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Compilation and Characterization of *Arabiopsis thaliana* Response Regulators Implicated in His-Asp Phosphorelay Signal Transduction

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His-Asp phosphorelays are evolutionary-conserved powerful biological tactics for intracellular signal transduction. Such a phosphorelay is generally made up of "sensor histidine (His)-kinases", "response regulators", and "histidine-containing (HPt) phosphotransmitters". In the higher plant, *Arabidopsis thaliana*, results from recent intensive studies suggested that His-Asp phosphorelays may be widely used for propagating environmental stimuli, such as phytohormones (e.g., ethylene and cytokinin). In this study, we first inspected extensively the occurrence of Arabidopsis response regulators in order to compile and characterize them. The results showed that this higher 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, biochemical properties, and expression profiles. Comparative studies were conducted for each representative (ARR3 and ARR4 for type-A, and ARR10 for type-B). It was suggested that expression of the type-A response regulator is cytokinin-inducible, while that of the type-B response regulator appears to be not. Results from yeast two-hybrid analyses suggested that the type-B response regulator may have an ability to stably interact with a set of HPt phosphotransmitters (AHPs). These and other results will be discussed with special reference to the His-Asp phosphorelay signaling network in *Arabidopsis thaliana*.

Key words: *Arabidopsis thaliana* — His to Asp phosphorelay — Response regulator — Signal transduction.

Widespread bacterial signal transduction circuits are sometimes referred to as "two component systems" or "phosphorelays", and they regulate a large variety of cellular responses, including bacterial chemotaxis, osmoregulation, photosensitivity, microbial pathogenesis (for reviews, see Stock et al. 1989, Bourret et al. 1991, Appleby et al. 1996). Such a phosphorelay signaling system is generally made up of two or more multidomain signal transducers (for a review, see Parkison and Kofoid 1992). They are generally referred to as "sensors" and "response regulators", each of which contains one or more phosphotransfer signaling domains (see Fig. 1). Namely, they are histidine (His)-kinases, phosphoaccepting-receivers, and histidine-containing phosphotransmitters (HPt) (for a review, see Mizuno 1998). The His-kinase domain in a given sensor has an ability to use ATP to *trans*-phosphorylate a specific substrate histidine residue on the adjacent subunit within the homodimer (reaction I in Fig. 1). The receiver domain in a given response regulator exhibits a phosphoaccepting ability to modify its own aspartate residue by acquiring a phosphoryl group from a cognate phosphohistidine phosphodonor (reactions II and V). This histidine to aspartate phosphotransfer reaction appears to be catalyzed by the receiver itself. The receiver is thus an enzyme capable of undergoing autophosphorylation with use of a phosphohistidine as substrate. However, some phosphorylated receivers are readily converted to their unphosphorylated form (reaction III). The dephosphorylation reaction also appears to be autocatalytic. In this sense, some receivers are enzymes capable of exhibiting a phosphohistidine phosphatase activity (reactions II+III, and reactions V+III). In fact, this autophosphatase activity confers a characteristic half-life on a given phosphoreceiver. Unlike His-kinases and receivers, the HPt phosphotransmitter does not exhibit any catalytic function in its own right (Ishige et al. 1994, Tsuzuki et al. 1995, Uhl and Miller 1996, Kato et al. 1997). Rather, it serves as a crucial intermediate in a histidine to aspartate phosphotransfer pathway by acquiring (reaction VI) and transferring (reaction V) a phosphoryl group from and to a receiver domain, respectively. In principle, such signaling pathways involving two or more of these phosphotransfer domains can be considered as "multistep histidine to aspartate (His-Asp) phosphorelays" (Appleby et al. 1996). The His-Asp phosphorelay mechanism appears to be an evolutionary-conserved biological tactics for intracellular signal transduction in response to an external or internal stimulus. In a given bacterial species, like *Escherichia coli*, these His-Asp phosphorelay systems involving a number of signal transducers are tuned to operate over a wide range of stimuli (Mizuno 1997, 1998). In the light of these conceptual views, here we attempt to char-

Abbreviations: AHP Arabidopsis histidine-containing phosphotransmitter; ARR, Arabidopsis response regulator; PCR, polymerase chain reaction.

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acterize a set of response regulators, recently found in the higher plant, *Arabidopsis thaliana*.

The first discovery of the Arabidopsis ethylene receptors immediately implied that the bacterial His-Asp phosphorelay mechanism may operate in this higher plant, because the structural designs of their primary amino acid sequences turned out to be very similar to those of bacterial sensor His-kinases (Chang et al. 1993, Hua et al. 1995, Gamble et al. 1998, Hua and Meyerowitz 1998, Sakai et al. 1998a). Such an assumption was further strengthened by the subsequent finding that a similar His-kinase is also implicated in a cytokinin-mediated signal transduction in this higher plant (Kakimoto 1996). These findings may or may not be surprising (for a review, see Chang and Stewart 1998), because many instances of such His-Asp phosphorelay pathways have been discovered in diverse eukaryotic species, including yeasts (Maeda et al. 1994, Posas et al. 1996), fungi (Alex et al. 1996), and slime molds (Wang et al. 1996, Schuster et al. 1996, Chang et al. 1998), suggesting that this particular signal transduction mechanism is not restricted to prokaryotes (for a reveiw, see Wurgler-Murphy and Saito 1997). However, discovery of the other crucial common components (i.e., response regulators and HPt phosphotransmitters) was a long time in coming for higher plants. It was recently demonstrated that *Arabidopsis thaliana* possesses a set of response regulators, each of which contains a typical receiver domain (Imamura et al. 1998, Sakai et al. 1998, Urao et al. 1998). A set of HPt phosphotransmitters was finally uncovered (Miyata et al. 1998, Suzuki et al. 1998), each of which has an ability to interact with a certain set of response regulators through a His-Asp phosphotransfer reaction (Suzuki et al. 1998). One can thus envisage that these uncovered lineups of His-Asp phosphorelay signal transducers must play important roles in concert by exhibiting their abilities to propagate environmental signals in this higher plant.

Up to now, several independent groups have demonstrated, by reporting their cDNA sequences, that *Arabidopsis thaliana* has a number of genes each encoding a putative response regulator (Brandstatter and Kieber 1998, Imamura et al. 1998, Sakai et al. 1998b, Urao et al. 1998). Furthermore, an inspection of the current Arabidopsis databases suggests that there must be more. Nonetheless, clarification of the Arabidopsis His-Asp phosphotransfer signal transduction mechanism is at a very early stage. To gain further insight into the presumed His-Asp phosphorelay signaling network, here we attempt to compile the members and properties of response regulators in *Arabidopsis thaliana*, on the bases of currently available databases as well as literatures. A new representative of such response regulators was also cloned and characterized, suggesting that it may interact stably with a certain set of HPt phosphotransmitters (AHPs).

Materials and Methods

Arabidopsis and related materials—The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was used in most experiments. Plants were grown with 16 h light/8 h dark fluorescent illumination at 22°C on soil or under continuous light on agar plates containing MS salts and 2% sucrose, unless otherwise noted. To examine the cytokinin-responsiveness of ARR expression, plants were grown hydroponically for four weeks with MGRL culture medium containing 2 mM $Ca(NO_3)_2$ and 3 mM KNO_3, and then they were transferred to the same fresh medium omitted the N-sources. After 11 days-cultivation, the plants were directly treated with a cytokinin (100 μM of *t*-zeatin) by spraying its fine mist onto the shoots. At time intervals (hour), total RNA fractions were isolated from the cytokinin-treated leaves (Taniguchi et al. 1998). The RNA samples were electrophoresed in 1.2% agarose gel containing formaldehyde, and then blotted onto a nylon membrane (Hybond-N+, Amersham). These blotted filters were subjected to Northern hybridization analyses, as described previously (Imamura et al. 1998), with each specific DNA probe that was appropriately designed for each response regulator cDNA. An Arabidopsis cDNA library in λgt11 was a gift from K. Shinozaki (RIKEN, Japan). To prepare cDNA, poly(A)$^+$ RNA was obtained from greening rosettes.

Escherichia coli and related materials—*E. coli* K-12 strain DZ225 (F-, *ΔenvZ, lacU169, araD139, rpsL, relA, flgB, thiA*) was used to prepare the cytoplasmic membranes (Nagasawa et al. 1993). Cells were grown in Luria broth, then the urea-treated cytoplasmic membrane was purified, as described previously (Tokishita et al. 1990). *E. coli* strain BL21 was used as a host for overexpression of Arabidopsis 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., Japan), with the recommended sequencing kits according to the manufacture's instructions.

PCR amplification—Using appropriate pairs of primers, polymerase chain reaction (PCR) was carried out to prepare DNA segments. The 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 ARR10 polypeptides, plasmids pET-ARR10 and pET-ARR10-R were constructed as follows: the ARR10 coding sequences were amplified from the corresponding cDNA clones by using appropriate pairs of PCR primers (see below). These amplified DNA segments were cloned into an *E. coli* expression vector, pET22b(+) (Novagen, Madison, WI., U.S.A.), so as to be placed under the T7 phage promoter. Each ARR10 coding sequence thus cloned has an initial methionine codon from the vector and a C-terminally extended histidine-tag. The PCR primers used are: ARR10, 5'-GACGCATATGACTATGGAGCAAG and 5'-AGGTTGAGCTCGCTGACAAAGAAAAGGG; ARR10-R, 5'-GACGCATATGACTATGGAGCAAG and 5'-CACAGGATCCTTATTCTTCTTAAGTTTGCTC.

Purification of Arabidopsis polypeptides with Ni-Column—*E. coli* BL21 cells carrying appropriate plasmids 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 (Tokishita et al. 1990). This sample was 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 plants 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, 1 mM EDTA, pH 7.4), 5×Denhardt's solution, and 0.5% SDS buffer 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 0.1% SDS for 15 min at room temperature, twice with 2×SSPE and 0.1% SDS for 30 min at 65°C, and then with 0.2×SSPE and 0.1% SDS for 30 min at 65°C. The washed membranes were exposed and analyzed on a phosphoimaging analyzer (BAS-2000II) (FujiXerox, Tokyo, Japan).

In vitro phosphotransfer experiment—The ARR and AHP polypeptides were purified, as described previously (Imamura et al. 1998, Suzuki et al. 1998)). Urea-treated membranes (10 μg of protein) were incubated with the purified AHP 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 AHPs, which were essentially free of membranes and ATP, were collected. This purified radioactively phosphorylated AHPs were incubated with purified ARR 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 phosphoimaging analyzer, as described above.

Yeast two-hybrid system—A kit for yeast two-hybrid analysis (MATCHMAKER^{TM}) (Clontech, Boston, U.S.A.) was obtained. This kit contains all tools essential for two-hybrid screening. They include the vectors: pGBT9 providing GAL4 DNA-binding domain (*TRP1* marker), and pGAD10 providing GAL4 activation domain (*LEU2* marker). They also include the yeast host strains: HF7c carrying both the *GAL1-HIS3* and (*GAL4* 17-mers)$_3$-*CYC1-lacZ* reporters, and SFY526 carrying the *GAL1-lacZ* reporter. Procedures of the two-hybrid screening are essentially according to the manual supplied from the manufacturer of the kit. An Arabidopsis cDNA-expression library was obtained from TOYOBO (Osaka, Japan), namely, Arabidopsis MATCHMAKER cDNA Library. Also, cDNAs were prepared from mRNA of 3 weeks-old green vegetative tissue of *Arabidopsis thaliana* (cv. Columbia). The following plasmids were constructed from pGBT9 for baits, namely, pGBT9-ARR10-ΔC, pGBT9-ARR10-R, pGBT9-ARR3, pGBT9-ARR4, pGBT9-AHP1, pGBT9-AHP2, and pGBT9-AHP3. The following plasmids were also constructed from pGAD424 for preys, namely, pGAD424-ARR10-DC, pGAD424-ARR10-R, pGAD424-AHP1, pGAD424-AHP2, and pGAD424-AHP3. To construct these plamsids, a number of appropriate PCR-primers were used (not shown), and the nucleotide sequences of the resulting plasmids were confirmed by sequencing.

Mapping of ARR genes—Genetic mapping of the ARR genes was carried out by Cleaved Amplified Polymorphic Sequences (CAPS) mapping method using recombinant inbred (RI) lines (Lister and Deam 1993).

Results and Discussion

Arabidopsis thaliana has a large number of genes each encoding a putative response regulator—Recently, several groups have demonstrated independently that this higher plant possesses a set of response regulator genes (see Table 1). First, Imamura et al. (1998) isolated five cDNAs (named ARR3 to ARR7), each of which encodes a small polypeptide that contains a typical receiver domain. Urao et al. (1998) reported four cDNAs (named ATRR1 to ATRR4), whose protein products have structural designs very similar to those of ARRs. Subsequently, Sakai et al. (1998b) characterized two more cDNA sequences (named ARR1 and ARR2), each of which encodes a relatively large polypeptide containing a typical receiver domain followed by a large C-terminal extension. A few more response regulator genes were identified through a different approach of Brandstatter and Kieber (1998). These were identified as cytokinin inducible cDNAs (named IBC6 and IBC7). Since one discovery of response regulator genes was followed by another in quick succession, here it was needed to extensively compare these reported sequences with each other in order to compile the minimal list of response regulator genes for *Arabidopsis thaliana*. The results from such an effort made in this study are summarized in Table 1. It was concluded that, at least, nine distinct response regulator cDNAs have so far been identified. Since each was labeled with each different name by each investigator, in this study we tentatively labeled them with serial-numbers (ARR1 to ARR9), solely for clarity of this text (Table 1).

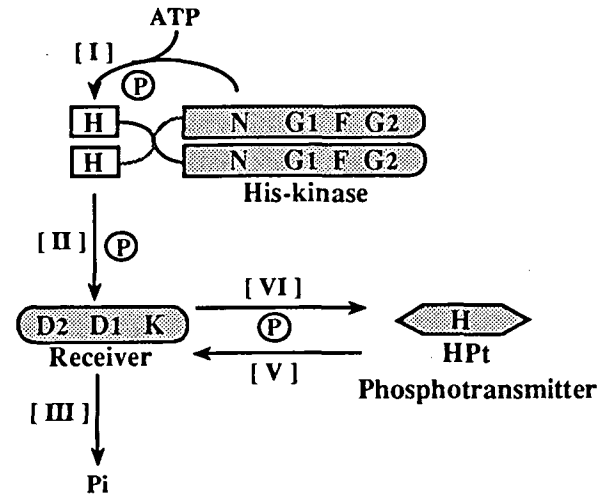

Fig. 1 A schematic representation of His-Asp phsphorelay reactions, observed among the three type of signal transducers. The typical structures of three types of common signal transducers are schematically shown. They are "His-kinases", "receivers", and "HPt phosphotransmitters" (Mizuno 1998). These exhibit each characteristic His-Asp phosphorelay reaction (reactions I through V), as indicated. Details are given in the text (see Introduction).

On the basis of such a critical compilation, we then extensively searched for new genes, each of which encodes an as yet uncharacterized response regulator, in the currently-available Arabidopsis databases. Such an inspection revealed the occurrence of five more genes, each of which most likely encodes a new response regulator (labeled with ARR10 to ARR14 in Table 1). Among them, ARR10 (or ARP4) and ARR11 (or ARP3) have been deposited, as cDNA sequences in the GenBank database, suggesting that they are transcribable. However, the other three (ARR12 to ARR14), found in the Arabidopsis genomic sequences, are purely hypothetical. All together, one can compile, at least, 14 response regulator genes at present (1999, April), and, for some of them, their occurrence can be assigned on each chromosome (Table 1).

Arabidopsis thaliana has two distinct types of response regulators—In general, a typical receiver of about 120 amino acids in a given signal transducer exhibits a phosphoaccepting ability to modify its own aspartate residue (invariant D1 site), which is located at the center (see Fig. 1). Together with this critical aspartate residue, two other amino acids are exclusively conserved among the receiver family of domains: another aspartate residue (acidic D2 site) at the N-terminal end, and a lysine residue (K site) at the very C-terminal end. Based on this general view and the results from analyses of the compiled data in Table 1, it was concluded that the members of response regulators (ARR1 to ARR14) can be classified into two distinct subtypes, as judged from their structural designs (type-A and type-B, see Table 1 and Fig. 2). Both the types of response regulators have a typical receiver domain at their N-terminal ends (Fig. 2A), but their amino acid contexts defer extensively form each other (Fig. 2B).

The members of type-A response regulators (ARR3 to ARR9) are relatively small in their amino acid lengths (see Table 1, e.g., 184 amino acids in ARR5, 259 amino acids in ARR4). They have highly homologous receiver sequences, as compared with each other (for the sequence alignment, see Fig. 3A), although each has a variable short amino acid insertion at its center (see the underlined region). It should be noted that each members has each characteristic very short C-terminal extension (data not shown). In this respect, it may also be noted that these type-A response regulators somewhat resemble the well-characterized *E. coli* CheY response regulator in the sense that they appear to contain only a receiver domain (Parkinson and Kofoid 1992). In sharp contrast, each member of the type-B response regulators (ARR1, ARR2, and ARR10-ARR14) has a relatively large C-terminal extension (Fig. 2A), which is preceded by a highly homologous receiver domain (for the sequence alignment, see Fig. 3B). Among the type-B response regulators, the amino acid sequences of the C-ter-

Table 1 Compiled list of Arabidopsis response regulators

Response regulators (serial-numbers)	Sizes (amino acids)	Types[a]	Reported cDNAs[b] (accession nos.)	Chro. location[c] (accession nos.)
ARR1	669	B	ARR1 (AB016471)	?
ARR2	664 or 644[d]	B	ARR2 (AB016472)/ARP5 (ATAJ5196)	IV (ATFCA5)
ARR3	231	A	ARR3 (AB008486)	I (AC007258)
ARR4	259	A	ARR4 (AB008487)/ATRR1 (AB010915)/IBC7 (AF057282)	I (AC007067)
ARR5	184	A	ARR5 (AB008488)/ATRR2 (AB010916)/IBC6 (AF057281)	III (ATT17F15)
ARR6	186	A	ARR6 (AB008489)	V (AB009053)
ARR7	206	A	ARR7 (AB008490)	?
ARR8	224	A	ATRR3 (AB010917)	II (AC005662)
ARR9	234	A	ATRR4 (AB010918)	?
ARR10	552	B	ARP4 (ATAJ5195)	IV (ATF10N7)
ARR11	444	B	ARP3 (ATAJ5194)	?
ARR12	573	B	Hypothetical	II (ATF13D4)
ARR13	575	B	Hypothetical	II (AC005623)
ARR14	382	B	Hypothetical	II (AC006069)

[a] This classification is based on our results in this study.
[b] Each credit should be: ARR1 and ARR2 (Sakai et al. 1998b), ARR3 to ARR7 (Imamura et al. 1998), ATRR1 to ATRR4 (Urao et al. 1998), IBC6 and IBC7 (Brandstatter and Kieber 1998), and ARP3 to ARP5 (Buchholz et al. 1998). For details, see the given GenBank accsession nos.)
[c] The chromosomal location of each gene was estimated from the available genomic sequences of *Arabidopsis thaliana* (see the given GenBank accession nos.). With regard to *ARR3* and *ARR4*, the locations were determined by Cleaved Amplified Polymorphic Sequences (CAPS) in our laboratory (see Materials and methods).
[d] There appears to be a discrepancy between the two reports (AB016472 and ATAJ5196).

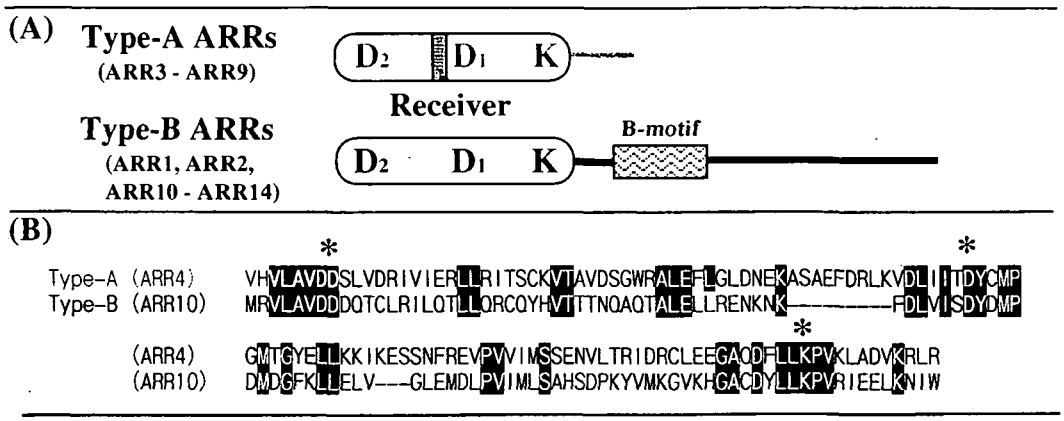

Fig. 2 Arabidopsis possesses two types of response regulators. (A) Fourteen members of response regulators were classified into two subtypes (type-A and type-B), based on their structural designs (see Table 1), Each member of the type-A response regulators has a short insertion at its center of the receiver domain (see the shaded box), whereas each member of the type-B response regulators has a large C-terminal extension, in which a highly conserved motif (referred to as B-motif) is exclusively found, as schematically shown. (B) The amino acid sequence of the type-A receiver (ARR4) was compared with that of the type-B (ARR10). Note that both of these have a set of crucially conserved amino acids (indicated by asterisks), yet their overall contexts are quite different form each other.

minal extensions are rather variable (note that ARR1 and ARR2 are highly similar throughout to each other, as mentioned previously, Sakai et al. 1998b, and ARR10 and ARR12 are somewhat similar to each other). However, a striking fact is that a common motif consisting of about 80 amino acids can be detected exclusively in their C-terminal extensions, as schematically shown in Fig. 2A (B-motif), and the amino acid sequence alignment of this motif is given in Fig. 3C. As has been pointed out by Sakai et al. (1998b), the amino acid sequences of this motif show a weak and limited similarity to those of the Myb-related motif that are found in various plant proteins with unknown function (Fig. 3C, bottom). This plant Myb-related motif was originally proposed for the putative potato homolog (MybSt1) of the mammalian DNA-binding Myb oncoproteins (Baranowskij et al. 1994). To avoid misleading, nonetheless, here we would like to simply refer to this exclusively-conserved motif in type-B response regulators as "B-motif". This B-motif appears to be a signature sequence of the family of type-B response regulators (Fig. 2B).

In short, 14 members of response regulators can be classified into two subtypes (so far, 7 members in type-A, and 7 in type-B), whose structural designs differ extensively form each other type. It is thus tempting to speculate that each type is functioning in each different manner in the presumed His-Asp phosphorelay signaling pathways. In this respect, it may be worth mentioning that Sakai et al. (1998b) previously proposed that the C-terminal region containing B-motif may function as a nuclear-localized DNA-binding domain. Clarification of this intriguing view must await further experimentation.

Characterization of a member of B-type response regulators—Previously, our group have extensively characterized some representatives belonging to the type-A response regulators (ARR3 and ARR4) (Imamura et al. 1998, Yamada et al. 1998), and provided evidence that they have an in vitro ability to acquire a phosphoryl group from certain HPt phosphotransmitters (AHP1 and AHP2) (Suzuki et al. 1998). Furthermore, it was demonstrated that expression of these genes are induced upon treatment of plants with cytokinins (e.g., *t*-zeatin) (Taniguchi et al. 1998). We needed to clone and characterize some representatives belonging to the type-B family of response regulators, in the hope of conducting comparative studies.

Thus, we attempted to clone the presumed ARR10 and ARR12 cDNAs with a PCR-based strategy with use of three independent sets of Arabidopsis cDNA banks. After intensive screening, we could clone ARR10 cDNA, but not ARR12 cDNA (data not shown). Its entire nucleotide sequence was determined, and the original exon-intron assignment, proposed in the current database (GenBank accession no. ATF10N7), was confirmed to be correct. To gain insight into the biochemical properties of ARR10, recombinant ARR10 polypeptides were expressed in *E. coli* with the pET histidine-tag system. The entire ARR10 polypeptide and its minimal receiver domain were overexpressed in an *E. coli* host strain. The former was found to be overexpressed in *E. coli* as an insoluble form (data not shown), whereas the latter was as a soluble form (Fig. 4A, lane 1). Thus, the ARR10 receiver domain (named ARR10-R) was homogeneously isolated as a soluble form to use for further biochemical analyses (lane 2). The previously characterized ARR3 and ARR4 polypeptides were also purified, as references of type-A receivers (lanes 3 and 4, respectively).

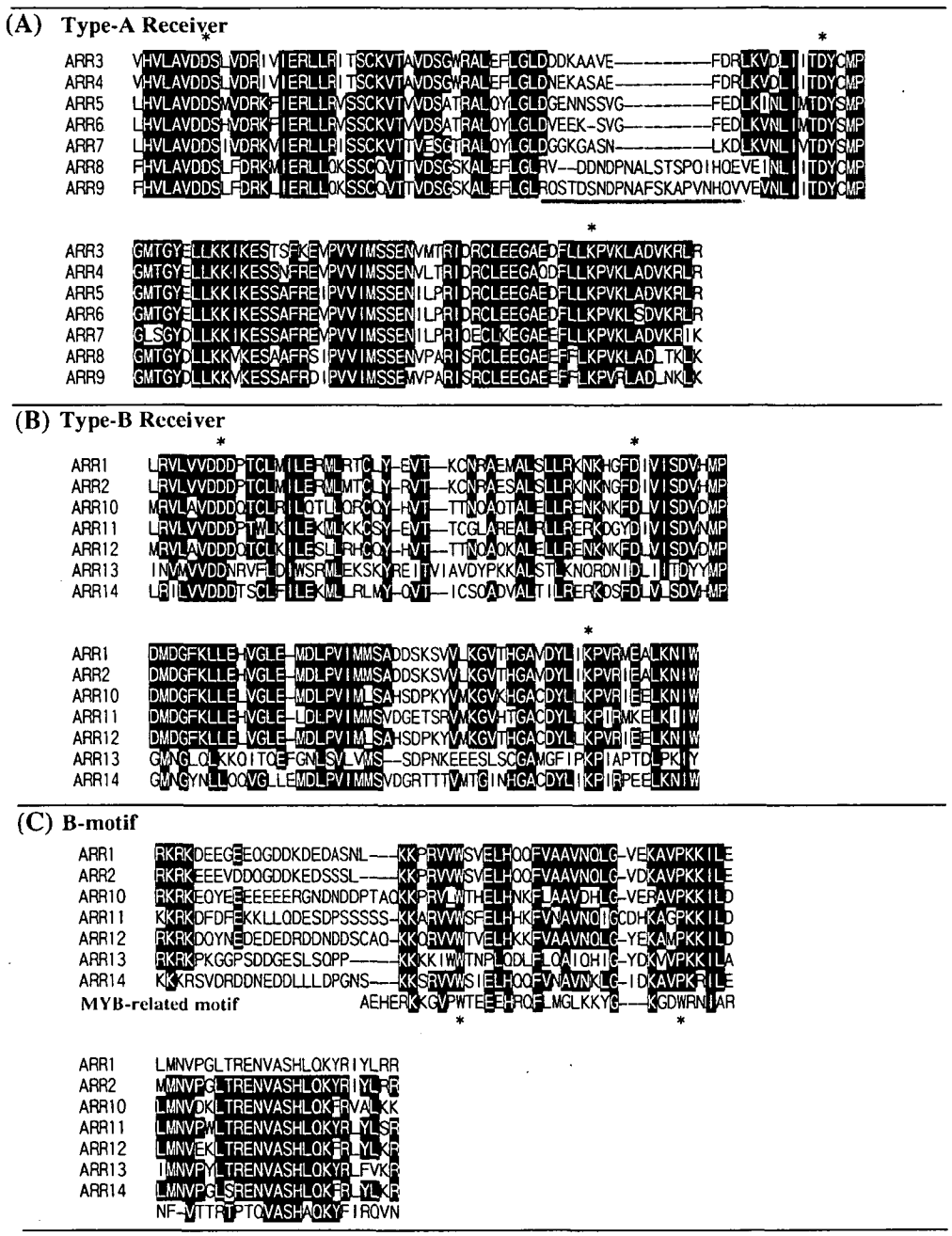

Fig. 3 Alignments of deduced amino acid sequences of Arabidopsis response regulators. The amino acid sequences were aligned for the following members; (A), type-A receivers; (B), type-B receivers; (C) B-motifs. These aligned regions correspond to those schematically indicated in Fig. 2A (amino acid numbers are omitted for clarity of these figures). Among each group, highly conserved amino acids are highlighted. In panel C, the partial sequence, denoted by "MYB-related motif", is from a putative Arabidopsis Myb-related transcriptional activator (GenBank Accession no. ATAC003028-2). Tryptophan residues that crucially conserved in a number of Myb-related proteins are indicated by asterisks.

As mentioned above, we have recently identified five distinct HPt phosphotransmitters (AHP1 to AHP5) in *Arabidopsis thaliana* (Suzuki et al. 1998). We demonstrated that the purified AHP1 and AHP2 polypeptides can be phosphorylated at a certain histidine residue in an *E. coli* in vitro system. Here we purified the radiolabeled phosphohistidine-containing AHP2 polypeptide, as described previously (Suzuki et al. 1998) (Fig. 4B, lane 1). When this

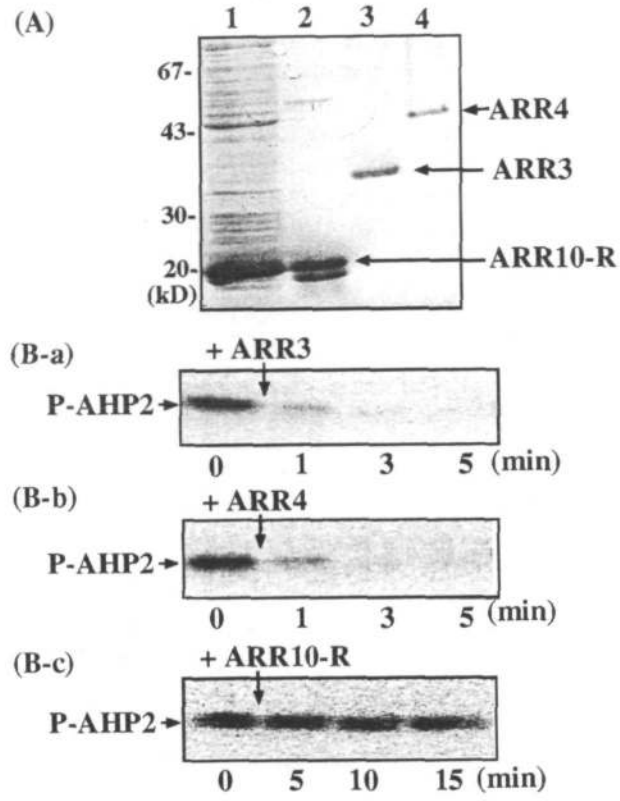

Fig. 4 Isolation of ARR polypeptides and characterization of their in vitro phosphorelay activity. (A) Isolation of a set of polypeptides used in this study. They were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue (see Materials and methods); lane 1, total soluble fraction of *E. coli* cells producing a recombinant ARR10 receiver domain (named ARR10-R) (80 μg); lane 2, isolated ARR10-R (3 μg); lane 3, ARR3 (1 μg); lane 4, ARR4 (1 μg). (B) In vitro analyses of phosphotransfer between a representative of HPt phosphotransmitter (AHP2) and ARRs. Radioactively phosphorylated AHPs were purified (denoted by P-AHP2, see Material and methods), and then the isolated ARR polypeptides were each added. At time intervals (min) indicated, the samples were analyzed by SDS polyacrylamide gel electrophoresis, followed by autoradiography (panel a for ARR3, panel b for ARR4, panel c for ARR10-R). Note that AHP2 and ARR10-R migrated to the positions different form each other in the gel.

radiolabeled AHP2 was incubated with the purified ARR3 and ARR4 polypeptides, respectively, the radioactive phosphoryl group on each AHP was very rapidly released (Fig. 4B-a and 4B-b, respectively). Essentially the same results were obtained, when such in vitro analyses were carried out for the following combinations (AHP1 vs. ARR3, and AHP1 vs. ARR4) (data not shown). These results suggest that both the ARR3 and ARR4 response regulators have an in vitro ability to function as a phosphohistidine phosphatase toward the AHP1 and AHP2 phosphotransmitters (see Fig. 1, reactions V+III). Essen-

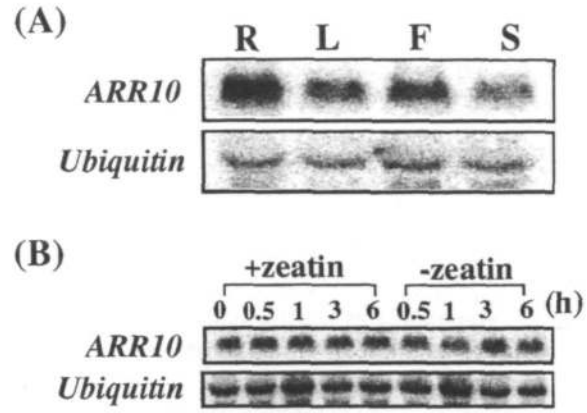

Fig. 5 Northern hybridization analyses and cytokinin responsiveness of the ARR10 transcripts. (A) Northern hybridization analyses were carried out to locate the ARR10 transcript, with use of the ubiquitin transcript as an internal and loading reference. Total RNAs were isolated from each organ indicated. (B) Cytokinin responsiveness of the accumulation of the ARR10 transcript in the leaves of N-starved plants. Total RNA fractions were prepared from leaves at indicated times after spraying with *t*-zeatine (100 μM) or 0.2% methanol solution alone (control). These RNA fractions (20 μg each) were subjected to Northern hybridization analyses with an appropriate and specific probe for ARR10. The hybridized filters were exposed and analyzed on a phosphoimaging analyzer. These procedures were essentially the same as those described previously (Taniguchi et al. 1998).

tially the same in vitro assay was conducted for the purified ARR10 receiver. But, nothing happened on both the radiolabeled AHP1 and AHP2 polypeptides (Fig. 4B-c for AHP2, and the data for AHP1 are not shown). Simply, our purified ARR10-R polypeptide may have had lost its native activity under our in vitro conditions. Rather, the following alternative view is also plausible, because the result, observed for ARR10, is not unexpected one. According to the general concept as to the function of the receivers (see Introduction, and Fig. 1), a given receiver is principally capable of exhibiting one or more of the following types of phosphotransfer activities: phosphoacceptor from histidine (reactions II and V), phosphodonor for histidine (reaction IV), and phosphatase toward phosphohistidine (reactions V+III). As far as our in vitro results are concerned, the ARR3 and ARR4 receivers exhibit a strong phosphohistidine phosphatase activity toward AHPs. The ARR10 receiver may alternatively serve as a phosphodonor for AHPs (note that this particular activity could not be assessed with our in vitro system used). In any event, an improved in vitro system will be needed to address this intriguing issue.

Expression of the ARR10 gene—Standard Southern hybridization analysis showed that an approprite ARR10 probe hybridized with each specific restriction fragment in the digests of Arabidopsis genomic DNA (data not shown).

Based on this, then, standard Northern hybridization analysis of the ARR10 transcript was carried out by using total RNA preparations from roots, leaves, flowers, and siliques (Fig. 5A). The results show that a certain amount of the ARR10 transcript could be detected in all organs tested, including stems (data not shown). A critical question was then arose as to if the expression of ARR10 is affected by a treatment with cytokinins (e.g., *t*-zeatin), as previously demonstrated in the case of ARR3 and ARR4 (Taniguchi et al. 1998). The shoots of N-starved plants, grown hydroponically, were sprayed with or without 100 μM *t*-zeatin. At time intervals (0 to 6 h), total RNA fractions from leaves were prepared, and then subjected to quantitative Nothern hybridization analyses (Fig. 5B). It was revealed that such a treatment did not affect the level of the ARR10 transcript. This result is in sharp contrast to the previous finding that the rapid accumulation of both the ARR3 and ARR4 transcripts in leaves was induced under essentially the same conditions (Taniguchi et al. 1998), suggesting that not all of the *ARR* genes are cytokinin-inducible in their transcription.

Together with the previous results by Taniguchi et al. (1998), the result in this study suggested to us a possibility that the expression of type-A response regulators is induced by cytokinins and nitrate, while that of type-B may be not. To test this idea, comprehensive and comparative Northern hybridization analyses were conducted with special reference to the expression profiles of both other members of type-A and type-B response regulators. The results showed that the above idea is the case (see the accompanying paper, Kiba et al. 1999). In this regard, it may be notable that Urao et al. (1998) reported that expression of the ATRR1 (identical to ARR4/IBC7) and ATRR2 (identical to ARR5/IBC6) transcripts are induced in response to stress treatments, such as low-temperature (4°C), dehydration and salt treatment (250 mM NaCl), while those of the ATRR3 (ARR8) and ATRR4 (ARR9) genes show no such responses. In any event, expression profiles of a series of ARR genes should be examined more extensively and comparatively under various growth conditions. This remains to be carried out.

ARR10 may interact physically with a set of AHPs— To gain further insight into the properties of ARR10, we finally adopted the widely used yeast two-hybrid system, and searched for Arabidopsis proteins that are capable of stably interacting with ARR10. A porting of the ARR10 coding sequence was first connected appropriately to the GAL4-binding domain in pGBT9, to yield a bait vector (named pGBT9-ARR10-ΔC). This ARR10 coding sequence corresponds the ARR10 polypeptide extending from Met-1

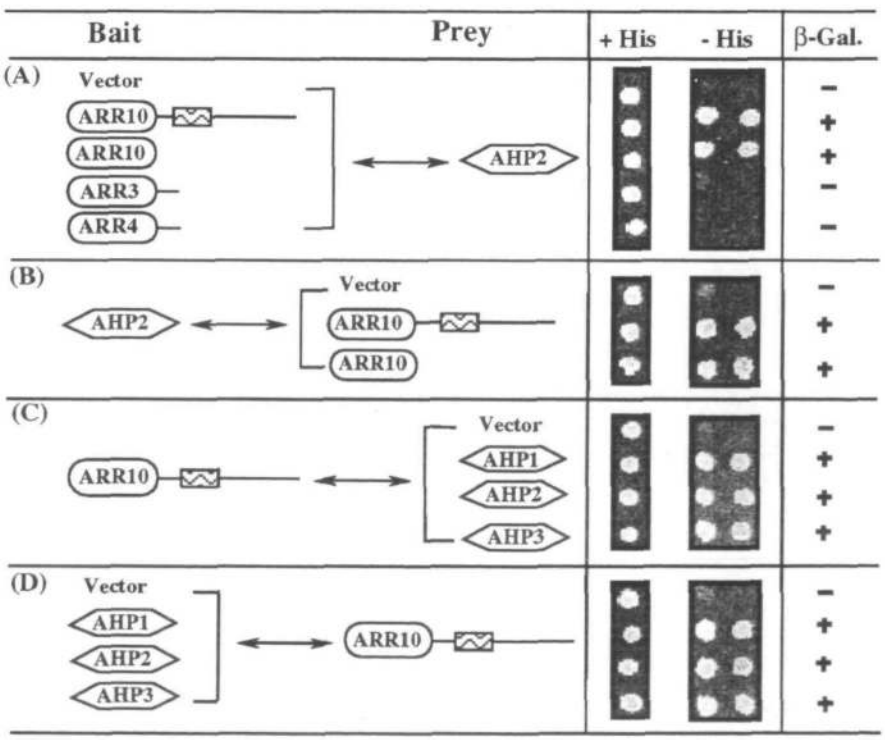

Fig. 6 Yeast two-hybrid analyses. Extensive two-hybrid analyses were conducted with use of a variety of pairs of bait/prey, as schematically shown. The results were first scored with special reference to the histidine-autotrophy on appropriate agar-plates (+His and −His, respectively) (see Material and methods). There are two spots in each −His column, and the right hand side one corresponds to 1/10 cells of the left. These results were then confirmed by the LacZ-reporter assay, as also indicated (β-Gal. + or −).

to Arg-442, thus lacking the C-terminal 110 amino acids. On the other hand, an Arabidopsis cDNA expression library was obtained, in which approximately 3×10^6 independent cDNA clones were connected to the GAL4 activation domain in the yeast prey vector pGAD10. Using these as baits and preys, respectively, an extensive two-hybrid screening was carried out with the yeast strain HF7c carrying both the GAL1-HIS and (GAL4 17-mers)$_3$-CYC1-*lacZ* fusion on the genome, on the bases of the histidine-autotrophy on 3-aminotriazole (3AT)-containing minimal plates as well as the LacZ-expression assay. Among 10^7 yeast transformants screened, we succeeded in isolating 11 strongly-positive clones, each of which was assumed to carry an Arabidopsis cDNA, whose translation product may interact with the ARR10 region in yeast cells. After extensive analyses, surprisingly, we found that all of these 11 positive clones carries the entire AHP2 coding sequence, which had been connected in frame to the GAL4 activation domain.

This finding was then evaluated by intensive two-hybrid analyses, as summarized in Fig. 6. A set of various baits were prepared, which included ARR10-ΔC, ARR10-R, ARR3, and ARR4. These baits were tested against AHP2 as prey (panel A). The results suggested that ARR10 has an ability to physically (or stably) interact with AHP2, and the ARR10 receiver domain is responsible for this interaction. Interestingly, the representatives (ARR3 and ARR4) of type-A receivers showed no such an ability. These views were confirmed by two-hybrid assays using AHP2 as bait (i.e., reciprocal two-hybrid assays) (panel B). We then asked if ARR10 can interact specifically with AHP2, or with other AHPs too. The results showed that ARR10 has an ability to interact with any AHPs tested (AHP1 to AHP3) (panels C and D, respectively). Since these yeast two-hybrid results do not necessarily indicate that they indeed do so in plants, the in vivo specificity of such a presumed interaction with amoung ARR10/AHPs remains to be examined carefully.

Implications—In this study, we inspected the occurrence of Arabidopsis response regulators, showing that this higher plant has, at least, 14 members of response regulators that can be classified into two distinct subtypes (Table 1). Although such members of response regulators must increase in future, the results in this study provided us with some clues for understanding the presumably-complex network of His-Asp phosphorelay signaling in *Arabidopsis thaliana*. Each representative of type-A and type-B response regulators, characterized in this and previous studies, showed distinct properties, as compared with each other type, in several facets [e.g., (i) structural designs, (ii) expression profiles, and (iii) biochemical properties]. (i) A type-B response regulator appears to have a certain functional domain(s) at its C-terminal part that might serve as a nuclear-localizing DNA-binding domain (so-called the output domain), whereas most of type-A response regulators seem to lack such a domain, thus they may function as a simple molecular switch, like the *E. coli* CheY protein (Parkinson and Kofoid 1992). (ii) Expression of all the type-A response regulator genes, characterized so far, is cytokinin-inducible in their transcription, while the type-B response regulator genes appears to be not. (iii) Type-A response regulators appear to have a strong phosphohistidine phosphatase activity toward AHPs, whereas those belonging to type-B may have an ability to make a stable complex with the same set of AHPs. It was recently found that another type-B respone regulator, ARR1, also exhibits an ability to interact with AHPs in yeast two-hybrid analyses (Suzuki et al. unpblished results). It was thus suggested that the type-A and type-B reponse regulators, respectively, interact with AHPs in a manner different from each other. In any case, verification of these simplified statements must await further experimentation. Nonetheless, the compiled and comparative results in this study should shed light on the presumed His-Asp phosphorelay signaling network that may be involved in adaptive responses to external and/or internal stimuli in *Arabidopsis thaliana*. If fact, our inspection of the current 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 (Hua and Meyerowitz 1998, and references therein), two (CKI1 and CKI2) were shown to be implicated in a cytokinin responsiveness (Kakimoto 1996, and personal communication), one (ATHK1) was proposed to be a putative osmosensor (Shinozaki, personal communication), and the occurrence of the other three were predicted in the Arabidopsis genomic sequences (note that we recently cloned the corresponding cDNAs, and confirmed their existence, Suzuki et al. unpublished data). Interestingly, most of these sensor His-kinases also contain a typical receiver domain at each C-terminal end, suggesting that they exert their His-Asp phosphorelay activites in a complicated fashion. Possible links between these 11 sensors, 14 ARRs, and 5 AHPs are entirely elusive.

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