Plant Cell Physiol. 41(9): 1002–1012 (2000)

Rapid Paper

Circadian Waves of Expression of the APRR1/TOC1 Family of Pseudo-Response Regulators in *Arabidopsis thaliana*: Insight into the Plant Circadian Clock

Akinori Matsushika [1], Seiya Makino [1], Masaya Kojima and Takeshi Mizuno [2]

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

The Arabidopsis pseudo-response regulator, APRR1, has a unique structural design containing a pseudo-receiver domain and a C-terminal CONSTANS motif. This protein was originally characterized as a presumed component of the His-to-Asp phosphorelay systems in *Arabidopsis thaliana*. Recently, it was reported that APRR1 is identical to the *TOC1* gene product, a mutational lesion of which affects the periods of many circadian rhythms in Arabidopsis plants. TOC1 is believed to be a component of the presumed circadian clock (or central oscillator). Based on these facts, in this study four more genes, each encoding a member of the APRR1/TOC1 family of pseudo-response regulators were identified and characterized with special reference to circadian rhythms. It was found that all these members of the APRR1/TOC1 family (APRR1, APRR3, APRR5, APRR7, and APRR9) are subjected to a circadian rhythm at the level of transcription. Furthermore, in a given 24 h period, the APRR-mRNAs started accumulating sequentially after dawn with 2–3 h intervals in the order of APRR9→APRR7→APRR5→APRR3→APRR1. These sequential events of transcription, termed 'circadian waves of APRR1/TOC1', were not significantly affected by the photoperiod conditions, if any (e.g. both long and short days), and the expression of APRR9 was first boosted always after dawn. Among these APRRs, in fact, only the expression of APRR9 was rapidly and transiently induced also by white light, whereas such light responses of others were very dull, if any. These results collectively support the view that these members of the APRR1/TOC1 family are together all involved in an as yet unknown mechanism underlying the Arabidopsis circadian clock. Here we propose that the circadian waves of the APRR1/TOC1 family members are most likely a molecular basis of such a biological clock in higher plants.

Abbreviations: APRR, Arabidopsis pseudo-response regulator; ARR, Arabidopsis response regulator; PCR, polymerase chain reaction.

Introduction

Common signal transduction mechanisms, generally referred to as histidine→aspartate (His-to-Asp) phosphorelay systems, are involved in a wide variety of cellular responses to environmental stimuli (Appleby et al. 1996, and references therein). A His-to-Asp phosphorelay system consists of two or more common signal transducers, a sensor exhibiting His-kinase activity, a response regulator containing a phospho-accepting Asp in its receiver domain, and a His-containing phosphotransmitter (HPt) (Parkinson and Kofoid 1992, Mizuno 1998, and references therein). To date, numerous instances of such His-to-Asp phosphorelay systems have been reported for not only many prokaryotic species (Parkinson and Kofoid 1992, Mizuno 1997), but also certain eukaryotic species (Wurgler-Murphy and Saito 1997, Chang and Stewart 1998). In the higher plant, *Arabidopsis thaliana*, the first discovery of the His kinase type of ethylene receptors immediately suggested that a His-to-Asp phosphorelay mechanism operates in this plant as a signaling system crucial for the ethylene (or phytophormone) response (Chang et al. 1993, Hua et al. 1995, Hua and Meyerowitz 1998, Sakai et al. 1998a). Many groups have subsequently shown that *A. thaliana* possesses a number of genes each encoding a member of the His-to-Asp phosphorelay signal transducer family (at least, 11 His-kinases, five HPt phosphotransmitters, and 20 response regulators) (Kakimoto 1996, Brandstatter and Kieber 1998, Imamura et al. 1998, Imamura et al. 1999, Miyata et al. 1998, Sakai et al. 1998b, Suzuki et al. 1998, Urao et al. 1998, Urao et al. 1999, D'Agostino and Kieber 1999). Collectively, His-to-Asp phosphorelay is a newly emerging paradigm of intracellular signal transduction network in *A. thaliana* (or higher plants). From the biological viewpoint, nevertheless, clarification of such a His-to-Asp phosphorelay network is at a very early stage.

We have been studying the His-to-Asp phosphorelay system in *A. thaliana*, and a number of genes, each encoding either His-kinase (designated as AHK), HPt phosphotransmitter (designated as AHP), or response regulator (designated as ARR), have been isolated and characterized extensively (Imamura et al. 1998, Imamura et al. 1999, Suzuki et al. 1998, Taniguchi et al.

[1] These two authors equally contributed to this work.
[2] Corresponding author: E-mail, tmizuno@agr.nagoya-u.ac.jp; Fax, +81-52-789-4089.

1998, Yamada et al. 1998, Kiba et al. 1999, Nakamura et al. 1999). During the course of these studies, we found that this plant possesses a set of unique genes encoding proteins very similar to, but clearly distinct from, the ARR family of response regulators (Makino et al. 2000). Notably, the former lacks the phospho-accepting aspartate site that is invariantly conserved in the receiver domain of the classical response regulators. Thus, these novel proteins were collectively designated as '*A*rabidopsis *p*seudo-*r*esponse *r*egulators (APRRs)'. They are further classified into two subgroups, based on the structural designs of their amino acid sequences: one is represented by APRR1 and the other by APRR2. Members of the APRR1 family of pseudo-response regulators contain a characteristic signature domain (named C-motif), whereas those belonging to the APRR2 family have another signature domain (named B-motif), which is common also to the type-B family of classical response regulators (Imamura et al. 1999, Jin and Martin 1999). Among these, APRR1 is particularly interesting for two reasons. First, the characteristic C-motif consisting of ~50 amino acids appears to be a widespread motif that found in many other apparently unrelated plants proteins (Makino et al. 2000). The well-known example is the CONSTANS (CO) family of proteins, which is crucially involved in the regulation of the flowering time in response to circadian-related environmental conditions (e.g. long-day photoperiod) (Putterill et al. 1995, Robert et al. 1998, Kobayashi et al. 1999). Second, the transcription of APRR1 in plants oscillates within a given 24 h period, and appears to be subjected to a circadian rhythm (Makino et al. 2000). It is thus tempting to envisage that there is a link between a His-to-Asp phosphorelay system and a circadian rhythm.

Circadian rhythms are presumably driven by an endogenous biological clock that regulates many biochemical, physiological and behavioral processes in a wide variety of organisms (Dunlap 1999, and references therein). In higher plants also, there is a wide range of processes that show a circadian rhythm (Kreps and Kay 1997, Koornneef et al. 1998, and references therein). They include movement of organs such as leaves and petals, stomatal opening, sensitivity to light of floral induction, and metabolic processes such as respiration and photosynthesis. Nevertheless, until recently, little was known about the molecular mechanisms underlying circadian rhythms in plants. In *A. thaliana*, however, a certain number of circadian-related genes were recently reported (for reviews, see Anderson and Kay 1996, Kreps and Kay 1997, Koornneef et al. 1998, Piechulla 1999, Somers 1999, Staiger and Heintzen 1999, Murtas and Millar 2000, Samach and Coupland 2000, and references therein). For example, light-driven entrainment of the presumed Arabidopsis circadian clock has been shown to be mediated by phytochrome A (PhyA), phytochrome B (PhyB), and cryptochromes (Cry1 and Cry2), thus affirming the roles of these photoreceptors as input regulators to the circadian clock. Similarly, the ELF3 gene has also been proposed to act in the input pathway (Hicks et al. 1996, Zagotta et al. 1996). With regard to the presumed circadian clock (or central oscillator), several genes were recently proposed to encode potential clock-related components. Both the *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) genes are implicated in a part of a feedback loop that is closely associated with the circadian clock (Wang et al. 1997, Wang and Tobin 1998, Schaffer et al. 1998). A mutation of the *TOC1* gene affects the periods of many circadian rhythms including expression of the *CAB* gene (Millar et al. 1995, Somers et al. 1998). Thus, this gene is also believed to encode a component of the central oscillator. Recently, the *TOC1* gene was cloned and characterized (Strayer et al. 2000). Surprisingly, the TOC1 protein is identical to the APRR1 pseudo-response regulator that we have been studying with special reference to the His-to-Asp phosphorelay, as mentioned above (Makino et al. 2000). Based on this newly emerging horizon, we here extensively characterized the APRR1/TOC1 family of pseudo-response regulators.

Materials and Methods

Arabidopsis and related materials

The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used. Plants were grown with 16 h light/8 h dark fluorescent illumination at 22°C on soil, or on agar plates containing MS salts and 2% sucrose, unless otherwise noted.

DNA sequencing

Sequencing of double-stranded DNA on plasmids was performed by using an automated DNA sequencer (Model 373A) (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions with the recommended sequencing kits.

PCR amplification

Using appropriate pairs of primers, polymerase chain reaction (PCR) was carried out to prepare DNA segments. An Arabidopsis cDNA library in λgt11 expression vector was a gift from Dr. K. Shinozaki (RIKEN, Japan). 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.

Preparation of RNA and hybridization

Total RNA was isolated from appropriate organs 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+ nylon membranes. The fixed membranes were hybridized with ^{32}P-labeled DNA fragments in 6× standard saline phosphate and EDTA (SSPE; 1× = 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-2000II) (FujiXerox, Tokyo, Japan).

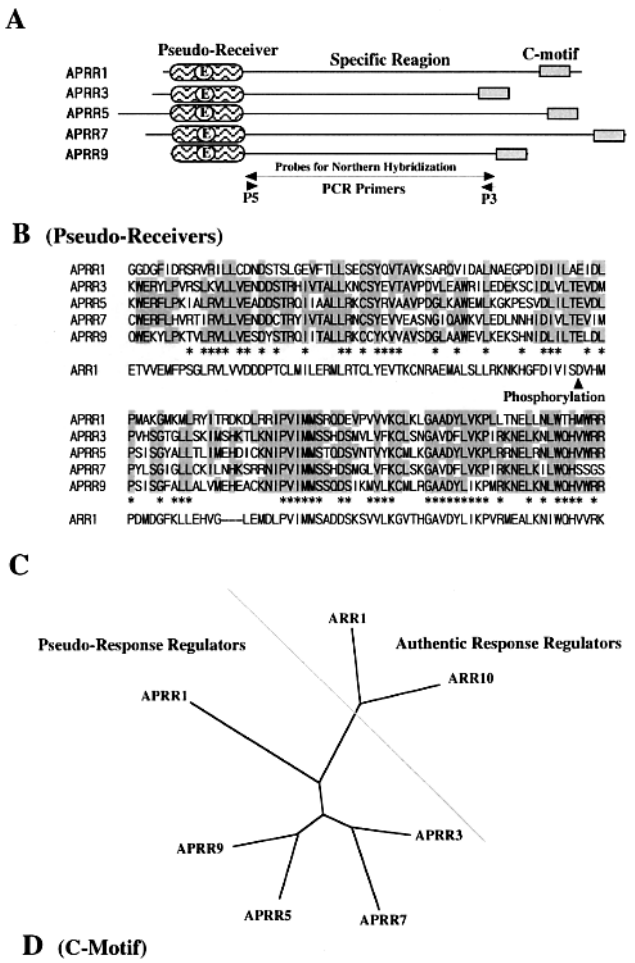

Fig. 1 Arabidopsis possesses at least five members belonging to the APRR1/TOC1 family of pseudo-response regulators. (A) Schematic representations of the structural designs of APRRs, which were predicted on the bases of inspection of the Arabidopsis genome sequences. The approximate positions of PCR primers, designed to amplify corresponding DNAs, are shown (see Fig. 2). Genomic DNAs were also amplified using these primer-sets, and they were used as each specific probe for northern hybridization analyses (see below). (B) The amino acid sequences of the pseudo-receiver domains of these APRR1 family members were aligned and compared with the authentic receiver sequence of ARR1. (C) A neighbor-joining tree indicating a phylogenetic relationship among these members of the APRR1 family and the authentic response regulators, ARR1 and ARR10, is shown. To construct this phylogenetic tree, the amino acid sequences that correspond to the pseudo-receiver (or receiver) domains were used. Branch lengths are proportional to sequence divergence (Jeanmougin et al. 1998). (D) The amino acid sequences of the C-motifs of these APRR1 family members were aligned and compared with that of CO.

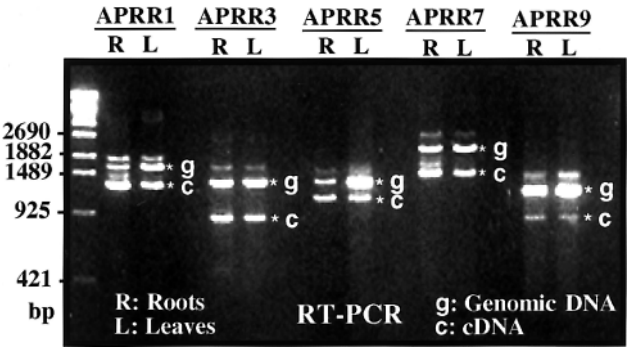

Fig. 2 Detection of cDNAs for the members of the APRR1/TOC1 family in Arabidopsis roots and leaves. RNA fractions both from roots and leaves were subjected to RT–PCR with the primer-sets, specifically designed for each member of the APRR1 family of pseudo-response regulators (see Fig. 1A). Resulting amplified DNAs were analyzed by agarose gel electrophoresis, followed by staining with ethidium bromide. Both cDNA and genomic DNAs were amplified, as indicated.

Results

Arabidopsis thaliana has a certain number of genes each encoding a pseudo-response regulator belonging to the APRR1/TOC1 family

As mentioned above, APRR1 has a pseudo-receiver domain at its N-terminal end and a characteristic C-motif at its C-terminus, as schematically illustrated in Fig. 1A. In this study we first searched Arabidopsis genome sequences for new genes, each of which was inferred to encode an as yet uncharacterized APRR1-like pseudo-response regulator. Such efforts allowed us to predict the occurrence of four more genes in the databases, each of which was assumed to encode a new APRR1-like pseudo-response regulator (Fig. 1A and Table 1). They were designated as APRR3, APRR5, APRR7, and APRR9, respectively (here we use odd numbers in order to distinguish these members from the other type of pseudo-response regulators, represented by APRR2; see Introduction). As judged from their predicted primary amino acid sequences, they all have a structural design very similar to that of APRR1 (Fig. 1).

Alignment of the amino acid sequences of their pseudo-receiver domains is shown in Fig. 1B, in a comparison with that of the authentic response regulator, ARR1. This alignment showed that the amino acid sequences of these pseudo-receiver domains are highly similar to each other, and more importantly they significantly resemble the receiver sequence of the ARR1 response regulator. However, it should be emphasized again that the former lacks the presumed phospho-accepting aspartate (D) site. Instead, each pseudo-receiver has a glutamate (E) residue at this particular position (see Fig. 3). In any case, to gain a statistical insight into the relationships among these APRRs and classical response regulators (ARRs), a phylogenetic tree was constructed by means of the neighbor-joining

Table 1 List of Arabidopsis pseudo-response regulators (APRRs)

Pseudo-response regulator	Size[a] (amino acids)	Chromosome location (GenBank accession nos)
APRR1	618	V (AB010073/AB041530/ATH251083)
APRR3	495	V (AB019231)
APRR5	667	V (AB025641)
APRR7	727	V (ATF9G14/AL162973)
APRR9	468	II (AC005310/T02680/AC005819)

[a] Note that each number of amino acids is solely the one predicted from the corresponding genomic sequence.

method (Fig. 1C). This tree clearly reveals very close relationships among APRRs and ARRs. However, it should be noted that the amino acid sequences of the long C-terminal extensions of APRRs, preceded by the common pseudo-receiver domains, appear to be highly different from each other, except for the conserved regions of C-motif (see Fig. 3).

Fig. 1D shows alignment of the amino acid sequences of their C-motifs, found commonly in all APRRs, in a comparison with that of the characteristic motif that is highly conserved within the CONSTANS (CO) family of plant-specific proteins (Robert et al. 1998). These sequences of ~50 amino acids are highly similar to each other, and are rich in positively charged amino acids (arginine and lysine). Note also that four aromatic amino acids (tyrosine and phenylalanine) are invariantly conserved at certain positions. From these results of extensive inspection (Fig. 1), we predict that *A. thaliana* possesses at least five members of the APRR1/TOC1 family of response regulators. The characteristics of these APRRs are summarized also in Table 1.

All the five APRR genes are transcribed

The above results are based on simple inspection of the Arabidopsis genome sequences, therefore, they do not necessarily indicate that these predicted APRR genes are indeed functional in the biological sense. To address this issue critically, we attempted to clone these corresponding cDNAs. To this end, a set of appropriately designed oligo-nucleotides was prepared for reverse transcriptase-based polymerase chain reactions (RT-PCR). Each pair of primers was designed so as to be highly specific to a certain APRR, as schematically indicated in Fig. 1A.

Total RNA fractions were prepared from both Arabidopsis roots and leaves (Columbia ecotype, 21 d grown). They were subjected to RT-PCR with the primer-sets specified above (Fig. 2). In every case, a major cDNA product was detected on agarose-gel electrophoresis for the RNA preparations from both roots and leaves. The size of each presumed cDNA was in very good agreement with that deduced from the inferred exon-intron arrangement in the corresponding genomic sequence (data not shown). Another major band corresponding to a larger size, found in each lane, was reasonably assumed to represent an amplified genomic DNA, probably due to contamination by genomic DNA in the RNA preparations. In any event, the corresponding cDNAs from the leaf preparation were cloned for APRR3, APRR5, APRR7, and APRR9, respectively. Their entire nucleotide sequences were then determined. Based on these experimentally confirmed cDNA sequences, the amino acid sequences were deduced, and then comparatively aligned (Fig. 3). The results clearly support the notion presented above, with regard to the characteristic natures of APRRs. However, it should be noted that we have not yet isolated each full-length cDNA; thus, the first methionine of each APRR is that inferred from the corresponding genomic sequence (this issue remains unresolved).

In short, we here conclude that *A. thaliana* has at least five genes each encoding a member of the APRR1/TOC1 family of pseudo-response regulators, and that they are transcribed in both roots and leaves.

All members of the APRR1/TOC1 family of pseudo-response regulators are subjected to a circadian rhythm at the level of transcription

The most critical question in this study is whether or not APRR3, APRR5, APRR7, and APRR9 are all subjected to a circadian rhythm at the level of transcription, as was demonstrated previously for APRR1 (Makino et al. 2000). Here it was demonstrated by extensive northern hybridization analyses that this is indeed the case, as demonstrated below (Fig. 4).

Each double-stranded DNA, amplified with the pairs of PCR primers specified above (Fig. 1), should give us a probe that is highly specific to a given APRR, as judged from their nucleotide sequence diversity (Fig. 3). These DNA probes should allow us to specifically detect each APRR transcript on standard northern hybridization analyses. Based on this rationale, the expression profile of each APRR gene was extensively characterized with special emphasis on circadian rhythms, as follows. Plants were grown for 20 d under conditions of 12 h light and 12 h dark. In one line of experiments, these photoperiod (or light/dark) conditions were maintained for the next 3 d, whereas in the other line of experiments the conditions were changed to constant-light, as schematically shown in Fig. 4.

Fig. 3 The amino acid sequences of the APRR1/TOC1 family of response regulators. The regions corresponding to the pseudo-receiver domains and the C-motifs are shaded and denoted, respectively. In the pseudo-receiver domains, the position of glutamate residue (E), which corresponds to the phospho-accepting aspartate residue in the authentic receiver, was indicated.

From these plants (leaves), RNA samples were prepared at 3 h intervals during the next 72 h. Northern hybridization analyses were performed on these RNA samples with each specific DNA probe for APRR3 and APRR9 (and UBQ10 as a loading reference). The results showed that the levels of both the APRR3 and APRR9 transcripts varied considerably in an oscillatory manner during the given 72 h period, under both the light/dark and constant-light photoperiod conditions (Fig. 4). These results were best interpreted by assuming that the expression of both APRR3 and APRR9 is indeed subjected to a circadian rhythm, as in the case of APRR1. Similar experiments, conducted with the probes specific to APRR5 and APRR7, respectively, gave the same results (Fig. 5, and data not shown). Perhaps more interestingly, the peak of the APRR3 transcript is at a quite different phase (or time) from that of the APRR9 transcript (~9 h difference). In other words, the level of the APRR3 transcript became its maximum upon the onset of evening, whereas that of the APRR9 transcript did so a few h after dawn, regardless of the objective and subjective photoperiod conditions. In conclusion, it was found that all the members of the APRR1/TOC1 family of pseudo-response regulators are subjected to a circadian rhythm at the level of transcription.

Sequential expression of the APRR1/TOC1 family of pseudo-response regulators

The above results prompted us to characterize in more detail the mRNA accumulation of the APRR1/TOC1 family of pseudo-response regulators, in terms of circadian rhythms. Plants were grown for 21 d under three different photoperiod conditions [16 h light/8 h dark corresponding to a long day (LD), 12 h light/12 h dark corresponding to an intermediate day (ID), 8 h light/16 h dark corresponding to a short day (SD)]. From these plants (leaves), total RNA samples were prepared at 3 h intervals during the next day (24 h, nine samples). All samples were analyzed by northern hybridization with probes specific for APRR1, APRR3, APRR5, APRR7, and APRR9, respectively. The results are shown in Fig. 5 (A for LD, and B for SD, the results from ID are not shown here, but see Fig. 4, 6). In every case, expression of the APRR1 family members rhythmically oscillated in the given 24 h period. Interestingly, the level of each mRNA became its maximum at a distinctive time. Specifically, the level of APRR9-mRNA became its maximum a few h after dawn, whereas that of APRR7-mRNA did so at ~6 h after dawn. Similarly, APRR5-mRNA accumulated to its maximum level at 8 h after dawn, APRR3 at 10 h after dawn, and finally APRR1 at 12 h after dawn. These events were explained by assuming that each mRNA started accumulating after dawn sequentially at 2–3 h intervals in the order of APRR9→APRR7→APRR5→APRR3→APRR1. As shown in Fig. 5B, these particular events were also seen for plants grown under the SD conditions. Regardless of the photoperiod conditions tested, the order of sequential expression and time intervals were little affected, if any.

It should be noted here that in the experiments shown in Fig. 5, each hybridized filter was prepared under appropriate conditions, slightly different from those for others, by exposure to a phosphoimage analyzer for an appropriate time, in order to obtain a clear picture. In this sense, the results did not

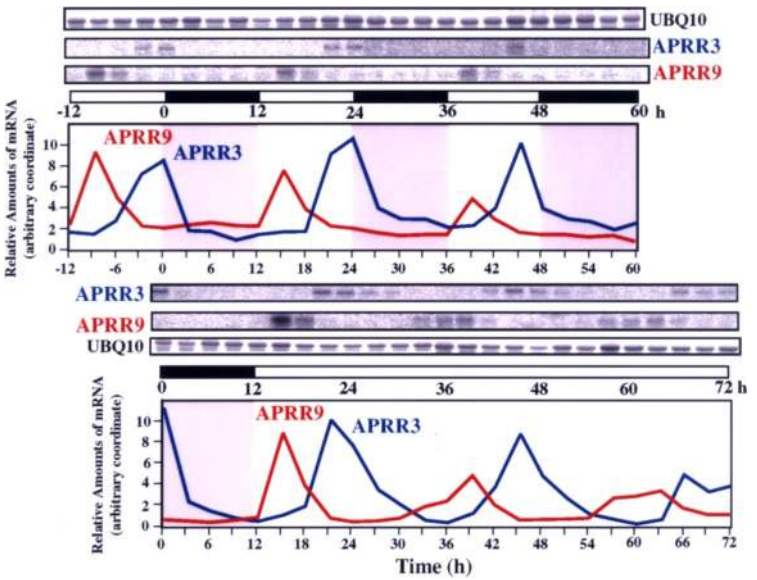

Fig. 4 Northern hybridization analyses for the expression of APRRs in the plants grown under the condition of constant light. Northern hybridization analyses were carried out, with each specific probe denoted (see Fig. 1A) (Taniguchi et al. 1998), to examine the fluctuation of expression of the APRR1/TOC1 family of pseudo-response regulators. RNA samples were prepared from plants (leaves) grown under the photo-conditions given in the text (also as shown schematically in these figures) (Makino et al. 2000). In order to quantify each transcript, the ubiquitin (UBQ10) transcript was used as an internal and loading reference.

give us any idea about the level of expression of a given APRR, relative to those of others. Then, the RNA fraction containing the largest amount of each corresponding mRNA was selected for each APRR (Fig. 5), and the fractions were analyzed simultaneously under essentially the same hybridization and exposure conditions (Fig. 6). Based on the intensities of the resulting radioactive bands, and the nucleotide lengths of randomly radiolabeled probes, the relative level of expression of each APRR was estimated. This gave us a rough idea that these APRR-mRNAs accumulate in plant leaves to a more or less similar order of magnitude, relative to each other.

Circadian waves of the APRR1/TOC1 family of pseudo-response regulators

To gain further insight into the circadian-related events, the experimental data were schematically treated by taking the level of UBQ10-mRNA as an internal and loading reference (Fig. 7A for LD, 7B for ID, and 7C for SD). The results of these analyses collectively supported the following views (for raw data, see Fig. 5). (i) Each mRNA of APRRs starts accumulating after dawn sequentially at approximately 2 h intervals in the order APRR9→APRR7→APRR5→APRR3→APRR1. (ii) First of all, the wave of APRR9-mRNA is boosted always immediately after dawn, irrespective of the entrained photoperiod conditions. (iii) The order and intervals of such sequential expression are little affected by the photoperiod conditions, if any. An intriguing result of these events is as follows. For example, APRR3-mRNA reaches its maximum level around af-

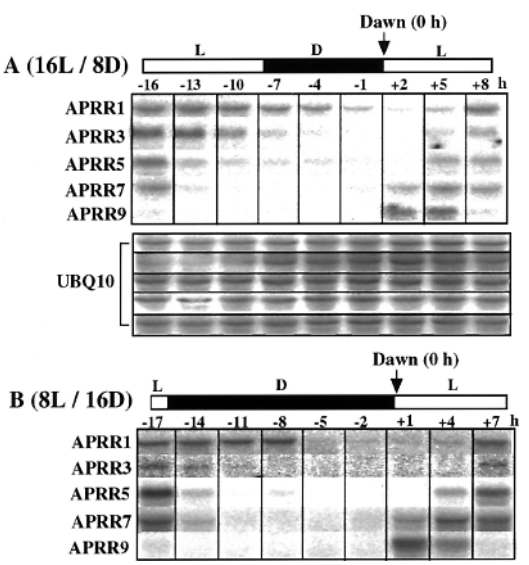

Fig. 5 Northern hybridization analyses for the APRRs in the plants grown under different photoperiod conditions. Northern hybridization analyses were carried out, with each specific probe denoted (see Fig. 1A), to examine the fluctuation of expression of the APRR1/TOC1 family of pseudo-response regulators. RNA samples were prepared from plants (leaves), grown under the two different photo-conditions given in the text, namely LD (A) and SD (B) (also as shown schematically in these figures). As an internal and loading reference, the ubiquitin (UBQ10) transcript was also detected in the washed filter (the data for SD was not shown for clarity, because the profile was essentially the same as that for LD).

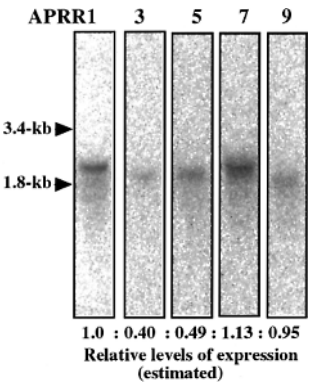

Fig. 6 Quantitative and comparative northern hybridization analyses for APRRs. Northern hybridization analyses were carried out, with each specific probe denoted (see Fig. 1A), to examine the maximum levels of expression of the APRR1/TOC1 family of pseudo-response regulators. RNA samples used were the same as those used in Fig. 5 (LD conditions: −10 h sample for APRR1, −13 h for APRR3, −16 h for APRR5, +8 h for APRR7, +5 h for APRR9). These were analyzed simultaneously under essentially the same hybridization and exposure conditions. The relative level of expression of each APRR was roughly estimated, relative to each other.

ternoon under the LD conditions, a few h before evening under the ID conditions, and around sunset under the SD conditions. (iv) Except for that of APRR1, the shape of each peak is apparently sharp, and does not change significantly, regardless of the photoperiod conditions. (v) In this respect, APRR1-mRNA is somewhat unique in that its expression (or accumulation) is prolonged for a while longer, as compared with in the case of others, as can be seen in Fig. 7. In other words, the peak of APRR1 does not drop sharply, rather it has an evident shoulder, regardless of the photoperiod conditions tested. As a result, although both APRR3-mRNA and APRR1-mRNA seem to start accumulating concomitantly, APRR1 reaches its plateau a few h later than APRR3.

In short, the expression of the APRR1/TOC1 family of pseudo-response regulators is subjected to a circadian rhythm in an oscillatory and sequential manner. To express concisely these particular events, which can be seen in Fig. 5, 7, hereafter we would like to introduce the term, 'circadian waves of the APRR1/TOC1 family members'.

Expression of the APRR1/TOC1 family of genes and light responses

Finally, a critical question arose as to whether or not the expression of the APRR1 family of genes is regulated by light. To test this, Arabidopsis seedlings were grown in the dark for 6 d, and then these etiolated plants were transferred to continuous white light. RNA samples were prepared from the whole plants at appropriate intervals, and then they were analyzed by northern hybridization with each specific probe (Fig. 8A, B). In the dark-grown etiolated plants, certain levels of mRNAs were detected for APRR1, APRR3, APRR5, and APRR7. In the case of APRR9, however, its expression was very low in the dark-grown plants, but upon the light treatment, it was induced very rapidly and transiently. The responses of other APRRs to such a light treatment appear to be very dull, at least where our experimental conditions are concerned. In any event, clarification of the relevant issues with regard to these light responses of APRRs must await more close experiments, which should include such analyses of red, far red, and blue light responses. However, one can at least conclude that, amongst APRRs, the expression of APRR9 is very sensitive to a light treatment of plants. This is consistent with the fact that the circadian wave of APRR9 is first boosted always after dawn (Fig. 7).

Discussion

Circadian rhythms are an old issue of plant physiology (Garner and Allard 1920), and a newly emerging paradigm of plant molecular biology, as one can learn from a number of recent review articles dealing with plant circadian rhythms (Anderson and Kay 1996, Kreps and Kay 1997, Thomas and Vince-Prue 1997, Koornneef et al. 1998, Piechulla 1999, Somers 1999, Staiger and Heintzen 1999, Murtas and Millar 2000, Samach and Coupland 2000, and references therein). According to current and general models, plant circadian clocks regulate many biological rhythms such as those corresponding photosynthetic activity, leaf movement, stomatal aperture, and flowering time. Such biological clocks presumably consist of input pathways, a central oscillator, and output pathways. The central oscillator is thought to generate a rhythm through a transcription-translation negative feedback loop. In this respect, the results as to plant molecular biology have begun to shed light on the molecular bases of circadian rhythms in higher plants, as mentioned above. To make a complicated story simple, we will focus here on the Arabidopsis *TOC1* gene, the product of which was previously proposed to be a component of the presumed central oscillator (Millar et al. 1995, Somers et al. 1998), keeping the fact that TOC1 is identical to APRR1 in mind (Strayer et al. 2000).

The *TOC1* gene is thought to play an important role in circadian regulation (Millar et al. 1995, Somers et al. 1998). This assumption is based on the fact that the semi-dominant *toc1-1* mutant has shortened periods of several rhythmic markers including the *CAB* gene, and also of the leaf movement and stomatal conductance rhythms. Furthermore, in some strains of Arabidopsis the *toc1-1* mutation results in early flowering. Since this single *toc1-1* mutation interferes with a wide range of clock-controlled output processes, TOC1 most likely functions upstream or within the oscillator itself. It was also shown that both light and temperature entrainment appears to be normal in *toc1-1*. Thus, TOC1 is believed to be a part of a central oscillator. Nevertheless, little is known about the molecular function of TOC1, and how does TOC1 play a role for the pre-

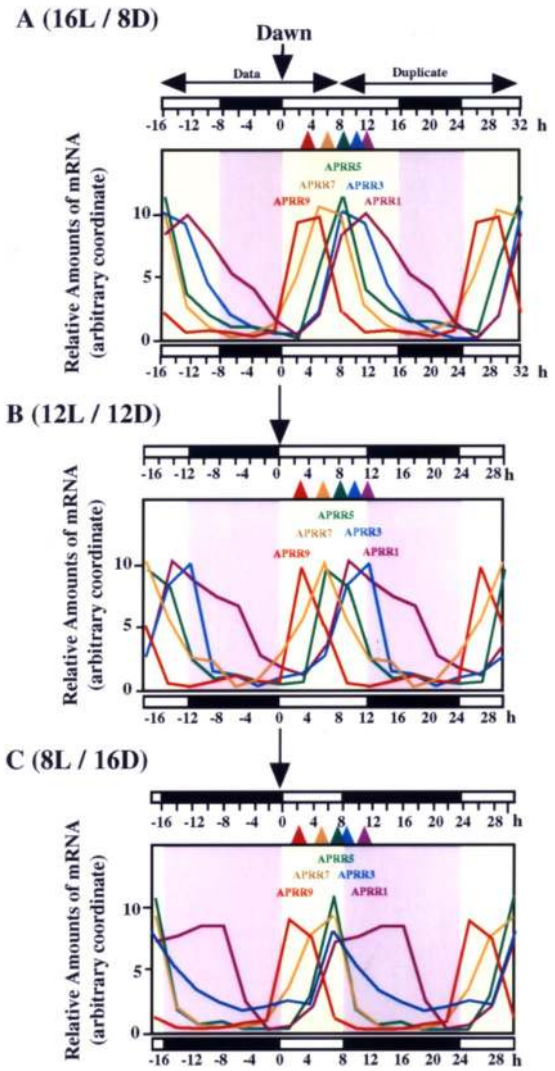

Fig. 7 Schematic representations of circadian waves that is exhibited by the five members of the APRR1/TOC1 family of pseudo-response regulators. To gain insight into the circadian rhythms, exhibited by the five members of the APRR1/TOC1 family, the experimental data (Fig. 4, 5) of northern hybridization were quantified with each UBQ10 band as an internal and loading reference. The raw data are shown in Fig. 4 (ID conditions) and Fig. 5 (both LD and SD conditions). To gain further insight into the observed events, each profile was duplicated, based on the reasonable assumption that each fluctuation profile should be repeated during the following 24 h period, as indeed demonstrated in Fig. 4 as an example. To clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, approximately and arbitrarily. With regard to this issue, see Fig. 6. In these figures, each potion of peaks of APRRs was roughly estimated and indicated by the arrowheads (thus, note that these positions do not correspond to the seeming peaks). Note also that we intended to align these three panels by taking the time of light-on (or dawn) as 0 h.

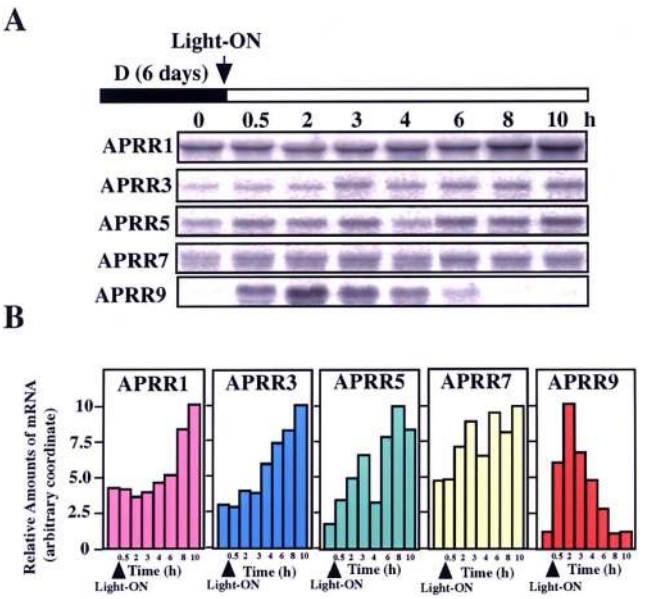

Fig. 8 Northern hybridization analyses for the APRRs in the etiolated plants treated white light. Plants were grown under dark conditions for 6 d, and then, the etiolated seedling was transferred to conditions of white light. RNA samples were prepared at intervals, as indicated. With these RNA samples, northern hybridization analyses were carried out, with each specific probe denoted (see Fig. 1A), to examine the light response expression of the APRR1/TOC1 family of pseudo-response regulators. Both the autoradiogram (A) and quantified data (B) are shown for each indicated APRR. In (B), to clarify the profiles, the maximum level (or amplitude) of each transcript was taken as 10, arbitrarily. With regard to this issue, see Fig. 6.

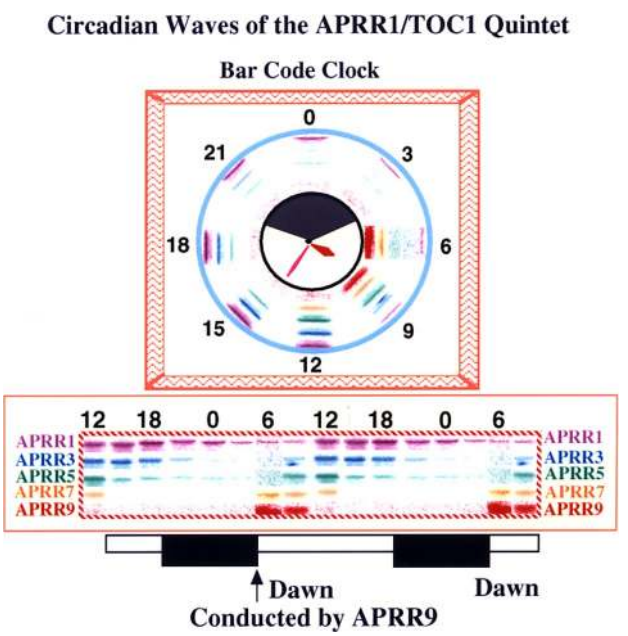

Fig. 9 A view of the hypothetical bar code clock that is based on the circadian waves of the APRR1/TOC family members. This picture made by a computer is solely intended to explain the conceptual view as to the circadian waves of the APRR1/TOC1 family members, and the hypothetical bar code clock. The raw data of Fig. 5 (the LD conditions) were used. Other details are given in the text.

sumed clock. In this respect, the results of this study provided new clues for understanding the molecular functions of the APRR1/TOC1 pseudo-response regulators.

One of the intriguing findings in this study is that APRR1/TOC1 most likely acts together with four other players belonging to the APRR1/TOC1 family of pseudo-response regulators. Another important finding is that these five APRRs together all exhibit rhythmic, sequential, and ordered expression profiles at the level of transcription, which is termed 'circadian waves'. This quintet of circadian waves seems to be independent of the entrained photoperiod conditions. However, the timing of the boost of the waves appears to be light-controlled. In this event, the conductor of the quintet appears to be APRR9, the expression of which is not only regulated rhythmically, but also induced by white light. In other words, APRR9 most likely serves as a conductor to maintain the punctual and robust circadian waves. These findings are intriguing from the viewpoint of circadian rhythms, because the circadian waves, exhibited by the APRR1/TOC1 family members, may be the crucial basis of the molecular mechanisms underlying biological oscillations in Arabidopsis. However, it should be noted that in this study we are mainly concerned about the expression profiles of APRRs at the level of transcription, but little about the accumulation profiles of these translation products in plants. It should be also noted that there might be as yet unidentified members that would join the circadian waves. To verify the intriguing views mentioned above, these crucial issues must be addressed next. It would be also of interest to examine the profiles of circadian waves in the *toc1-1* mutant.

The APRR1/TOC1 family of proteins is also intriguing from other viewpoints. They all have a pseudo-receiver domain that might be involved in His-to-Asp phosphorelay signal transduction, as pointed out previously (Makino et al. 2000). They have a common signature motif, named the C-motif, which is shared by the well-known CO family of proteins, which are believed to play an important role, together with GIGANTEA, in circadian-controlled flowering (Putterill et al. 1995, Kobayashi et al. 1999, Park et al. 1999). There might be a functional linkage between APRRs and CO through their common structural motifs. In this context, we previously showed that this C-motif in APRR1 is responsible for its nuclear-localization (Makino et al. 2000). Therefore, it is reasonably assumed that the quintet of APRR1/TOC1 family members act in concert in the nuclei. In this respect, it remains to be determined if APRRs serve as DNA-binding transcription factors, because it is generally believed that the circadian rhythm of gene regulation per se is a part of the underlying mechanisms of many, if not all, of the resulting biological rhythms. In fact, two other genes, *CCA1* and *LHY*, were recently identified as potential clock genes, both of which encode MYB-related DNA-binding transcription factors (Wang et al. 1997, Wang and Tobin 1998, Schaffer et al. 1998). It is also of interest to determine whether or not there is a genetic and/or biochemical interaction between APRRs and CCA1/LHY, both of which are potential clock components. In particular, it may be noted that the expression of CCA1 is induced rapidly by a light treatment of etiolated seedlings (Wang et al. 1997), and the kinetics of such a light-induction of CCA1 is very similar to that observed here for APRR9 (Fig. 8). It was recently demonstrated that the PHYTOCHROME INTERACTING FACTOR 3 (PIF3) protein is at least partly responsible for this light-responsive induction of CCA1, through binding directly to the CCA1 promoter (Martinez-Garcia et al. 2000). PIF3 is presumably a MYC-related basic helix–loop–helix (bHLH) DNA-binding protein, which can recognize a G-box-like sequence, CACGTG. Interestingly, an inspection revealed that two CACGTG sequences are found in the presumed promoter region of APRR9 (−551 to −506, and −439 to −434, the first nucleotide of the inferred ATG codon of APRR9 being taken as +1), as in the case of the CCA1 promoter (−607 to −602). Taken these together, it is tempting to speculate that PIF3 might be involved in the light-responsive induction of APRR9, which might result in a coordinate regulation of both APRR9 and CCA1. Finally, it would be worth mentioning that it was recently suggested that APRR1 has the ability to interact with the gene product of *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), which encodes a transcription factor crucial for late seed development (Kurup et al. 2000, Rohde et al. 2000). So far, we have no idea about this particular issue.

In summary, our findings in this study collectively led us to propose a priori that the circadian waves of the APRR1/TOC1 quintet is the crucial basis of the molecular mechanisms underlying biological oscillations in *A. thaliana*. If this is indeed the case, then one can envisage attractive mechanisms, by which the APRR1/TOC1 family of pseudo-response regulators can measure and tell the time. For example, the circadian waves of APRRs may provide parameters (or dials) for measurement of time, as schematically illustrated in Fig. 9. As can be seen, a given time can be defined by a combinatorial profile of the levels of APRR-mRNAs (i.e. the combinatorial intensities of five hybridized bands). Each distinctive profile at each given time looks like 'a bar code' (see each vertical column in Fig. 5, 9). Taking the external and/or internal coincidence models into consideration (for a review, see Thomas and Vince-Prue 1997), one can easily envisage several molecular mechanisms, by which 'the bar code clock that is based on the circadian waves' or 'the APRR1/TOC1 quintet that is conducted by the light-regulated APRR9′ can somehow tell the time (Fig. 9). At this particular time, nevertheless, it is too early and premature to discuss such hypothetical views in details. However, our findings in this study through reverse-genomics, together with those from forward-genetics of Kay and collaborators, should provide us with clues for understanding the molecular mechanism underlying the circadian clock in higher plants.

Acknowledgements

This study was supported by Grants-in-Aid (09274101 and 09274102 to T.M.) for scientific research on a priority area from the Ministry of Education, Science, Sports and Culture of Japan.

References

Anderson, S.L. and Kay, S.A. (1996) Illuminating the mechanism of the circadian clock in plants. *Trends Plant Sci.* 1: 51–57.

Appleby, J.L., Parkinson, J.S. and Bourret, R.B. (1996) Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. *Cell* 86: 845–848.

Brandstatter, I. and Kieber, J.J. (1998) Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in *Arabidopsis*. *Plant Cell* 10: 1009–1019.

Chang, C., Kwok, S.F., Bleeker, A.B. and Meyerowitz, E.M. (1993) *Arabidopsis* ethylene-response gene ETR1: similarity of product to two-component regulators. *Science* 262: 539–544.

Chang, C. and Stewart, R.C. (1998) The two-component system: regulation of diverse signaling pathways in prokaryotes and eukaryotes. *Plant Physiol.* 117: 723–731.

D'Agostino, I.B. and Kieber, J.J. (1999) Phosphorelay signal transduction: the emerging family of plant response regulators. *Trends Biol. Sci.* 24: 452–456.

Dunlap, J.C. (1999) Molecular bases for circadian clocks. *Cell* 96: 271–290.

Garner, W.W. and Allard, H.A. (1920) Effects of the relative length of night and day and other factors of the environment on growth and reproduction in plants. *J. Agric. Res.* 18: 553–606.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the *Arabidopsis* ABI3 gene by positional cloning. *Plant Cell* 4: 1251–1261.

Hicks, K.A., Millar, A.J., Carre, I.A., Somers, D.E., Straume, M., Meeks-Wagner, D.R. and Kay, S.A. (1996) Conditional circadian dysfunction of the Arabimutant. *Science* 274: 790–792.

Hua, J., Chang, C., Sun, Q. and Meyerowitz, E.M. (1995) Ethylene insensitivity conferred by *Arabidopsis ERS* gene. *Science* 269: 1712–1714.

Hua, J. and Meyerowitz, E.M. (1998) Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* 94: 261–271.

Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Kiba, T., Ueguchi, C., Sugiyama, T. and Mizuno, T. (1999) Compilation and characterization of *Arabidopsis* response regulators implicated in His-Asp phosphorelay signal transduction. *Plant Cell Physiol.* 40: 733–742.

Imamura, A., Hanaki, N., Umeda, H., Nakamura, A., Suzuki, T., Ueguchi, C. and Mizuno, T. (1998) Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 95: 2691–2696.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G. and Gibson, T.J. (1998) Multiple sequence alignment with Clustal X. *Trends Biol. Sci.* 23: 403–405.

Jin, H. and Martin, C. (1999) Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol. Biol.* 41: 577–585.

Kakimoto, T. (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274: 982–985.

Kiba, T., Taniguchi, M., Imamura, A., Ueguchi, C., Mizuno, T. and Sugiyama, T. (1999) Differential expression of genes for response regulators in response to cytokinins and nitrate in *Arabidopsis thaliana*. *Plant Cell Physiol.* 40: 767–771.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999) A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286: 1960–1962.

Koornneef, M., Alonso-Blanco, C., Peeters, A.J.M. and Soppe, W. (1998) Genetic control of flowering time in Arabidopsis. *Annu. Rev. Plant Physiol.* 49: 345–370.

Kreps, J.A. and Kay, S.A. (1997) Coordination of plant metabloism and development by the circadian clock. *Plant Cell* 9: 1235–1244.

Kurup, S., Jones, H.D. and Holdsworth, M.J. (2000) Interactions of the developmental regulator ABI3 with proteins identified from developing *Arabidopsis* seeds. *Plant J.* 21: 143–155.

Makino, S., Kiba, T., Imamura, A., Hanaki, N., Nakamura, A., Suzuki, T., Taniguchi, M., Ueguchi, C., Sugiyama, T. and Mizuno, T. (2000) Genes encoding pseudo-response regulators: Insight into His-to-Asp phosphorelay and circadian rhythm in *Arabidopsis thaliana*. *Plant Cell Physiol.* 41: 791–803.

Martinez-Garcia, J.F., Huq, E. and Quail, P.H. (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science* 288: 859–863.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.H. and Kay, S.A. (1995) Circadian clock mutatns in *Arabidopsis* identified by Luciferase imaging. *Science* 267: 1161–1163.

Miyata, S.-I., Urao, T., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) Characterization of genes for two-component phosphorelay mediators with a single HPt domain in *Arabidopsis thaliana*. *FEBS Lett.* 437: 11–14.

Mizuno, T. (1997) Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res.* 4: 161–168.

Mizuno, T. (1998) His-Asp phosphotransfer signal transduction. *J. Biochem.* 123: 555–563.

Murtas, G. and Millar, A.J. (2000) How plants tell the time. *Curr. Opin. Plant Biol.* 3: 43–46.

Nakamura, A., Kakimoto, T., Imamura, A., Suzuki, T., Ueguchi, C. and Mizuno, T. (1999) Biochemical characterization of a putative cytokinin-responssive His-kinase, CKI1, from *Arabidopsis thaliana*. *Biosci. Biotechnol. Biochem.* 63: 1627–1630.

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim H.K., Soh, M.S, Kim H.J, Kay, S.A. and Nam, H.G. (1999) Control of circadian rhythms and photoperiodic flowering by the Arabidopsis *GIGANTEA* gene. *Science* 285: 1579–1582.

Parkinson, J.S. and Kofoid, E.C. (1992) Communication modules in bacterial signalling proteins. *Annu. Rev. Genet.* 26: 71–112.

Piechulla, B. (1999) Circadian expression of the light-harvesting complex protein genes in plants. *Chronobiol. Int.* 16: 115–128.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995) The *CONSTANS* gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell* 80: 847–857.

Robert, L.S., Robsons, F., Sharpe, A., Lydiate, D. and Coupland, G. (1998) Conserved structure and function of the *Arabidopsis* flowering time gene *CONSTANS* in *Brassica napus*. *Plant Mol. Biol.* 37: 763–772.

Rohde, A., Rycke, R.D., Beeckman, T., Engler, G., Van Montagu, M. and Boerijan, W. (2000) ABI3 affects plastid differentiation in dark-grown *Arabidopsis* seedlings. *Plant Cell* 12: 35–52.

Sakai, H., Aoyama, T., Bono, H. and Oka, A. (1998b) Two-component response regulators from *Arabidopsis thaliana* contain a putative DNA-binding motif. *Plant Cell Physiol.* 39: 1232–1239.

Sakai, H., Hua, J., Chen, Q.G., Medrano, L.J., Bleecker, A.B. and Meyerowitz, E.M. (1998a) *ETR2* is an *ETR1*-like gene involved in ethylene signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 95: 5812–5817.

Samach, A. and Coupland, G. (2000) Time measurement and the control of flowering in plants. *Bioessays* 22: 38–47.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carre, I.A. and Coupland, G. (1998) The late elongated hypocotyle mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93: 1219–1229.

Somers, D.E. (1999) The physiology and molecular bases of the plant circadian clock. *Plant Physiol.* 121: 9–20.

Somers, D.E., Webb, A.A.R., Pearson, M. and Kay, S.A. (1998) The short-period mutant, *toc1-1*, alters circadian clock regulation of multiple outputs throughout development in *Arabidopsis thaliana*. *Development* 125: 485–494.

Staiger, D. and Heintzen, C. (1999) The circadian system of *Arabidopsis thaliana*: forward and reverse genetic approaches. *Chronobiol. Int.* 16: 1–16.

Strayer, C., Oyama, T., Schultz, T.F., Raman, R., Somers, D.E., Mas, P., Panda, S., Kreps, J.A. and Kay, S.A. (2000) Cloning of the *Arabidopsis* clock gene *TOC1*, an autoregulatory response regulator homolog. *Science* 289: 768–771.

Suzuki, T., Imamura, A., Ueguchi, C. and Mizuno, T. (1998) Histidine-containing phosphotransfer (HPt) signal transducers implicated in His-to-Asp phosphorelay in *Arabidopsis*. *Plant Cell Physiol.* 39: 1258–1268.

Taniguchi, M., Kiba, T., Sakakibara, H., Ueguchi, C., Mizuno, T. and Sugiyama, T. (1998) Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate. *FEBS Lett.* 429: 259–262.

Thomas, B. and Vince-Prue, D. (1997) *Photoperiodism in Plants.* Academic

Press, San Diego, U.S.A.

Urao, T., Yakubov, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell* 11: 1743–1754.

Urao, T., Yakubov, B., Yamaguchi-Shinozaki, K. and Schinozaki, K. (1998) Stress-responsive expression of genes for two-component response regulator-like proteins in *Arabidopsis thaliana*. *FEBS Lett.* 427: 175–178.

Wang, Z.-Y., Kenigsbuch, D., Sun, L., Harel, E., Ong, M.S. and Tobin, E.M. (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an *Arabidopsis Lhcb* gene. *Plant Cell* 9: 491–507.

Wang, Z.-Y. and Tobin, E.M. (1998) Constitutive expression of the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) gene disrupts circadian rhythms and suppresses its own expression. *Cell* 93: 1207–1217.

Wurgler-Murphy, S.M. and Saito, H. (1997) Two-component signal transducers and MAPK cascades. *Trends Biol. Sci.* 22: 172–176.

Yamada, H., Hanaki, N., Imamura, A., Ueguchi, C. and Mizuno, T. (1998) An *Arabidopsis* protein that interacts with the cytokinin-inducible response regulator, ARR4, implicated in the His-Asp phosphorely signal transduction. *FEBS Lett.* 436: 76–80.

Zagotta, M.T., Hicks, K.A., Jacobs, C.I., Yound, J.C., Hangarter, R.P. and Meeks-Wagner, D.R. (1996) The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *Plant J.* 10: 691–702.

(Received July 27, 2000; Accepted August 7, 2000)