Plant Cell Physiol. 41(6): 791–803 (2000)

Genes Encoding Pseudo-Response Regulators: Insight into His-to-Asp Phosphorelay and Circadian Rhythm in *Arabidopsis thaliana*

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In the higher plant, *Arabidopsis thaliana*, results from recent intensive studies suggested that His-to-Asp phosphorelay mechanisms are involved presumably in propagation of environmental stimuli, such as phytohormones (e.g. ethylene and cytokinin). Here we identified and characterized a set of novel Arabidopsis genes whose products considerably resemble the authentic response regulators (ARR-series) of Arabidopsis in the sense that they have a phospho-accepting receiver-like domain. However, they should be discriminated from the classical ones in the strict sense that they lack the invariant phospho-accepting aspartate site. They were thus named APRRs (*A*rabidopsis *p*seudo-*r*esponse *r*egulators). Two such representatives, APRR1 and APRR2, were characterized extensively through cloning of the corresponding cDNAs, in terms of their structural designs, biochemical properties, subcellular localization in plant cells, and expression profiles at the transcriptional level. The result of in vitro phosphorylation experiment with the Arabidopsis AHP phosphotransmitter suggested that the pseudo-receivers have no ability to undergo phosphorylation. The result of transient expression assay with onion epidermal cells showed that the GFP-APRR1 fusion protein has an ability to enter into the nuclei. The C-terminal domain of APRR1, termed CONSTANS-motif, appears to be responsible for the nuclear-localization. The most intriguing result was that the accumulation of APRR1 transcript is subjected to a circadian rhythm. The APRR1 protein is identical to the one that was recently suggested to interact with the ABI3 (ABISCISIC ACID INSENSITIVE3) protein. These are discussed with special reference to the His-to-Asp phosphorelay signal transduction and circadian rhythm in *Arabidopsis thaliana*.

Key words: *Arabidopsis thaliana* — Circadian rhythm — His-kinases — His-to-Asp phosphorelay — Response regulators.

Abbreviations: APRR, Arabidopsis pseudo-response regulator; ARR, Arabidopsis response regulator; AHP, Arabidopsis histidine-containing phosphotransmitter; GFP, green fluorescent protein; PCR, polymerase chain reaction.

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Common prokaryotic signal transduction mechanisms are generally referred to as "histidine-to-aspartate (His-to-Asp) phosphorelay systems" (or "two-component regulatory systems") (Appleby et al. 1996, and references therein). Such a His-to-Asp phosphorelay involves two or more of common signal transducers, a sensor exhibiting histidine (His)-kinase activity, a response regulator containing a phospho-accepting receiver domain, and a histidine-containing (HPt) phosphotransmitter (Parkinson and Kofoid 1992, Mizuno 1998, and references therein). To date, numerous instances of His-to-Asp phosphorelay systems, involved in a wide variety of adaptive responses to environmental stimuli, have been reported for not only prokaryotic species, but also eukaryotic species (Mizuno 1997, Wurgler-Murphy and Saito 1997, Chang and Stewart 1998).

The first discovery of the Arabidopsis ethylene receptors immediately implied that such His-to-Asp phosphorelay mechanisms most likely operate in this higher plant, because the structural designs of their primary amino acid sequences are very similar to those of classical His-kinases (Chang et al. 1993, Hua et al. 1995, Gramble et al. 1998, Hua and Meyerowitz 1998, Sakai et al. 1998a) Furthermore, it was recently demonstrated that *Arabidopsis thaliana* possesses a number of response regulators (ARR-series), each of which contains a typical phospho-accepting receiver domain (Brandstatter and Kieber 1998, Imamura et al. 1998, Sakai et al. 1998b, Urao et al. 1998). A set of HPt phosphotransmitters was also uncovered (AHP-series), each of which has an ability to interact with a certain set of response regulators through a His-to-Asp phosphotransfer reaction (Miyata et al. 1998, Suzuki et al. 1998). In sum, our inspection of the Arabidopsis databases revealed that this plant has, at least, 11 sensor His-kinases. Five (ETR1, ETR2, ERS1, ERS2, and EIN4) have been demonstrated to be ethylene receptors, two (CKI1 and CKI2) were assumed to be involved in a cytokinin responsiveness (Kakimoto 1996), and one (ATHK1) was proposed to be a putative osmosensor (Urao et al. 1999). The other three putative His-kinases (AHK2 to AHK4) were predicted in the Arabidopsis genome sequence databases, and the existence of each corresponding cDNA was confirmed by

cloning and sequencing (Suzuki, Ueguchi and Mizuno, unpublished data). This plant has, at least, 5 HPt phosphotransmitters, and the amino acid sequences of these are very similar to each other (Suzuki et al. 1998). And also, this plant has, at least, 14 members of the family of response regulators that can be classified into two distinct subtypes (type-A and type-B), as judged from their structural designs and expression profiles (Imamura et al. 1999, D'Agostino and Kieber 1999). The type-A family of response regulators is induced by cytokinin-treatment of plants at the transcriptional level (Brandstatter and Kieber 1998, Taniguchi et al. 1998, Kiba et al. 1999). Collectively, we now know much about the presumed members of His-to-Asp phosphorelay signal transducers of Arabidopsis. From the biological viewpoint, nevertheless, clarification of the Arabidopsis His-to-Asp phosphorelay mechanism is at a very early stage. In particular, biological links between these 11 His-kinases, 5 HPt phosphotransmitters, and 14 response regulators are entirely elusive.

We have been studying such His-to-Asp phosphorelay systems in *Arabidopsis thaliana*, and a number of Arabidopsis genes each encoding either a His-kinase, an HPt phosphotransmitter, or a response regulator, have been isolated and characterized, as mentioned above (Imamura et al. 1998, Suzuki et al. 1998, Taniguchi et al. 1998, Yamada et al. 1998, Imamaura et al. 1999, Kiba et al. 1999, Nakamura et al. 1999). However, nothing is so far special in the sense that they all are very similar to bacterial ones, as far as their fundamental structural designs and His-to-Asp phosphorelay reactions are concerned. During the course of such studies, however, we found that *Arabidopsis thaliana* has another set of genes whose predicted products significantly resemble the authentic response regulators (ARR-series) in their amino acid sequences, at a first glance. Yet, they differ from the typical ones in a strict sense, as will be discussed. Here we characterized these intriguing genes extensively in the hope of finding new facets with special reference to the Arabidopsis His-to-Asp phosphorelay signal transduction mechanism.

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. To examine the cytokinin-responsiveness of APRR-transcripts in plants, N-starved plants were first grown on 0.4% Gelrite plates for 2 weeks in a growth chamber at 22°C under fluorescence light at an intensity of approximately 120 μE m^{-2} s^{-1}. The photoperiod was 16 h-light/8 h-dark. The Gelrite plates contained a half concentration of MS salts and 2% sucrose. The source of N-nutrient and its concentration was 4.5 mM potassium nitrate. The plants grown under these conditions contained only 2% of nitrate in leaves compared to the plants grown under nitrate-sufficient conditions. These plants were used for assaying the level of APRR-transcripts, after cytokinin treatment, as described previously (Taniguchi et al. 1998).

Escherichia coli and related materials—*E. coli* K-12 strain DZ225 (F^-, *ΔenvZ, lacU169, araD139, rpsL, relA, flgB, thiA*), carrying the plasmid-born multicopy *arcB* gene, was used to prepare the cytoplasmic membranes (Nagasawa et al. 1993). Cells were grown in Luria-broth, and then, the urea-treated cytoplasmic membrane was purified, as described previously (Tokishita et al. 1990). This cytoplasmic membrane was used to phosphorylate the Arabidopsis AHP phosphotransmitter proteins in vitro. *E. coli* strain BL21(DE3) was also used as a host for overexpression of Arabidopsis APRR1, APRR2, ARR10, and AHP2 polypeptides (Novagen, Madison, WI, U.S.A.).

DNA sequencing—Sequencing of double-stranded DNA on plasmids was performed by using an automated DNA sequencer (Model 373A) (Applied Biosystems, Foster City, CA., U.S.A.), according to the manufacture'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.

Plasmid construction—For overproduction of the APRR1 and APRR2 polypeptides, plasmids pET-APRR1-R and pET-APRR2-RB were constructed in this study, as follows: the APRR1-R and APRR2-RB coding sequences were amplified from the corresponding cDNA clones by using appropriate pairs of PCR primers. These amplified DNA segments were cloned onto an *E. coli* expression vector, pET22b(+) (Novagen, Madison, WI, U.S.A.), so as to be placed under the T7 phage promoter. Each coding sequence thus cloned on the expression vector has an initial methionine codon from the vector and a C-terminally extended histidine-tag.

Purification of polypeptides with Ni-Column—*E. coli* BL21(DE3) cells carrying appropriate plasmids, described above, were grown in M9-glucose medium containing 0.2% casamino acids and 15% sucrose. A cleared cell lysate was obtained by use of an Aminco French pressure cell (FA#073), followed by centrifugation (100,000 × *g* × 3 h) (Tokishita et al. 1990). These soluble protein samples were applied onto a Ni-column with the rapid affinity purification pET His-Tag^R system, (Novagen, Madison, WI, U.S.A.). Other details were those recommended by the supplier.

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 (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-2000II) (FujiXerox, Tokyo, Japan).

In vitro phosphotransfer experiments—The APRR1-R, APRR2-RB, ARR10-RB, and AHP2 polypeptides were each purified, as previously described (Imamura et al. 1998, Suzuki et al. 1998)). Urea-treated membranes (10 μg) were incubated with the purified AHP2 polypeptides (4 μg) at 37°C in the presence of 0.05 mM [γ-^{32}P]ATP (10,000 cpm pmol^{-1}), 50 mM KCl, and 5 mM $MgCl_2$ in TEDG buffer (Tris-HCl, EDTA, DTT, glycerol) (Aiba et al. 1989). The reaction mixture was immediately applied onto a Sephadex G-75 column previously equilibrated with TEDG buffer. Fractions containing the labeled AHP2 protein, which were essentially free of membranes and ATP, were collected. This purified radioactive phospho-AHP2 was incubated with the purified APRR2-RB polypeptides for short periods in TEDG buffer (30 μl) containing 50 mM KCl and 7 mM $MgCl_2$. After incubation, the samples were immediately subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (Laemmli 1970), followed by autoradiography. The gels were exposed and analyzed on a phosphoimage analyzer (BAS-2000II) (FujiXerox, Tokyo, Japan).

Transformation of onion cells and histochemical observation—The green fluorescent protein (GFP) expression vector, p221-EGFP-C1 is a recombinant derivative of pBI221/pEGFP-C1 (a gift from K. Hiratsuka, Nara Institute of Science and Technology, Japan) (Clontech, Palo Alto, CA, U.S.A.) (Chiu et al. 1996). The entire APRR1 coding sequence was inserted into p221-EGFP-C1 so as to be fused in-frame to the C-terminal end of the GFP coding sequence. Similarly, another plasmid was constructed to fuse the C-motif of APRR1 to GFP. These plasmid DNAs encompassing either the *GFP-APRR1* or *GFP-APRR1-C* fusion genes (0.4 μg each) were introduced to onion epidermal cells using a particle gun (GIE-III, TANAKA CO., Sapporo, Japan). The condition of bombardment was: vaccum of 600 mm Hg, helium pressure of 4.0 kg cm^{-2}, target distance of 3 cm. After bomberdment, tissues were incubated for 12 h at 25°C in the dark. Histochemical observation was made with the Olympus fluorescent microscopy (BH2-RFCA) with a filer (380–490 nm excitation).

Results

Genes each encoding a pseudo-response regulator—Based on an extensive inspection of the current Arabidopsis genome and cDNA sequence databases, we have previously compiled all the members of Arabidopsis response regulators (ARR-series) (Imamura et al. 1999). They were classified into two distinct subtypes: type-A response regulators consisting of seven members, and type-B including another seven. At that time, we intended to exclude several other candidates from these compiled members of ARRs, although they each have a domain very similar to the phospho-accepting receiver in their amino acid sequences. The reason why we did exclude these was that the amino acid sequences of their "receiver-like domain" are atypical in the following critical sense (Fig. 1A). Namely, they do not have the crucial and invariant aspartate residue that should serve as the phospho-accepting site in the classical receiver domain. One of these atypical examples was found as a predicted open-reading-frame in the genome sequence of AB010073 (chromosome V, GenBank accession no.). This hypothetical gene was inferred to encode a protein of 613 amino acids, in which a receiver-like sequence is located at its N-terminal end, as schematically shown (Fig. 1A, the predicted product was designated as APRR1) (see also Table 1). Similarly, the other (APRR2) was found in the genome sequence of ATT6K21 (chromosome IV, GenBank accession no.) (Fig. 1A and Table 1). Such receiver-like amino acid sequences of these predicted proteins are aligned with that of the typical receiver domain of the ARR10 response regulator of Arabidopsis (Fig. 1B).

As aligned in Fig. 1B, at a first glance, the amino acid sequences of the receiver-like domains of APRR1 and APRR2, respectively, are considerably similar to that of ARR10 (and those of other Arabidopsis response regulators that are not shown here). A classical receiver domain should contain three invariant amino acid residues, which all together are important for its phospho-accepting function (Parkinson and Kofoid 1992, Mizuno 1998). They are: the N-terminal aspartate (D) surrounded by negatively charged amino acids; the central aspartate site (D) that acquires a phosphoryl group; and the C-terminal lysine (K) followed often by proline (P), as indeed seen in the receiver domain of ARR10. As judged by these criteria, the receiver-like sequences of APRR1 and APRR2 are atypical, because, the phospho-accepting aspartate site (D) is replaced by glutamate residue (E) in both the amino acid sequences. Furthermore, the N-terminal aspartate is also

Table 1 List of Arabidopsis genes each encoding a pseudo-response regulator

Pseudo-response regulators	Sizes (A.A.)	Chromo. location (GenBank accession nos.)	EST (accession nos.)
APRR1	613 (618)	V (AB010073)	F20076
Three related ones		II (AC005310)	None
		V (AB019231)	None
		V (AB025641)	None
APRR2	483 (487)	IV (ATT6K21)	T76266
Another related one		V (AB016872)	None

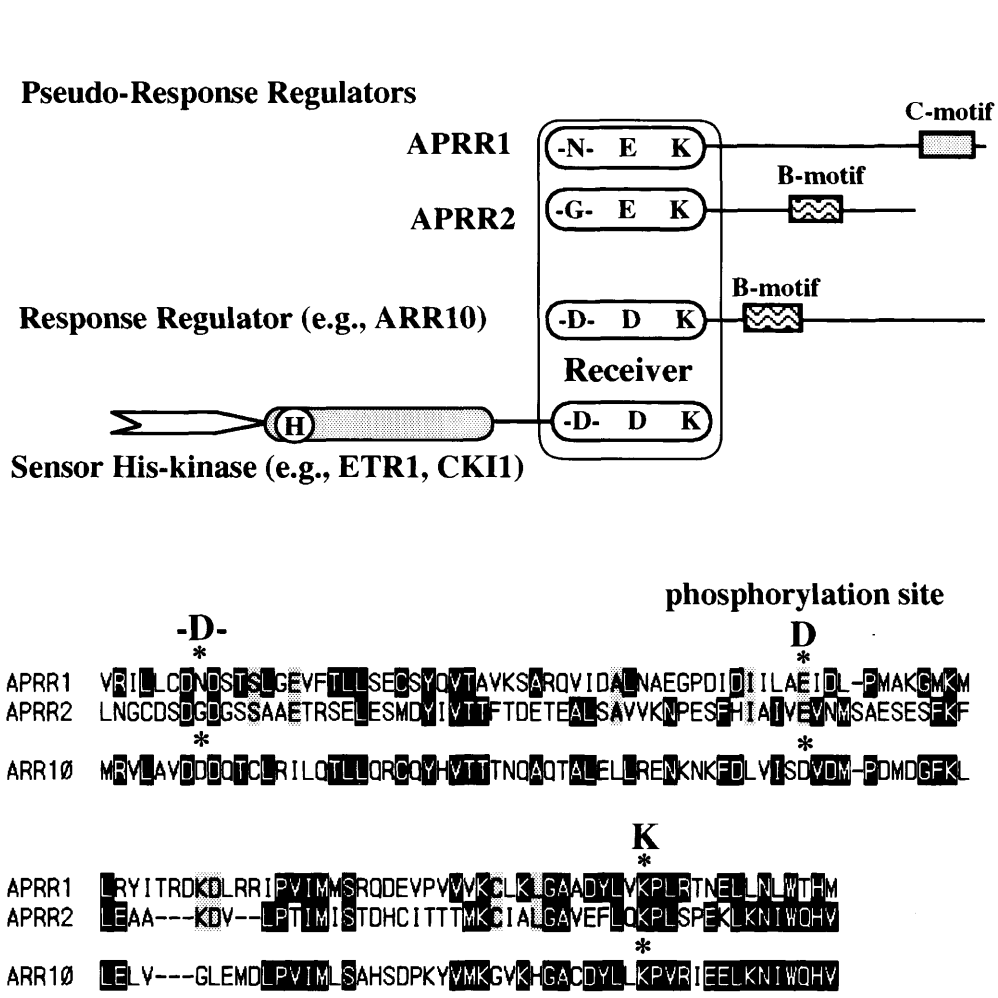

Fig. 1 Arabidopsis possesses a set of pseudo-response regulators. (A) The structural designs of the pseudo-response regulators (named APRR1 and APRR2), characterized in this study, were compared schematically with those of the authentic response regulators (e.g. APRR10) and the sensor His-kinase (e.g. ETR1 and CKI1). (B) The amino acid sequences of the pseudo-receiver domain of APRR1 and APRR2 were compared with that of ARR10. Other details are given in the text.

missing, although the negatively charged neighbors are present. Based on these considerations, in the strict sense, APRR1 and APRR2 should be discriminated from the authentic members of response regulators. They were thus designated as *A*rabidopsis *p*seudo-*r*esponse *r*egulators, APRRs. However, it would be tempting to speculate that these APRRs may play a biological role in concert with the authentic members of the ARR family of response regulators in an as yet unknown His-to-Asp phosphorelay system. In any case, we wanted to characterize these novel proteins (or genes) closely. It should be noted that an extensive inspection of the Arabidopsis databases revealed the existence of several other members of the APRR family of proteins, as listed in Table 1. We focused our attention on APRR1 and APRR2, because, their corresponding cDNA sequences are found also in the Arabidopsis EST databases, but others are not.

Characterization of APRR1—With appropriately designed oligo-nucleotides as primers, the APRR1 cDNA segment was cloned by polymerase chain reaction (PCR) method from an Arabidopsis cDNA bank, and then, the nucleotide sequence was determined. The result showed that the predicted APRR1 amino acid sequence in the database is essentially correct, but, a short stretch of 5 amino acids should be inserted at the amino acid position of around 250 (Fig. 2A). This is simply due to an incorrect assignment of the splicing sites in the database. The structural features of APRR1 are schematically shown (Fig. 2B). As emphasized above, APRR1 has a pseudo-receiver domain at its N-terminal end. Two stretches of about 50 amino acids, nearly identical to each other, are found in a tandem manner around the center. More interestingly,

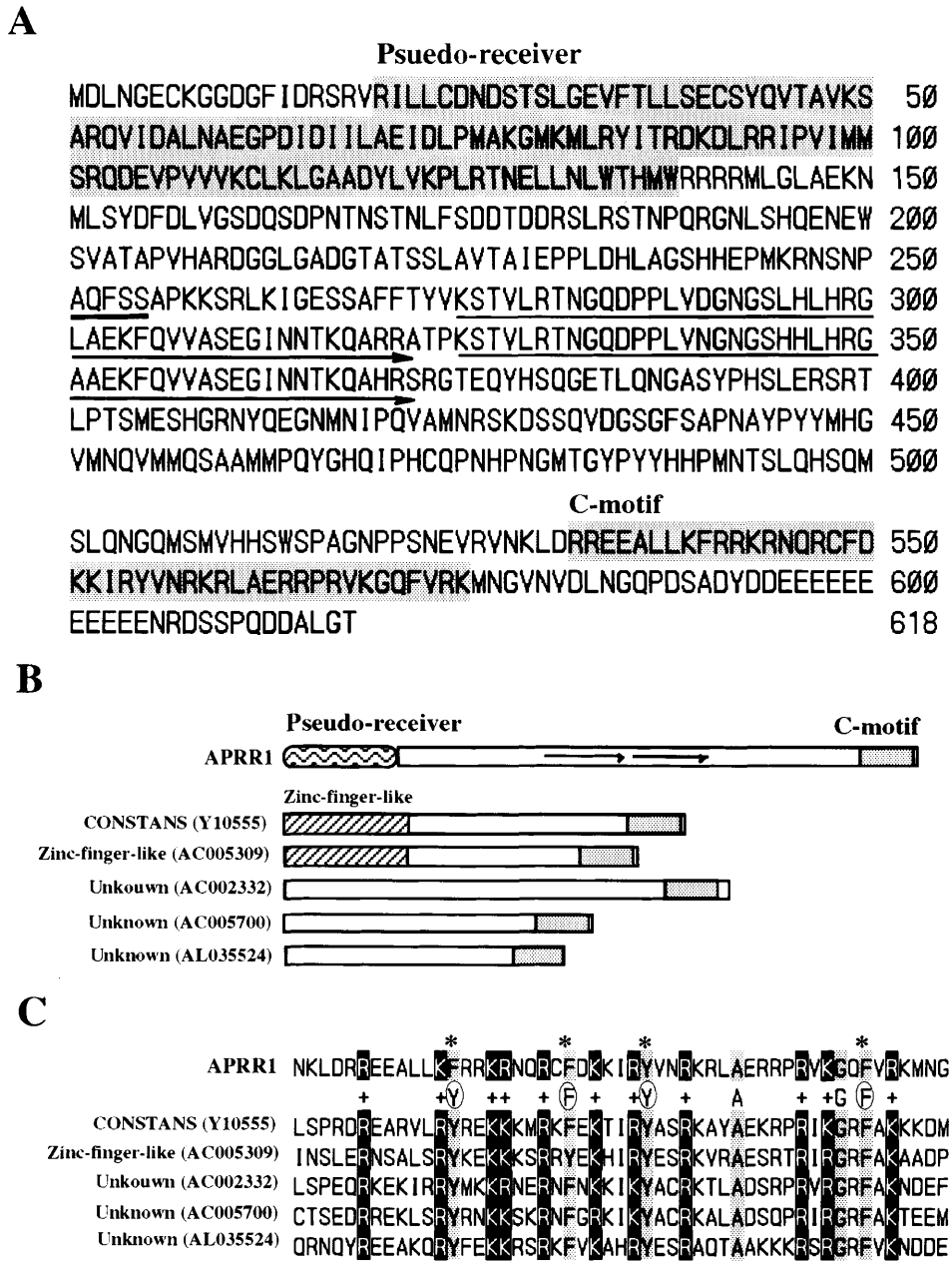

Fig. 2 Structure of APRR1. (A) The amino acid sequence of APRR1 was deduced from the determined nucleotide sequence of the APRR1 cDNA. The regions corresponding to the pseudo-receiver domain and the C-motif, respectively, are shaded. A repetitive sequence of about 50 amino acid is underlined with horizontal arrows. An insert of 5 amino acids, deduced from the cDNA sequence, is also underlined. (B) Such a structural design of APRR1 is schematically shown in the comparison with those of other Arabidopsis proteins (with each GenBank accession no.). All of them contain the highly conserved C-motif. (C) The amino acid sequences of these C-motifs, found in the proteins listed in (B), were aligned, in which the conserved positively charged and aromatic amino acids are highlighted.

when a similarity search was done with the entire APRR1 sequence as the query, it was found that APRR1 contains a short motif-like sequence consisting of about 50 amino acids at its C-terminal end (Fig. 2). Amino acid sequences very similar to this motif-like sequence were found in a number of proteins that have been deposited in the Arabidopsis databases. Most of them are found in unknown (or hypothetical) proteins, as schematically shown in Fig. 2B (note that there are many others that are not listed here). Importantly, the well-characterized *CONSTANS* (*CO*)

gene product of Arabidopsis also has this motif at its C-terminal end (Fig. 2B) (Robert et al. 1998). *CONSTANS* is the gene whose mutations cause delayed flowering under the control of circadian rhythm (Putterill et al. 1995, Kobayashi et al. 1999). As aligned in Fig. 2C, these amino acid sequences are highly similar to each other, and located in the C-terminal end of a given protein. They are rich in positively charged amino acids (arginine and lysine). Note also that four aromatic amino acids (tyrosine and phenylalanine) are invariantly conserved. We named these sequences collectively as "C-motif" (C-terminal *CONSTANS*-motif), based on the assumption that a C-motif in a given protein may play a common role. It is also worth mentioning that three other APRR1-like proteins, listed in Table 1, also contain not only a pseudo-receiver domain, but also this C-motif in their C-terminal ends.

Characterization of APRR2—The APRR2 cDNA segment was also cloned, and then, the nucleotide sequence was determined (Fig. 3). The predicted APRR2 amino acid sequence in the databases was experimentally confirmed, but, our data again showed that a short stretch of 4 amino acids should be added at the amino acid position of around 350 (Fig. 3A), due to an incorrect assignment of the splicing sites in the databases. The structural design of APRR2 is schematically shown in the comparison with that of the authentic ARR10 response regulator (Fig. 3B). Interestingly, APRR2 has a B-motif, which was previously proposed as the signature motif that is found exclusively in the

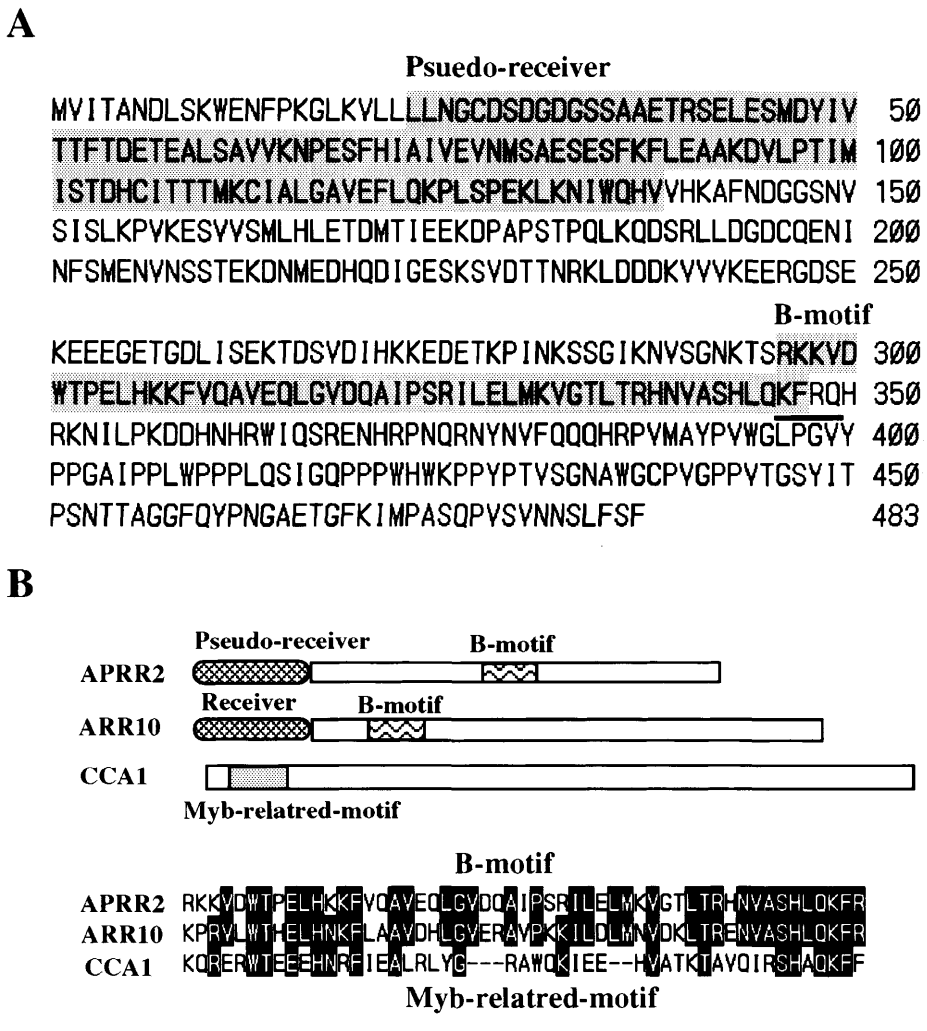

Fig. 3 Structure of APRR2. (A) The amino acid sequence of APRR2 was deduced from the determined nucleotide sequence of the APRR2 cDNA. The regions corresponding to the pseudo-receiver domain and the B-motif, respectively, are shaded. An insert of 4 amino acids, deduced from the cDNA sequence, is underlined. (B) Such a structural design of APRR1 is schematically shown in the comparison with those of Arabidopsis ARR10 and CCA1. Both APRR1 and ARR10 contain the highly conserved B-motif, whereas CCA1 contains the Myb-related-motif that is distantly related to the former. (C) The amino acid sequences of the B- and Myb-related-motifs were aligned, in which the conserved amino acids are highlighted.

type-B family of response regulators (including ARR10) (Imamura et al. 1999). As aligned in Fig. 3C, APRR2 contains such a B-motif whose amino acid sequence is highly similar to that found in ARR10. This B-motif seems to be distantly related to the Myb-related motif that is found in certain plant proteins (Jin and Martin 1999), including the well-documented CIRCADIAN CLOCK ASSOCIATED1 (CCA1) protein of Arabidopsis (Fig. 3C). The *CCA1* gene was suggested to encode a Myb-related transcription factor (Wang and Tobin 1998). In any case, at a first glance, the APRR2 pseudo-response regulator has a structural design very similar to those of the type-B response regulators (see Fig. 1). However, as emphasized above, it was confirmed by our cDNA sequencing that the receiver-like domain in APRR2 indeed lacks the crucial phospho-accepting aspartate site. In the current database, another APRR2-related protein can be predicted, which also contains a B-motif as well as a pseudo-receiver domain, as listed in Table 1.

Phosphorylation experiment—In general, a given receiver domain has an ability to acquire a phosphoryl group from a cognate phospho-histidine phospho-donor at the conserved central aspartate site (Parkinson and Kofoid 1992, Mizuno 1998). With regard to APRR1 and APRR2, it was thus interesting to ask the simple question of whether or not their pseudo-receiver domains have such an ability to acquire a phosphoryl group from an appropriate phospho-donor, for example, the Arabidopsis HPt phosphotransmitter (Suzuki et al. 1998). To answer this, a recombinant APRR1 polypeptide was purified with the *E. coli* pET histidine-tag system (Fig. 4A). This purified polypeptide, designated as APRR1-R, contains the pseudo-receiver domain extending from Val-19 to Trp-138, followed by a His-tag (see Fig. 2). Similarly, a recombinant APRR2 polypeptide was also purified (Fig. 4B). This polypeptide, designated as APRR2-RB, contains the amino acid sequence from Met-1 to Asn-353, which encompasses both the pseudo-receiver domain and B-motif (see Fig. 3). As an appropriate reference, a recombinant ARR10 polypeptide was also purified (Imamura, Tachiki and Mizuno, manuscript in preparation). This polypeptide, designated as ARR10-RB, contains both the authentic receiver domain and B-motif (see Fig. 3B). We previously demonstrated that the purified AHP2 phosphotransmitter is phosphorylated at a certain histidine residue in an *E. coli* in vitro system (Suzuki et al. 1998). Here we purified the ^{32}P-labeled phospho-histidine-containing AHP2 polypeptide, as described previously (Fig. 4C, lane 1). When this radiolabeled AHP2 was incubated with the purified ARR10-RB polypeptide, the radioactive phosphoryl group on AHP2 was rapidly transferred onto ARR10-RB (Fig. 4C, lanes 2 and 3). It should be noted that in the previous paper we failed to demonstrate this in vitro event with regard to the purified receiver domain of ARR10 (named ARR10-R,

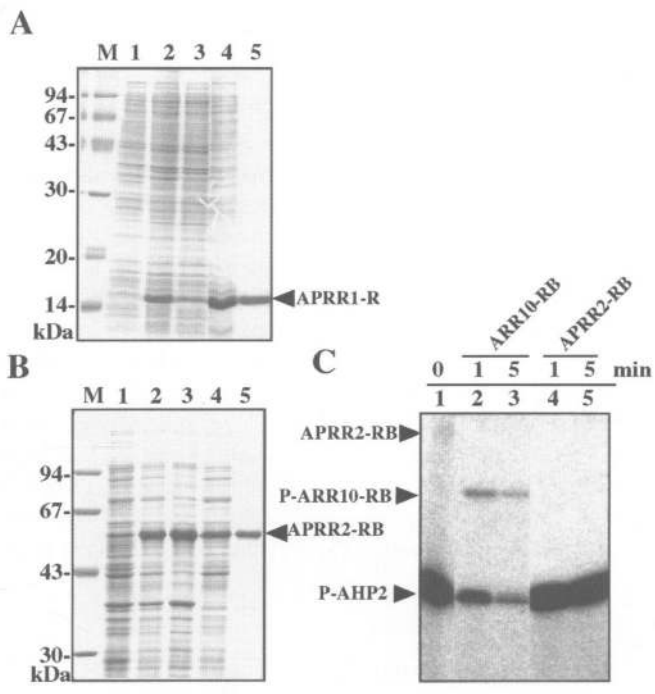

Fig. 4 Isolation of the APRR1, APRR2, and ARR10 polypeptides and characterization of their in vitro phosphorelay activity. (A) Isolation of the APRR1-R polypeptide in the *E. coli* expression system. The samples were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue; lane 1, total protein fraction of *E. coli* cells (non-induced); lane 2, total protein fraction (induced); lane 3, soluble protein fraction; lane 4, insoluble protein fraction; lane 5, isolated APRR1-R; lane M, molecular markers. (B) Isolation of the APRR2-RB polypeptide in the *E. coli* expression system; lane 1, total protein fraction of *E. coli* cells (non-induced); lane 2, total soluble protein fraction (induced); lane 3, membrane protein fraction; lane 4, soluble protein fraction; lane 5, isolated APRR2-RB. Other details are given in the text. (C) In vitro analyses of phosphotransfer between a representative of Arabidopsis HPt phosphotransmitter (AHP2) and the isolated polypeptides. Radioactively phosphorylated AHPs were purified (denoted by P-AHP2, see Material and Methods), and then the isolated ARR10-RB and APRR2-RB polypeptides were each added. At time intervals (min) indicated, the samples were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by autoradiography.

Imamura et al. 1999) (this issue will be addressed elsewhere, Imamura, Tachiki and Mizuno, manuscript in preparation). In any event, under the same in vitro conditions, the APRR2-RB polypeptide showed no ability to acquire the phosphoryl group from AHP2 (Fig. 4C, lanes 4 and 5). Essentially the same result was obtained, when the APRR1-R polypeptide was examined (data not shown). Although these results may or may not be reasonable, it provided us with a hint to understand the function of the pseudo-receiver domains.

Expression of the APRR1 and APRR2 genes in

plants—Results of standard Southern hybridization analyses showed that an appropriate APRR1 DNA probe hybridized with each specific restriction fragment in the digests of Arabidopsis genomic DNA (data not shown). The same was true in the case of the APRR2 gene. With use of these specific probes, Northern hybridization analyses of both the APRR1 and APRR2 transcripts were done by using total RNA preparations from roots, leaves, flowers, and siliques (Fig. 5A). The results show that a certain amount of both the APRR1 (ca. 2.1-kb) and APRR2 (ca. 1.6-kb) transcripts could be detected in all the organs tested. Both the transcripts seemed to be abundant in flowers. However, we must be cautious to evaluate this particular data in terms of "organ-specificity", because of the reason that will be emphasized below. In any event, it has been reported that the expression of all the members of the type-A family of response regulators is induced by cytokinin-treatment of plants (Taniguchi et al. 1998, Kiba et al. 1999). It would be thus interesting to ask the question of whether or not the expression profiles of either APRR1 or APRR2 is affected by such a treatment with cytokinins (e.g. *t*-zeatin). The shoots of N-starved plants, grown hydroponically, were sprayed with 100 μM *t*-zeatin. At time intervals (0 h to 6 h), total RNA fractions from leaves were prepared, and then, they were analyzed by quantitative Northern hybridization analyses. It was found that such a treatment does not affect the levels of the APRR1 and APRR2 transcripts (data not shown).

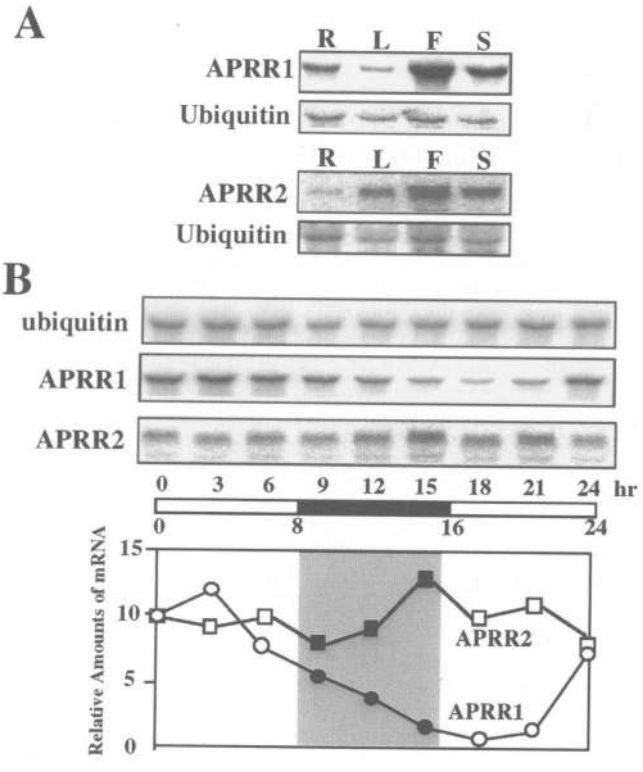

Fig. 5 Northern hybridization analyses. (A) Northern hybridization analyses were carried out to locate the APRR1 and APRR2 transcripts, respectively, with use of the ubiquitin transcript as an internal and loading reference. Total RNAs were isoalted from each organ indicated, R (roots), L (leaves), F (flowers), and S (siliques). (B) Northern hybridization analyses were carried out, with each probe indicated, to examine the fluctuation of transcript within a given day. RNA samples were prepared from plants (leaves), grown under the photo-conditions given in the text (also shown schematically). In order to quantify each transcript, the ubiquitin transcript was used as an internal and loading reference.

During the course of these Northern hybridization analyses, however, we noticed a curious phenomenon. With regard to the APRR1 transcript, particularly, the levels of RNA transcript varied considerably from a sample to a sample, even in the cytokinin non-treated plants (such an event was not seen in the case of the APRR2 transcript) (data not shown). *A priori*, we thought that it may be due to the timing to prepare RNA from plants within a day-period. It was thus needed to characterize the APRR1 transcript more closely with RNA samples prepared at serial time-intervals within a certain day-period. The results of such comparative Northern hybridization analyses are shown (Fig. 5B). Arabidopsis plants were grown for 20 d under the conditions of long-day photo-period (16 h-light and 8 h-dark). Throughout the next day, total RNA samples were prepared at each 3 h-interval, and then, they were analyzed by Northern hybridization with the APRR1 and APRR2 cDNAs as the probes. The APRR2 transcript was detected constantly. However, the levels of the APRR1 transcript varied considerably in an oscillated manner. This event may not be simply due to a light-response, because, the level of the APRR1 transcript reached at its maximum at few hours before evening, and it was markedly down-regulated at few hours after dawn. Rather, this is indicative of a circadian rhythm in plants (Kreps and Kay 1997, Dunlap 1999).

Expression of APRR1 is under the control of circadian rhythm—To address the intriguing issue mentioned above, expression profile of the APRR1 transcript was examined with special reference to circadian rhythm (Fig. 6). Arabidopsis plants were grown for 20 d under the conditions of 12 h-light and 12 h-dark. Note that the photo-period conditions were changed from those of the former experiment, on purpose (Fig. 5, 18 h-light/6 h-dark). In one line of experiment, the conditions of photo-period (or light/dark interval) were kept for the next 3 d, whereas in the other line of experiment the conditions were changed so as to be constant-light, as schematically shown in Fig. 6. From these plants (leaves), RNA samples were prepared at each 3 h-interval during the 3 d, to see whether or not the expression of APRR1 is indeed under the control of circadian rhythm. To this end, Northern hybridization analyses were done with these RNA samples (Fig. 6A, B). The results were best interpreted by assuming that the expres-

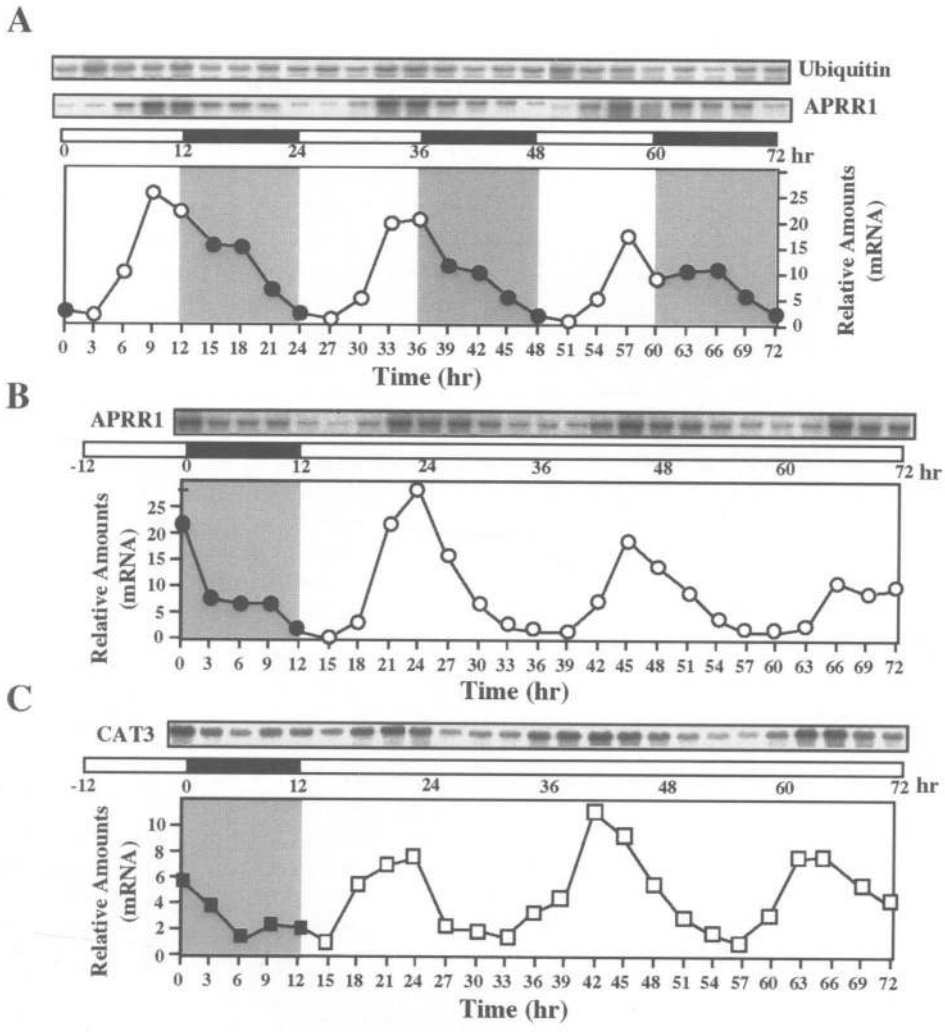

Fig. 6 Northern hybridization analyses. Northern hybridization analyses were carried out, with each probe indicated, to examine the fluctuation of the APRR1 and CAT3 transcripts. RNA samples were prepared form plants (leaves), grown under the photo-conditions given in the text (also shown schematically). In order to quantify each transcript, the ubiquitin transcript was used as an internal and loading reference.

sion of APRR1 is indeed subjected to a circadian rhythm.

A number of Arabidopsis genes whose transcriptions are under the control of circadian rhythm have been reported. Amongst them, the well-characterized *CAT3* gene was employed as a positive reference (Zhong and McClung 1996, Polidoros and Scandalios 1998), in order to support the above notion as to the expression of APRR1. As shown in Fig. 6C, the rhythmic expression profile of CAT3, under the conditions of constant-light, was very similar to that of APRR1 (although not exactly the same). In both cases, the level of each transcript reached at its maximum before evening, and reached at its minimum after dawn. In this respect, it would be worth mentioning that such a property of oscillated phase was independent upon the conditions of light/dark photo-period (i.e. regardless of either 18 h-light/6 h-dark or 12 h-light/12 h-dark) (cf. Fig. 5, 6). This is characteristic of a circadian rhythm, the phase of which can be reset presumably through phototransduction (Kreps and Kay 1997, Dunlap 1999).

Subcellular localization of APRR1—Since APRR1 is particularly interesting for us, as described above, we finally wanted to gain a hint with regard to its subcellular localization. This was done with a conventional and heterologous transient expression assay in onion epidermal cells (Fig. 7). A recombinant DNA encompassing a *GFP-APRR1* fusion gene was constructed and introduced into onion epidermal cells by using a particle-mediated DNA delivery procedure. The expressed GFP-APRR1 fusion protein was examined under a fluorescence microscopy.

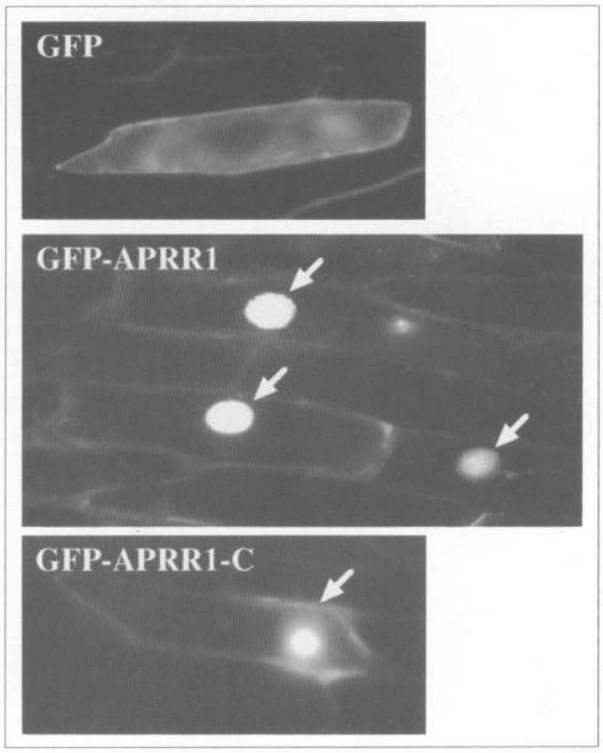

Fig. 7 Nuclear-localization of the GFP-APRR1 fusion protein. Recombinant DNA constructs carrying (*35S-GFP*), (*35S-GFP-APRR1*) and (*35S-GFP-APRR1-C*) were respectively bombarded into onion skin epidermal cells. The expression and localization of each product was observed after 24 h incubation (for details, see Materials and Methods).

Based on the nuclear and cytoplasmic fluorescence intensities, together with the result from an appropriate reference (GFP alone), it was found that a majority of GFP-APRR1 was predominantly (if not exclusively) localized to the nuclei of onion epidermal cells. We constructed also an appropriate plasmid carrying another fusion gene, in which the region specifying the C-motif was fused to GFP (Met-509 to Gly-578 of APRR1, see Fig. 2). This particular GFP-fusion protein (named GFP-APRR1-C) was also found in the nuclei (Fig. 7). These results are compatible with the idea that APRR1 has a nuclear-localization signal in its own right, and the C-motif appears to be responsible for the nuclear-localization.

Discussion

A typical His-to-Asp phosphorelay receiver domain is found in the response regulator family of Arabidopsis proteins, and in some case, in the His-kinase family of sensors (see Fig. 1A). In this study, we showed that Arabidopsis possesses a set of genes each encoding a protein with "a receiver-like domain". The structural designs of these proteins (APRRs) do not resemble those of His-kinases (e.g. ETR1 and CKI1). Rather, they indeed resemble those of response regulators (e.g. ARR10). However, APRRs should be discriminated from the authentic ARRs, because the formers lack the phospho-accepting aspartate site. As indeed demonstrated (Fig. 4), the isolated receiver-like domain of APRR2 showed no ability to acquire a phosphoryl group from the AHP2 phosphotransmitter, as far as our in vitro phosphotransfer conditions are concerned. We thus designated these novel proteins collectively as "pseudo-response regulators". They can be further classified into two groups (Table 1). The members that belong to the one group, including APRR1, have a characteristic signature motif (C-motif), whereas the members of the other group, including APRR2, have another characteristic signature motif (B-motif). The C-motif is intriguing in that it can be found in many other apparently unrelated Arabidopsis proteins. Most of them are hypothetical ones in the databases. However, it should be noted that the well known CONSTANS (CO) family of proteins have this motif (Robert et al. 1998). The product of the *CO* gene is important in the regulation of flowering in response to environmental conditions (Putterill et al. 1995). In these CONSTANS-related proteins, the C-motif was previously suggested to contain a nuclear-localization signal (Robert et al. 1998). In this context, APRR1 containing this C-motif was indeed demonstrated to have an ability to enter into the nuclei, and the C-motif appears to be responsible for the nuclear-localization (Fig. 7). The B-motif is also intriguing in the sense that this is the signature motif of the type-B family of Arabidopsis response regulators (Imamura et al. 1999). Furthermore, this motif appears to be distantly related to the Myb-related motif found in many plant proteins (Jin and Martin 1999). Such examples are the Arabidopsis CCA1 (CIRCADIAN CLOCK ASSOCIATED1) and LHY (LATE ELONGATED HYPOCOTYL) proteins, both of which were characterized as circadian clock associated DNA-binding transcription factors (Wang et al. 1997, Schaffer et al. 1998). These B- and C-motifs may play each characteristic common function in a given protein. Although our studies here revealed these intriguing properties of the newly identified APRR proteins, unfortunately, a clear linkage between APRRs and other authentic His-to-Asp phosphorelay signal transducers has not yet been emerged. However, it is tempting to speculate that these APRRs may play a biological role in concert with the authentic members of the ARR family of response regulators in an as yet unknown His-to-Asp phosphorelay mechanism, as discussed below.

In general, a receiver domain in a multi-domain signal transducer is considered to function as "a reversible ON/OFF molecular switch" that can be regulated by its own phosphorylation/dephosphorylation state (in this respect,

one can recall the GTP/GDP Ras molecular switch in eukaryotes) (Parkinson and Kofoid 1992). For example, the well-studied *E. coli* OmpR response regulator has a C-terminal DNA-binding domain, preceded by an N-terminal receiver domain (Mizuno and Mizushima 1990). Phosphorylation of the receiver domain results in the activation of the DNA-binding ability of the C-terminal domain, presumably, through induction of an OmpR-OmpR interaction (Nakashima et al. 1991). In any case, it can be generally assumed that the state of phosphorylation/dephosphorylation in a receiver domain may regulate an activity of a given signal transducer, primarily, by modulating a state of intra- and/or inter molecular protein-protein interaction (like GTP/GDP Ras). In this respect, there are several interesting reports that when the phospho-accepting aspartate site in a response regulator was changed to a certain amino acid, the resulting mutant regulator became "constitutive-active or locked-ON", albeit with a reduced activity (Klose et al. 1993, Brown et al. 1994, Wingrove and Gober 1994, Domian et al. 1997). This amino acid is glutamate. It should be remembered that each Arabidopsis APRR protein has this particular amino acid at the presumed phosphorylation site. In this sense, one can envisage that an APRR protein may be "a locked-ON version" of a response regulator. It would be worth mentioning also that it is well known that the higher plant phytochrome photoreceptors have a "pseudo-His-kinase domain" at their C-terminal ends (Elich and Chory 1997, and references therein). The amino acid sequences of the C-terminal domains of phytochromes significantly resemble those of the authentic His-kinase domains, but the formers lack the autophosphorylated histidine site. Collectively, it is tempting to speculate that in higher plants these "pseudo-receiver domains" and "pseudo-His-kinase domains" had evolved from the authentic ones so as to exert each unique function in an as yet unknown signaling network involving a His-to-Asp phosphorelay.

APRR1 is particularly interesting, because its transcription appears to be subjected to a circadian rhythm (Fig. 6). In plants, there is a wide range of processes that show a circadian rhythm (Kreps and Kay 1997, Koornnef et al. 1998, and references therein). It is believed that the circadian rhythm of gene expression is a part of the underlying mechanism for many, if not all, of the rhythms in metabolic and developmental processes in plants. These include movement of organs such as leaves and petals, stomata opening, sensitivity to light of floral induction, metabolic processes such as respiration and photosynthesis. In *Arabidopsis thaliana*, a number of circadian-related genes have been reported. Some genes (e.g. *CCA1* and *LHY*) have been suggested that they appear to be implicated in a part of a feedback loop that is closely associated with the Arabidopsis circadian clock, as mentioned above. Furthermore, 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 (Koornneef et al. 1998, and references therein). In this respect, some other genes (*DET1, COP1* and *TOC1*) are also involved in photoregulation of the Arabidopsis circadian clock (Kreps and Kay 1997, and references therein). Nonetheless, clarification of the plant clock component at the molecular level is at a very early stage. In any event, in plants, most of the circadian-regulated genes studied previously are related to photosynthesis and have a peak level of expression in the morning (for an example, see Park et al. 1999). A few exceptions include the COLD CIRCADIAN RHYTHM RNA binding protein 2 (*CCR2/AtGRP7*) gene and Catalase3 (*CAT3*), which peak in the evening (Carpenter et al. 1994, Zhong and McClung 1996, Heintzen et al. 1997). In this study, it was demonstrated that APRR1 appears to belong to the latter type of circadian rhythmic proteins. Collectively, it is tempting to speculate that APRR1 might play an imortant role in the Arabidopsis circadian rhythm per se, and there may be a link between the signal transduction through a His-to-Asp phosphorelay and the regulation of circadian rhythm. In any case, the intriguing findings in this study should give us a hint to understand the biological function of APRR1.

Finally, it should be noted the following fact. After we completed this study and when we performed a final search in the Arabidopsis databases for proteins similar to APRR1 and APRR2 in their amino acid sequences, we noticed that the amino acid sequence identical to APRR1 was recently deposited in the Arabidopsis databases (AJ251086, GenBank accession no.). The cDNA sequence was proposed to encode an ABI3-interacting protein (Kurup et al. 2000). The Arabidopsis *ABSCISIC ACID INSENSITIVE3* (*ABI3*) gene encodes a putative transcription factor that functions as a crucial regulator of late seed development, which shows a significant similarity to the maize *viviparous-1* protein (VP-1) (Giraudat et al. 1992, Parcy et al. 1997, Luerßen et al. 1998, Rohde et al. 2000). At present, we have no idea about a possible link between our results and this intriguing report. In this context, it is also worth mentioning that we have recently been searching proteins that interacts with APRR1 by means of yeast two hybrid system. One of the candidates thus identified is an uncharacterized protein with a Myc-related nuclear basic helix-loop-helix motif (Makino and Mizuno, unpublished data). Its basic helix-loop-helix region is very similar to that found in the Arabidopsis PIF3 protein. The PIF3 protein has recently been identified as a protein that can bind to the C-terminal fragments of phytochromes A and B, and it was suggested to function in phytochrome signaling (Ni et al. 1998, 1999). Clarification of these intriguing facets with regard to APRR1 should also shed

light on the biological function of this presumed His-to-Asp phosphorelay-associated protein.

This study was supported by Grants-in-Aid (09274101 and 09274102 to TS and TM) for scientific research on a priority area from the Ministry of Education, Science, Sports, and Culture of Japan. Thanks are also due to K. Hiratsuka (Nara Institute of Science and Technology, Japan) for his kind gift (the GFP expression vector) and help to conduct the GFP-experiment.

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(Received March 6, 2000; Accepted April 7, 2000)