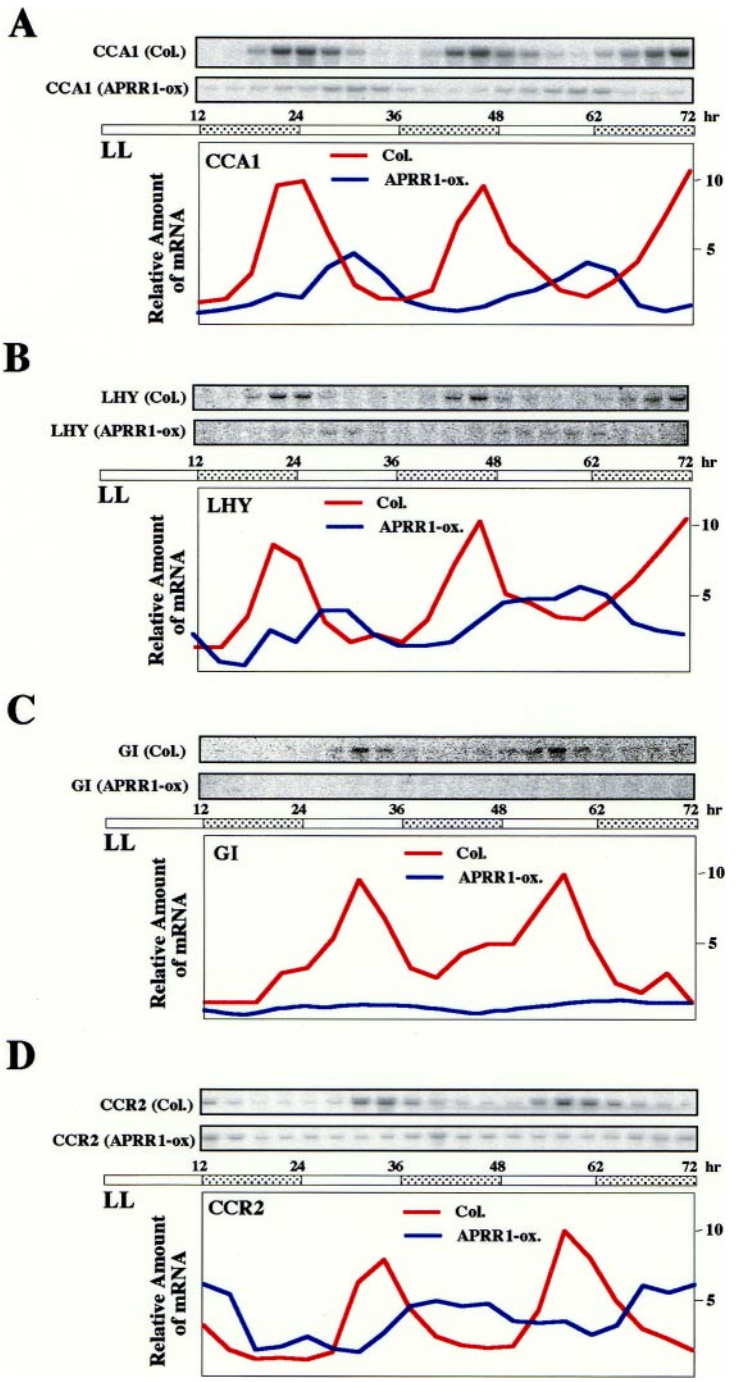

Fig. 5 The free-running circadian rhythms of certain circadian-associated genes, *CCA1*(A), *LHY* (B) *GI* (C) and *CCR2* (D) are markedly altered in APRR1-ox plants. Both wild-type (Col.) and APRR1-ox plants were grown under LD conditions of 12 h light/12 h dark for 20 d. They were further grown under continuous light (LL), as schematically shown (the shaded bars indicate subjective night). RNA samples were prepared from leaves at the intervals indicated. They were analyzed by Northern hybridization with a probe each specific for *CCA1*, *LHY*, *GI*, and *CCR2*, as indicated. These raw data are shown (each upper panel). The *UBQ10*-transcript was also analyzed as an internal and loading reference in these experiments, but they are not shown for clarity of the figures. The quantified data are also shown schematically (each lower panel). For other experimental details, see the legends to Fig. 2 and Fig. 3.

around subjective noon under LL conditions, as reported previously (Fowler et al. 1999). In APRR1-ox plants, however, the *GI*-transcript was hardly detected at any time. It may be noted that this event is similar to that observed for *APRR9* in APRR1-ox plants (see Fig. 3). *CCR2* was also examined (Fig. 5D). In wild-type plants, the *CCR2*-transcript peaked rhythmi-

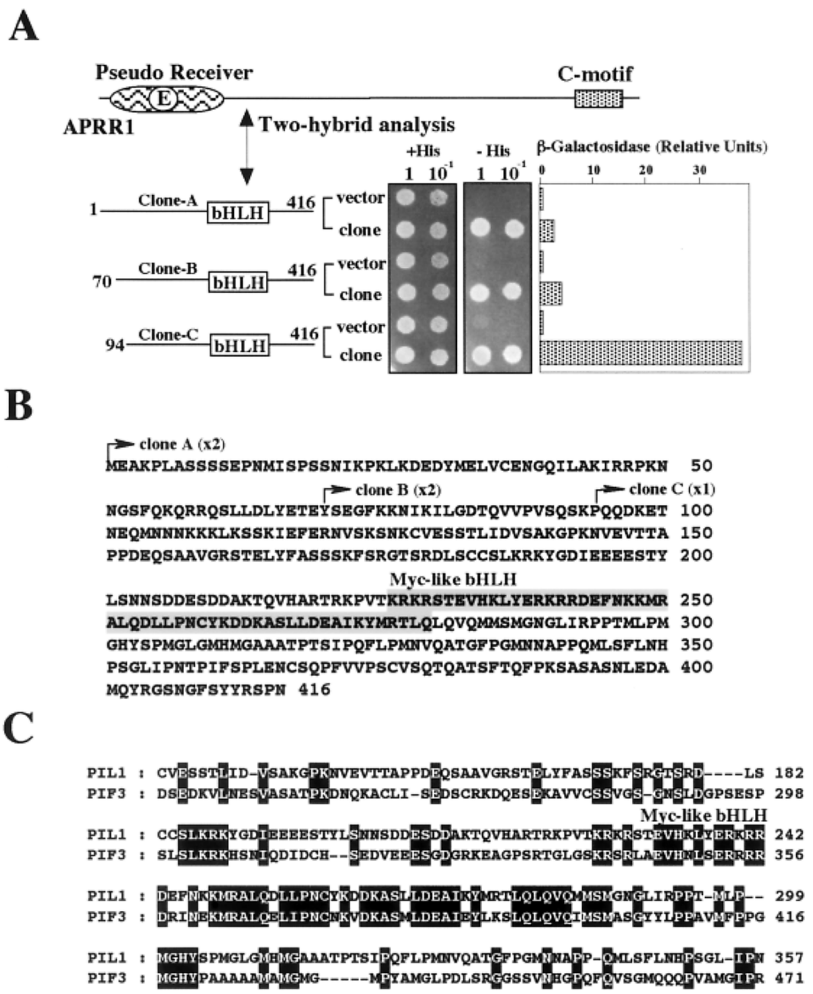

Fig. 6 Identification of a protein that physically interacts with APRR1 by yeast two-hybrid analyses. (A) Yeast two-hybrid analyses were carried out with the use of APRR1 as bait. The bait contained the entire region of the APRR1 protein, as schematically shown. Three types of positive clones (Clone-A to Clone-C) were isolated, each of which was found to carry a common open-reading-frame (ORF), as schematically shown. This ORF was found to specify a protein with a basic helix-loop-helix (bHLH) motif, as also schematically shown. These positive clones, together with an appropriate reference (vector), were first scored with regard to the histidine-autotrophy on appropriate His-containing agar-plates (+His and –His, respectively) with appropriate numbers of yeast cells (10^{-1} indicated "ten-times dilution"). These results were further confirmed by β-galactosidase assays, as also shown. (B) The above results allowed us to identify an Arabidopsis protein that might be able to physically interact with APRR1. This protein was designated PIL1 (PIF3-LIKE 1) (see the text and below). The deduced amino acid sequence of PIL1 is shown. A Myc-related bHLH motif, found in PIL1, is highlighted in its entire sequence. The corresponding gene has already been registered in the GenBank database (The Arabidopsis Genome Initiative, gene At2g46970, accession AC004411, chromosome II). However, it should be noted that the exon-intron alignment of the At2g46970 gene in the database differs slightly from the one experimentally determined here for our c-DNA clones. Thus, our determined amino acid sequence is adopted. (C) The amino acid sequence of PIL1 was aligned with that of PIF3 (PHYTOCHROME INTERACTING FACTOR 3). The Arabidopsis PIF3 protein has been identified as the one that specifically associates with red light-activated PHYB (Ni and Quail 1998, Martinez-Garcia et al. 2000). Only the amino acid regions similar to each other were aligned, in which the highly homologous bHLH motifs are denoted. The identical amino acids are highlighted.

cally around subjective evening, as also reported previously (for a review see Staiger and Heintzen 1999). This robust circadian rhythm of *CCR2* was no longer evident in APRR1-ox. The rhythm of *CCR2* was dampened under LL conditions, and the *CCR2*-transcript was more or less constitutively detected at a lower level in APRR1-ox plants under these conditions.

In summary, the circadian rhythms of certain potential clock components that function within, or close to, the central oscillator were severely disordered in APRR1-ox plants. The amplitudes and periods of the rhythmic profiles of *CCA1* and *LHY* were markedly altered. *GI* was severely depressed, whereas the rhythmic expression of *CCR2* was rapidly dampened.

APRR1 physically associates with a PIF3-like protein

The above results are fully compatible with the idea that

APRR1 plays an important role for an as yet unknown mechanism underlying generation of circadian rhythmicity in Arabidopsis. To gain further insight into the molecular function of APRR1, finally, we carried out yeast two-hybrid screenings to search for Arabidopsis proteins that physically interact with the APRR1 protein. The entire *APRR1*-cDNA sequence was connected to the GAL4 DNA-binding domain of pGBT9, to yield pGBT9-APRR1 (bait vector). On the other hand, an Arabidopsis cDNA-expression library was obtained, in which cDNAs were connected to the GAL4 activation domain in pGAD424 (prey library). Among 10^7 yeast transformants thus screened, five positive clones were obtained. Each of these was assumed to carry an Arabidopsis cDNA, protein product of which might interact with the APRR1 protein. Analyses involving restriction endonuclease digestion of these cloned DNAs showed that they were classified into three groups [clone-A (two clones), clone-B (two clones), and clone-C (one clone)] (Fig. 6A). Sequencing of each cDNA revealed that they were all derived from the same gene, which has been registered in the GenBank database (gene At2g46970, accession AC004411, chromosome II). In clone-B and clone-C, 5′-proximal regions (or N-terminal portions) had been truncated. In any case, the results of these two-hybrid assays showed that APRR1 might be able to interact with this particular gene product. Sequencing of the entire cDNA of this gene showed that it encodes a protein of 416 amino acids (Fig. 6B). This inferred protein contains a typical Myc-related basic helix-loop-helix (bHLH) motif, which is commonly found in some transcription factors of plants (The Arabidopsis Genome Initiative 2000). Interestingly, the amino acid sequence of this putative bHLH domain is highly similar to that found in the well-characterized Arabidopsis bHLH protein named PIF3 (PHYTOCHROME INTERACTING FACTOR 3) (Fig. 6C). PIF3 has been identified as the protein that specifically associates with red light-activated PHYB (Ni et al. 1998, Martinez-Garcia et al. 2000). Thus, the identified protein that might be able to associate with the APRR1 protein was hereafter designated PIL1 (PIF3-LIKE 1).

To verify the above notion, two-hybrid analyses were further carried out (Fig. 7A), in which PIF3 and APRR9 were also examined as alternative bait and prey, respectively. The results showed that the C-terminal domain of APRR1, lacking a pseudo-receiver domain, has a stronger ability to interact with PIL1. It should be emphasized that APRR1 showed the ability to interact with PIF3 as well. No interaction between APRR9

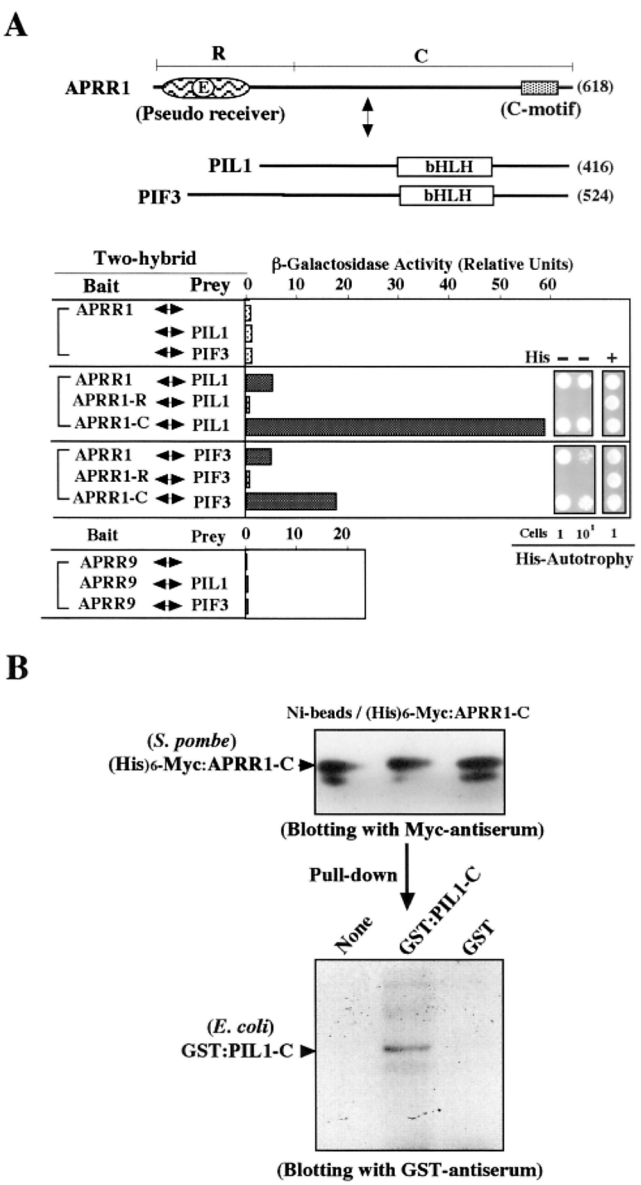

Fig. 7 Characterization of interaction between APRR1 and PIL1. (A) Yeast two-hybrid analyses were carried out with the use of a set of bait/prey pairs, as schematically shown (upper part). The results were scored with special reference to both the histidine-autotrophy (+His and –His) and the β-galactosidase activity (lower part). Other details are the same as those given in Fig. 6 (B) In vitro interaction between APRR1 and PIL1. In *S. pombe*, the C-terminal portion of APRR1 (named APRR1-C, see Fig. 7A), fused to a domain containing both the His-tag and Myc-epitope, was expressed. To do so, an *S. pombe* versatile expression vector (pREP1) was employed. In *E. coli*, on the other hand, the C-terminal portion of PIL1 (named PIL1-C, see Fig. 6A, clone-C), fused to glutathione S-transferase (GST), was expressed. To this end, an *E. coli* versatile expression vector was used (Toyobo Co. Ltd., Japan). First, Ni-beads carrying the (His)6-Myc:APRR1-C polypeptide were prepared from the *S. pombe* cell extract. The (His)6-Myc:APRR1-C polypeptide on Ni-beads were purified and detected by immunoblotting assay with an anti-Myc-epitope antiserum (upper panel). Then, in vitro pull-down assays were carried out with these Ni-beads against the *E. coli* cellular proteins containing the GST:PIL1-C polypeptide (the lane denoted by GST:PIL1). Appropriate references examined, used here, were the *E. coli* cellular proteins containing the GST polypeptide alone (the lane denoted by GST) and blank (the lane denoted by None). The Ni-beads were recovered by a centrifugation, and then washed extensively. The resulting samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting assays with an anti-GST antiserum (lower panel).

and PIL1 was observed in this yeast two-hybrid assay. It was thus suggested that APRR1 can physically associate with the putative Myc-related bHLH transcription factors, PIL1, and also PIF3. This was verified by an in vitro protein–protein interaction assay (Fig. 7B). In *Schizosaccharomyces pombe*, the C-terminal portion of APRR1 (named APRR1-C, see Fig. 6A), fused to a His-tag and Myc-epitope region, was expressed. In *Escherichia coli*, on the other hand, the C-terminal portion of PIL1 (named PIL1-C, see Fig. 6A, clone-C), fused to glutathione S-transferase (GST), was expressed. With Ni-beads carrying the (His)6-Myc:APRR1-C polypeptide, in vitro pull-down assays were carried out against *E. coli* cellular proteins containing the GST:PIL1-C polypeptide. The in vitro results supported the view that APRR1 can physically associate with PIL1 (Fig. 6B).

Discussion

Clarification of plant biological clocks is a paradigm of the current Arabidopsis molecular biology (see Introduction). In this respect, the rhythmic waves of the APRR1/TOC1 quintet are intriguing, as addressed above. In this study, certain circadian-regulated genes were extensively examined in Arabidopsis APRR1-ox transgenic plants, mainly, by determining the level of each transcript by means of Northern hybridization analyses. The level of transcript, determined by Northern hybridization, generally does not necessarily give us a direct idea about the level of a given protein that eventually plays its own physiological role. Also, a given circadian profile consists of amplitude, periodic length, and phase angle. However, Northern hybridization mainly used in this study does not allow us to precisely explain the results of this study with special reference to each rhythm component. Nevertheless, the results of this study provided us with much insight into the possible interplay of potential circadian components in Arabidopsis, as addressed below.

Our results with APRR1-ox plants are fully compatible with the current idea that APRR1 (TOC1) plays a crucial role within, or close to, the Arabidopsis central oscillator. The following findings of this study supported this current idea. (1) The free-running and robust circadian rhythms of the APRR1/TOC1 family of genes, including *APRR1* itself, are dampened in APRR1-ox plants. (2) A hallmark of clock-regulated circadian rhythms is the free-running rhythmicity of *CAB2*, a downstream target of the output pathways. This robust rhythmicity of *CAB2* is also dampened in APRR1-ox plants. (3) Circadian profiles of some clock-associated genes are also significantly altered in APRR1-ox plants. These genes include *CCA1*/*LHY*, *GI*, and *CCR2*. The effect on *CCA1* and *LHY* are particularly interesting, because these proteins are the potential components of the central oscillator (for a review, see Barak et al. 2000). Namely, expressions of *CCA1* and *LHY* seem to keep their rhythmicities, but their robustness and period are markedly altered, resulting in a longer period in APRR1-ox plants under LL conditions. In the accompanying paper (Matsushika et al. 2002), in fact, we showed also that constitutive overexpression of *CCA1* in plants dampened the free-running rhythmic expression of *APRR1* (and other *APRRs*), and vice versa. These results support the view that there are intimate interactions between *APRR1* and certain circadian-associated components, including *CCA1* and *LHY*.

In APRR-ox plants, the *APRR9*-transcript was hardly detected in RNA samples prepared at any time, irrespective of LD or LL conditions. Otherwise, it was suggested that constitutive expression of *APRR1* does not significantly affect the expression profiles of other APRR1/TOC1 family members (*APRR7*, *APRR5*, and *APRR3*), under LD conditions. However, it should be emphasized that their free-running circadian waves are severely dampened under LL conditions in APRR1-ox plants. This suggests that the seemingly sequential events of the *APRR9*→*APRR7*→*APRR5*→*APRR3*→*APRR1/TOC1* waves may not be such a simple unidirectional linear pathway. Rather, they may form interlocking loops more complex than we originally thought (Matsushika et al. 2000), in such a manner that *APRR1* and *APRR9* are more closely linked, as discussed further below.

One of the most intriguing findings of this study is that constitutive expression of *APRR1* results in a severe depression of *APRR9*. The fact that *APRR9* is induced by white light (or red light pulse) previously led us to speculate that the APRR9 protein might act at an interface between the light signal input pathways and the oscillator components (Matsushika et al. 2000). In wild-type plants the *APRR9*-transcript was rapidly accumulated when dark-grown young seedlings were exposed to light, it peaks within 2 h, and has decreased by 6 h (Makino et al. 2001), whereas the level of the *APRR9*-transcript was dramatically reduced on a *phyB* background (Matsushika et al. unpublished result). Such a light-dependent induction of *APRR9* was also no longer seen in APRR1-ox (Matsushika et al. unpublished result). These events are indicative of a negative *APRR1*→*APRR9* regulation. It is thus of interest to know how APRR1 and PHYB (or PHYA) regulate the expression of *APRR9*. In this connection, another intriguing finding of this study is that APRR1 can interact with a Myc-related bHLH factor, named PIL1, which is similar to PIF3. APRR1 appears to bind to PIF3 as well. The *PIF3* gene has been identified as the one encoding a phytochrome-interacting transcription factor. In plants with reduced levels of PIF3, in fact, there is a decrease in red-light induction of *CCA1* and *LHY* (Martinez-Garcia et al. 2000). Taking these together, one can assume that PIL1 (and/or PIF3) might also be involved in the phytochrome-dependent induction of *APRR9* by light, and that APRR1 negatively affects the expression of *APRR9* through its interaction with PIL1 (and/or PIF3). Although these views are speculative at present, they are worth being tested because the findings of this study addressed above are best explained by such a hypothesis.

It is premature to discuss in details about the molecular

bases of the Arabidopsis central oscillator(s) solely based on the results of this study. However, we have provided several insights into the importance of the APRR1/TOC1 circadian waves. These views will provide at least a preliminary framework for better understanding the molecular mechanisms underlying the circadian rhythms in Arabidopsis.

Materials and Methods

Plant growth conditions and related materials

Arabidopsis thaliana (Columbia, hereafter designated Col.) was mainly used as wild-type plants. Seeds were imbibed and cold treated at 4°C for 2 d before germination and growth at 22°C. To grow plants in the dark, the imbibed seeds were exposed to white light for 30 min before growth in the dark. Otherwise, plants were grown in a chamber with conditions of light from fluorescent lights (150–200 μmol m^{-2} s^{-1}) at 22°C on soil, or on agar-plates containing MS salts and 2% sucrose, as described previously (Taniguchi et al. 1998). Light/dark conditions used were either 16 h light/8 h dark (long-day conditions), 12 h light/12 h dark, or 8 h light/16 h dark (short-day conditions), as specifically noted for each experiment in the text.

Preparing RNA, and Northern blotting

Total RNA was isolated from appropriate organs (mainly leaves) of Arabidopsis by the phenol-sodium dodecyl sulfate (SDS) method (Taniguchi et al. 1998). For Northern hybridization, RNA was separated in agarose gels (1%) containing 2.2 M formaldehyde, then transferred to Hybond-N^+ membranes. The fixed membranes were hybridized with ^{32}P-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× Denhardt's solution, and 0.5% SDS containing 10% dextran sulfate and 100 μg ml^{-1} salmon sperm DNA, at 65°C for 18 h. The membranes were washed twice with 2× SSPE and 0.5% SDS for 15 min at room temperature, twice with 2× SSPE and 0.5% SDS for 30 min at 65°C, and then with 0.2× SSPE and 0.5% SDS for 15 min at 65°C. The washed membranes were exposed and analyzed on a phosphoimage analyzer (BAS-2500II) (FujiXerox, Tokyo, Japan).

PCR amplification

Using appropriate pairs of primers, if necessary, polymerase chain reaction (PCR) was carried out to prepare DNA segments. The standard conditions were primarily 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, 25 cycles with Thermal Cycler 480 (Takara Shuzo, Kyoto, Japan). A PCR kit was used, according to the supplier's instructions (Takara Shuzo, Kyoto, Japan).

Probes for Northern blotting

Several double-stranded ^{32}P-labeled DNA probes were used to detect each specific mRNA. The probes used were amplified by PCR with appropriate sets of primers. They were designed appropriately, according to the previous reports, including *UBQ10* (Taniguchi et al. 1998), *CCA1*, *GI*, *CCR2* (Wang and Tobin 1998), *LHY* (Schaffer et al. 1998), *CAB2* (Brusslan and Tobin 1992), *APRR1*, *APRR3*, *APRR5*, *APRR7*, and *APRR9* (Matsushika et al. 2000). They include: *APRR9* (5′-CAAGATTCGATAAAAATGGTGTTG and 5′-TCTCTGGCTTCTACTCCAACG), *APRR7* (5′-ATGTCATCTCATGACTCAATGGGG and 5′-AGAGATCTTATTTTCATCCGC), *APRR5* (5′-ACACAGGACTCGGTGAATACTGTG and 5′-TTCTCGTTGAAGAGATTGCTG), *APRR3* (5′-TCACATGATTCTATGGTTCTGGTC and 5′-TTGTGCCCATCGGTCACTTCC), *APRR1*-coding (5′-TCGAGGCAAGACGAAGTCCCTGTC and 5′-TCTTCTGTCAAGTTTATTTACCC), *APRR1*-3′-non-coding (5′-AGATACACCAAGAACTGAAAACCGTTG and 5′-CAAGATTCAAGTAAATGATACTGGAAGG), *CCA1* (5′-GGCCTAAGCGTAAACCAAACAATCC and 5′-GGGATCTTTCTGTTCCACATGAATG), *LHY* (5′-TGGACATAGAAATTCCGCCTCCTCG and 5′-GCTTTTGAAATTAGGAGCCAATGGC), *GI* (5′-GAGTTTTACCACAAGATCAGCGACC and 5′-GATGTCACCGCTGTATCAAAGCATG), *CCR2* (5′-GCTCTTGAGACTGCCTTCGCTC and 5′-CTCGTTAACAGTGATGCTACGG), *CAB2* (5′-GGGAATTCAAATCCAATGAGTAGAGAGA and 5′-GGGGATCCTCACACGGCCGCTTCCGAGGA). Each ^{32}P-labeled probe was prepared with a random-labeling kit (Takara Shuzo, Kyoto, Japan).

Constructing APRR1-ox transgenic plants

The entire coding sequence of the *APRR1* cDNA (Makino et al. 2000) was cloned by PCR-aided cloning into the pSK1 vector (Kojima et al. 1999) at the *Xba*I site downstream of the CaMV 35S promoter, to yield pSK1-APRR1. This construct was transformed into *Agrobacterium tumefaciens* strain EHA101, and then Arabidopsis plants (Columbia) was transformed by vacuum infiltration procedures, as described previously (Bechtold et al. 1993). Only transgenic lines segregating the hygromycin resistance as a single locus were used in further analysis, after establishing homozygous T3 seeds.

Yeast two-hybrid screening

A kit for two-hybrid analysis (MATCHMAKER™, Clontech) was obtained through TOYOBO Co. This kit contained all tools essential for two-hybrid screening, including the vectors: pGBT9 providing the GAL4 DNA-binding domain (TRP1 marker), and pGAD424 providing the GAL4 activation domain (LEU2 marker). The kit also included the yeast host strains: HF7c carrying both the GAL1-HIS3 and $(GAL4\ 17\text{-}mers)^3$ CYC1-lacZ reporters, and SFY526 carrying the GAL1-lacZ reporter. Two-hybrid screening was carried out essentially according to the manual accompanying the kit. An Arabidopsis cDNA-expression library was obtained from Clontech, namely, *A. thaliana* MATCHMAKER cDNA Library. cDNAs were prepared from mRNA of 3-week-old green vegetative tissue of Arabidopsis (Columbia). The following plasmid were constructed from pGBT9 for baits, namely, pGBT9-APRR1, pGBT9-APRR1-R, pGBT9-APRR1-C, and pGBT9-APRR9. The plasmid pGAD424-PIF3 was constructed from pGAD424 for preys. To construct these plasmids, we designed each appropriate set of PCR-primers for PCR-aided cloning, and the nucleotide sequences of the resulting plasmids were confirmed by sequencing. Plasmids, pGAD10-PIL1-A, pGAD10-PIL1-B, pGAD10-PIL1-C were isolated by screening (see Fig. 6).

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