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Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*

(*Escherichia coli*)

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ABSTRACT **The His to Asp phosphotransfer signal transduction mechanism involves three common signaling domains: the transmitter (or His-kinase), the receiver, and the histidine-containing phototransfer (HPt) domain. Typically, a sensor kinase has a His-kinase domain and a response regulator has a receiver domain containing a phosphoaccepting aspartate, whereas a histidine-containing phototransfer domain serves as a mediator of the histidine-to-aspartate phosphotransfer. This signal transduction mechanism was thought to be restricted to prokaryotes. However, many examples have been discovered in diverse eukaryotic species including higher plants. In *Arabidopsis*, three sensor kinases have been characterized, namely, ETR1, ERS, and CKI1, which were suggested to be involved in ethylene- and cytokinin-dependent signal transduction pathways, respectively. To date, no response regulator has been discovered in higher plants. We identify five distinct *Arabidopsis* response regulator genes, each encoding a protein containing a receiver-like domain. *In vivo* and *in vitro* evidence that ARRs can function as phosphoaccepting response regulators was obtained by employing the *Escherichia coli* His-Asp phosphotransfer signaling system.**

Bacteria have devised phosphotransfer signaling systems to elicit a variety of adaptive responses to their environment (refs. 1–4 and references therein). These signal transduction systems were originally referred to as ''two-component regulatory systems,'' typically consisting of two types of signal transducers, a ''sensor kinase'' and a ''response regulator'' (5, 6). The sensor kinase monitors an environmental stimulus, and modulates the function of a response regulator, which mediates changes in gene expression or cell behavior. Molecular communication between the sensor and its cognate regulator is based on a His-to-Asp (His-Asp) phosphotransfer. These signal transduction proteins contain one or more of the following common phosphotransfer signaling domains: the ''transmitter (or His-kinase)'' and ''receiver'' domain (3). A typical sensor kinase contains a C-terminal transmitter domain, whereas a response regulator contains an N-terminal receiver domain. The transmitter contains an invariant histidine residue, which is autophosphorylated in an ATP-dependent manner, while the receiver contains an invariant aspartate residue, which can acquire a phosphoryl group from the phosphohistidine of its cognate transmitter. Besides these two signaling domains, another common domain has been identified (7, 8). This histidine-containing phosphotransfer (HPt) domain contains a crucial histidine residue, which serves as a mediator of phosphotransfer by acquiring/transferring a phosphoryl group from/to another component (8–12).

Instances of His-Asp phosphotransfer systems have been found in a large number of bacterial species (3). Inspection of the nucleotide sequence of *Escherichia coli* reveals at least thirty different sensor-regulator pairs in this single species (13). This mechanism was once thought to be restricted to prokaryotes. However, many instances have been discovered in diverse eukaryotic species including higher plants (14–23). The most striking example is the yeast osmo-responsive system (23). Three components, Sln1p (sensor kinase)-Ypd1p (HPt domain)-Ssk1p (response regulator), constitute this osmo-responsive signaling pathway. This pathway is initiated by the autophosphorylation of the Sln1p osmosensor in the transmitter domain (at His-576). This phosphate is transferred to its own receiver domain (at Asp-1114), then to the Ypd1p-HPt domain (His-64), and finally to the receiver domain (at Asp-554) in Ssk1p. Interestingly, this His-Asp pathway is directly linked to a mitogen-activated protein kinase signaling cascade (23–25).

In higher plants, several sensor kinases have been reported (14, 18, 20), namely, for *Arabidopsis* (ETR1, ERS, and CKI1), and tomato (NR, Never-ripe). The ETR1, ERS and NR sensors are involved in the ethylene signal transduction pathway (14), whereas the CKI1 sensor is implicated in the cytokinin signal transduction pathway (20). However, neither a response regulator nor a HPt domain has been described, so far. It is plausible that these components also operate in higher plants. Here we address this issue in *Arabidopsis*, and find a group of response regulators that exhibit the activity expected for a common His-Asp phosphotransfer mechanism.

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. An *Arabidopsis* cDNA library in λgt11 was a gift from Shinozaki (RIKEN, Japan). To prepare cDNA, poly(A)$^+$ RNA was obtained from greening rosettes. An *Arabidopsis* genomic DNA library in λFIX was a gift from H. Kobayasi (Shizuoka Prefecture University, Japan).

***E. coli* and Related Materials.** *E. coli* K-12 strain DZ225 (F-, Δ*envZ*, *lacU169*, *araD139*, *rpsL*, *relA*, *flgB*, *thiA*) was used primarily in this experiment (26). This strain carries an *ompC*-

Abbreviations: His-Asp, His to Asp; ARR, *Arabidopsis* response regulator; HPt, histidine-containing phosphotransfer; β-gal, β-galactosidase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AB008486–AB008491).

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lacZ fusion gene on the chromosome. Bacteria were grown in Luria broth or on Luria agar plates at 37°C, unless otherwise noted.

PCR Amplification. Using appropriate pairs of primers, PCR amplification was carried out with the cDNA λ phage bank of *Arabidopsis*. The conditions were 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, 25 cycles [Takara Shuzo (Kyoto) Thermal Cycler 480]. 5′-Primers: 5′-CGTTGATGACAGTC(AT)(GC)GTTGATCG, 5′-CATGTTCTTGCTGTTGATGATAG, and 5′-CATGTTCTTGCCGTCGACGACAG. 3′-Primers: 5′-AGTTTCACCGGTTTCA(AG)TAAGAAATC.

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

Preparation of DNA/RNA and Hybridization. Total genomic DNA was isolated from *Arabidopsis* leaves by the method of Murray and Thompson (27). For Southern hybridization, DNA was digested with restriction endonucleases, subjected to electrophoresis in an 0.8% agarose gel, and then transferred to a Hybond-N+ nylon membrane (Amersham). Hybridization was carried out with ^{32}P-labeled DNA fragments as probes in a buffer containing 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA at 65°C for 18 h (28). The membranes were washed with 40 mM Na_2HPO_4 (pH 7.2) and 1% SDS for 5 min at 65°C three times, then for 15 min at 65°C, and then with 0.2 × standard saline citrate (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7), and 0.1% SDS for 30 min at 65°C., then autoradiographed (BAS-2000II, FUJIX, Tokyo). Total RNA was isolated from *Arabidopsis* roots or leaves by the phenol-SDS method (29). For Northern hybridization, RNA was separated in a 1% agarose gel 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/EDTA (SSPE; 0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution, and 0.5% SDS buffer containing 10% dextran sulfate and 100 μg/ml 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 autoradiographed as above.

Plasmid Constructions. The plasmid pET-ARR series was constructed as follows: A portion of each ARR coding sequence was amplified from the corresponding cDNA clone by using an appropriate pair of PCR primers. These amplified segments were cloned into an *E. coli* expression vector, pET22b(+) (Novagen), so as to be placed under the T7 phage promoter. Each ARR coding sequence thus cloned has an initial methionine codon from the vector and a C-terminally extended histidine-tag (see Fig. 3*A*). The PCR primers used are: ARR3, 5′-GGATCCATATGAGCGTCGGTGGTATCGGAGGA and 5′-GGATCCTTCCGTTTGTTTCCGTTGGAAAG; ARR4, 5′-GGATCCATATGATCGGTATAGGAATCGGAGAA and 5′-GGATCCAGCTTCCGTTTGTTTCCTTCGGC; ARR6, 5′-GAATTCCATATGGAGATTATCAACCATTCTTCA and 5′-GAATTCTCTCTCTTCTGAATACTCTTTGTGAA.

pET-ARR3, pET-ARR4, and pET-ARR6 were introduced into *E. coli* BL21, to overproduce the corresponding polypeptides with a histidine-tag (see Fig. 3*B*).

The plasmid pIN-ARR series were constructed as follows: The *Xba*I-*Hin*dIII segments containing the ARR coding sequences were isolated from the pET-ARR series (see Fig. 3*A*). These were inserted into the corresponding sites in an *E. coli* expression vector, pIN-III-A2 (26), to be placed under the *lpp-lac* promoter. Plasmid pIN-ARR3 was further subjected to site-directed mutagenesis with an oligonucleotide, 5′-TCATCACTAATTACTGTATGCC, to yield pIN-ARR3-ΔD.

Purification of ARR-H6 with a Ni Column. *E. coli* BL21 cells carrying pET-ARR plasmids were grown in M9-glucose medium containing 0.2% casamino acids and 15% sucrose. A cleared cell lysate was obtained after extensive sonication of the cells. This sample was applied to a Ni column with the rapid affinity purification pET His-Tag^R system, supplied by Novagen. Other details were those recommended by the supplier.

***In Vitro* Phosphotransfer Experiments.** The ArcB^c polypeptide (HPt domain) was purified as described (7). Urea-treated membranes were also prepared from *E. coli* cells overproducing the ArcB kinase, as described (30). The urea-treated membranes (25 μg) were incubated with the purified ArcB^c (5 mg) at 37°C for 30 min in the presence of 0.05 mM [γ-^{32}P]ATP (10,000 cpm/pmol), 200 mM KCl, and 5 mM $MgCl_2$ in TEDG buffer (Tris·HCl, EDTA, DTT, glycerol), as described (31, 32). The reaction mixture was immediately applied onto a Sephadex G-75 column previously equilibrated with TEDG buffer. Fractions containing the labeled ArcB^c, which were essentially free of membranes and ATP, were collected. This purified phospho-ArcB^c (1 μg) was incubated with purified ARR-H6 polypeptides (0.5 μg) for short periods in TEDG buffer (10 μl) containing 66 mM KCl and 7 mM $MgCl_2$ at 37°C. After incubation, the samples were immediately subjected to SDS/PAGE. Other details were the same as those described previously for the *in vitro* EnvZ-OmpR phosphotransfer experiment (31, 32). SDS/PAGE was carried out according to the method of Laemmli (33). The gels were exposed on a phosphoimager (BAS-2000II, FUJIX).

Enzyme Assays. *E. coli* DZ225 cells carrying appropriate plasmids were grown to the mid-logarithmic phase in Luria broth. The cells were collected and suspended in 250 mM sodium phosphate (pH 7.1) for determination of the cell density. β-Galactosidase (β-gal) activity was determined by Miller's method, after permeabilization with toluene (34).

RESULTS

Isolation of a Set of cDNA Clones That Could Specify Response Regulator-Like Proteins. The genomic sequence of the cyanobacterium *Synechocystis* PCC 6803 contains many ORFs, each of which could encode a response regulator containing a receiver domain (35). By using this set of deduced amino acid sequences as probes, we searched the EST database for sequences similar to those of the bacterial receivers (http://www.tigr.org/, The Institute for Genomic Research). This inspection led to several *Arabidopsis* sequences (e.g., accession numbers TC10702, TC9791, and TC8770), each of which seemed to specify a sequence similar to those of the bacterial receivers. Based on these EST cDNA sequences, several pairs of oligonucleotide primers were designed and used for PCR amplification of a λgt11 bank of *Arabidopsis* cDNA. Many amplified PCR fragments were cloned and their nucleotide sequences were determined. The results showed at least five distinct *Arabidopsis* cDNA fragments (≈340 bp each). Their deduced amino acid sequences suggested that each might correspond to a portion of a receiver-like sequence. To isolate the entire cDNAs, the λgt11-bank of *Arabidopsis* cDNA was further screened with each of the five PCR fragments as probes. In each case, positive cDNA sequences with a poly(A)$^+$ tail were identified. Among them, the largest representatives (≈750–1,200 bp) were sequenced . These five cDNA clones were each assumed to encode an entire ORF. Their amino acid sequences are highly similar to, but clearly distinct from, each other (Fig. 1*A*).

These predicted amino acid sequences were compared with that of the classical *E. coli* response regulator, CheY (Fig. 1, note that the CheY response regulator contains only a receiver domain) (37, 38). The five *Arabidopsis* sequences are somewhat

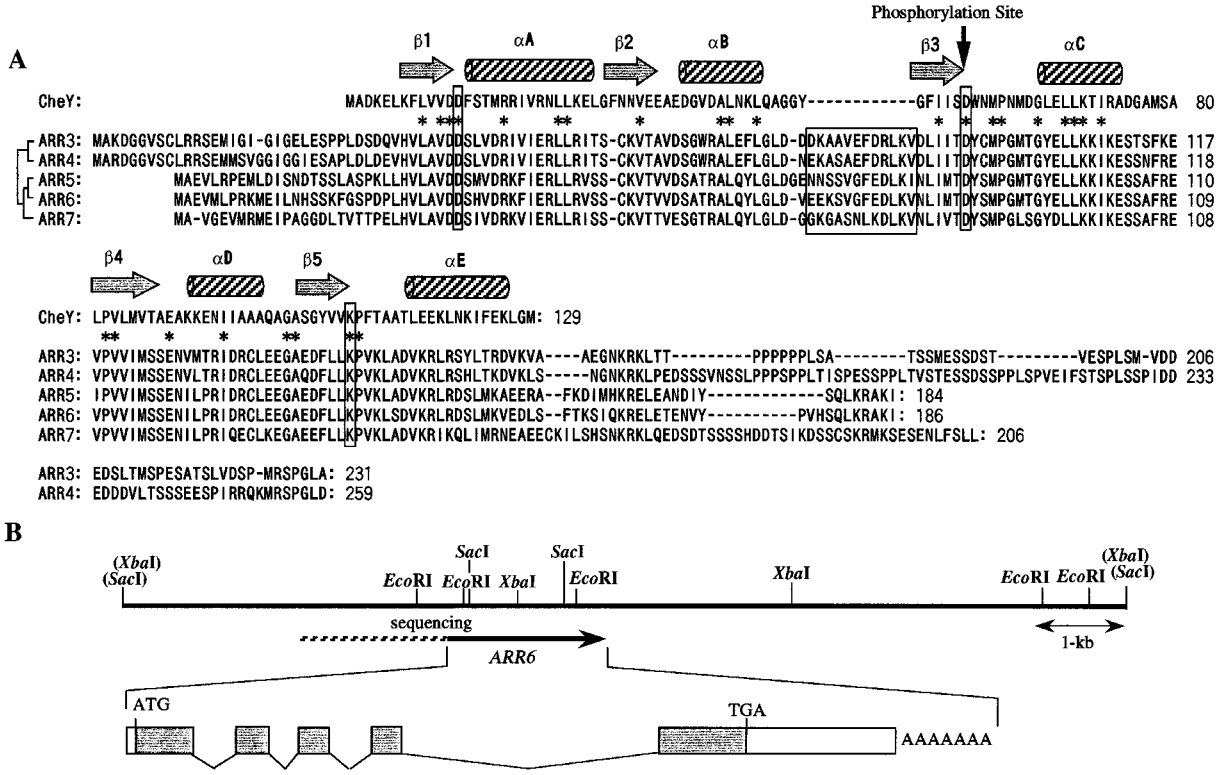

FIG. 1. Alignments of deduced amino acid sequences of *Arabidopsis* ARRs with that of the *E. coli* CheY response regulator. (*A*) The CheY amino acid sequence is shown at the top. Gaps (– – –) were introduced for optimal alignment. Three amino acid residues, which are invariant in the bacterial receiver sequences, are boxed, and the phosphoaccepting aspartate residue is indicated. The extra sequences found in ARRs are also boxed. (*B*) The genomic structure determined for the ARR6 gene; the sequenced region is indicated by the arrow. The restriction sites in parentheses are from the λgt11 phage vector.

similar to that of the CheY receiver. Although the similarities are not so strikingly evident, they all possess a set of critical amino acid residues that are known to be conserved invariantly in bacterial receivers (e.g., Asp-13, Asp-57, and Lys-109 in CheY, which are crucial for CheY phosphorylation, with Asp-57 the phosphorylation site). We supposed that these five cDNA clones each specify distinct response regulator-like polypeptides in *Arabidopsis* (named for *Arabidopsis* response regulator, or ARR-series). Compared with CheY, each ARR has a characteristic stretch of 12 extra amino acids at the center of the presumed receiver sequence. They also have extended amino acid sequences at both the N and C termini. These extra sequences show no significant similarity to those of any other proteins in the current databases. The C-terminal extra sequences differ from each other. As a whole, however, ARR3 (231 amino acids) is similar to ARR4 (259 amino acids), and ARR5 (184 amino acids) is similar to ARR6 (186 amino acids).

Structure and Expression of the ARR Genes. The copy numbers of the ARR genes in the *Arabidopsis* genome were examined by Southern blot analysis. Genomic DNA was isolated from *Arabidopsis* leaves, digested and analyzed as described in *Materials and Methods* (Fig. 2*A*). In each case, only one positive band was detected for each ARR probe. Furthermore, the hybridization profiles were quite different from one another. These findings confirmed the existence of a multigene family of ARRs in this plant. Standard Northern blot analyses of the ARR transcripts were also carried out, by using total RNA preparations from seedlings, roots, leaves, stems, flowers, and siliques of *Arabidopsis* grown under the standard laboratory conditions. Such analyses revealed that ARR transcripts could be detected for all of the ARR genes, which appeared to be expressed predominantly in the roots (Fig. 2*B*). Compared with roots, ARR expression in the other organs was much lower, although significant levels of expression were detected in other organs, e.g., in leaves in some cases (data not shown). The sizes of the transcripts (≈1 kb each), detected by Northern analysis, were consistent with those of the cDNA clones analyzed.

We cloned and determined the entire genomic sequence for ARR6, as an example (Fig. 1*B*). These results confirmed the cDNA sequence determined for ARR6, and also revealed that the coding sequence of ARR6 is interrupted by four introns.

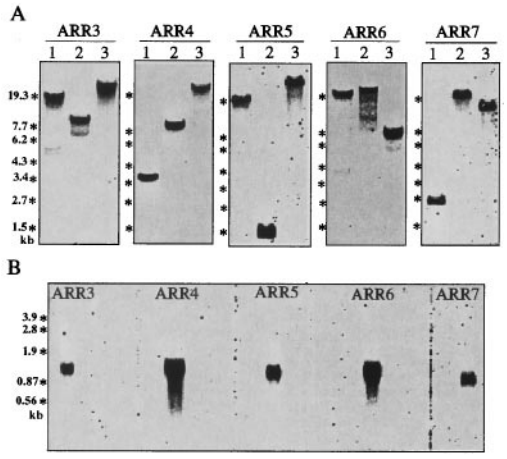

FIG. 2. Southern and Northern blot analysis. (*A*) Southern blot analysis was carried out for the ARR genes, by using each specific probe indicated, under high-stringency conditions. Genomic DNA was digested with *Bam*HI (lane 1), *Eco*RV (lane 2), and *Pst*I (lane 3). (*B*) Northern blot analysis to locate the ARR transcripts, by using each specific probe indicated. Total RNA was isolated from *Arabidopsis* roots. The positions on the gels were determined by using appropriate standards (indicated in kb at left side).

A 1.7-kb sequence was also determined for the 5′-upstream region of the ARR6 coding sequence, which should include the promoter region of the ARR6 gene. Physical or genetic mapping of these ARR genes on the *Arabidopsis* genome remains to be done.

ARRs Exhibit *in Vitro* Activity Characteristic of Bacterial Receivers. A critical issue is whether the *Arabidopsis* ARR gene products are indeed functional. For ARR3, ARR4 and ARR6, appropriate regions of the cDNA sequences were placed in an *E. coli* overexpression vector (Fig. 3*A*). The cloned regions corresponded to each presumed receiver domain (ARR3, amino acid positions Met-15 to Leu-175; ARR4, Met-16 to Leu-175; ARR6, Met-10 to Arg-167, see Fig. 1*A*). By using these plasmids, the ARR polypeptides with a histidine tag were expressed in *E. coli*, and then purified as described (Fig. 3B). These ARR polypeptides were examined in terms of their ability to acquire a phosphoryl group *in vitro*. The phosphodonor protein was the purified *E. coli* ArcB^c polypeptide, which is a typical HPt domain derived from the ArcB sensor kinase (7). The nature of this HPt domain has been well characterized (9). *In vivo* and *in vitro*, this domain is phosphorylated at His-717, and is capable of transferring its phosphoryl group to certain receivers (e.g., the *E. coli* OmpR response regulator, see Fig. 4*B*). Based on this example, we determined whether the purified ARR polypeptides (designated as ARR-H6) can acquire the phosphoryl group from a labeled phospho-HPt domain in a purified system (Fig. 3*C*). The labeled HPt domain was purified (lane 0), and then ARR3-H6 was suspended in a buffer (pH 7.8) containing 66 mM KCl and 7 mM $MgCl_2$. At short time intervals, the samples were analyzed by SDS/PAGE, followed by autoradiography. The radiolabel on phospho-ArcB^c disappeared very rapidly, and phospholabeled ARR3-H6 was detected transiently. Essentially the same reaction profiles were observed for ARR4-H6 and ARR6-H6 (data not shown). Control samples were prepared from *E. coli* lysates through essentially the same Ni-column procedures, except that the lysates were of cells carrying the control vector. Nothing happened in this case (Fig. 3*C*). When these purified ARR-H6 polypeptides were incubated alone with free ^{32}P-ATP, they incorporated no radiolabel (data not shown). Based on our current knowledge of the His-Asp phosphotransfer mechanism, these findings are best interpreted to mean that the phosphoryl group on the HPt domain was transferred rapidly to an aspartate residue of ARR-H6, and then the phosphoryl group was released from ARR-H6. In other words, the purified ARR-H6 polypeptides exhibit *in vitro* activity characteristic of the common bacterial receivers.

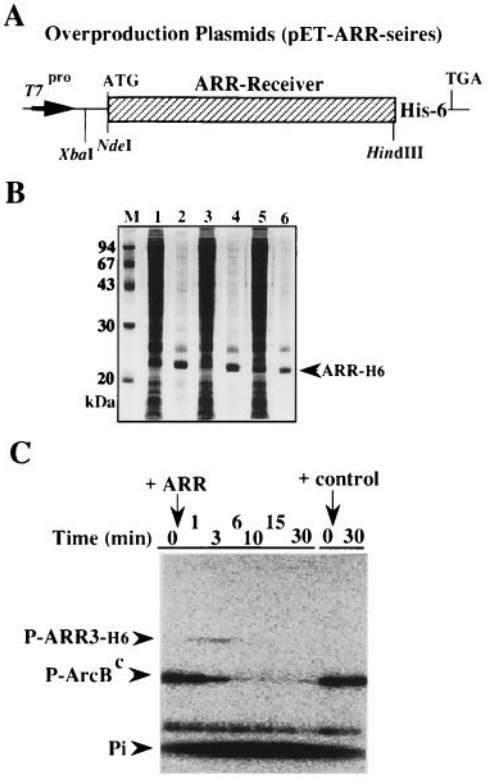

FIG. 3. Isolation of the ARR receiver domains and characterization of their *in vitro* activity. (*A*) Plasmids (pET-ARR series) were used to overexpress the ARR receiver domains in *E. coli*. The ARR genes can be expressed from plasmids under the T7 phage promoter, and the presumed ARR translation products were followed by a histidine tag (His × 6) at their C termini. (*B*) These overexpressed ARR products (designated as ARR-H6) were analyzed by SDS/PAGE (lane 1, ARR3-H6; lane 3, ARR4-H6; lane 5, ARR6-H6). They were then purified by Ni-column chromatography (lane 2, ARR3-H6, lane 2, ARR4-H6; lane 6, ARR6-H6). These samples were used for the following experiment. (*C*) *In vitro* analyses of phosphorylation and dephosphorylation of the ARR receiver domains. The purified ArcB^c polypeptide (HPt domain) was ^{32}P labeled (lane 0, designated as P-ArcB^c), as described (7, 9). This preparation was incubated with ARR3-H6. At intervals (indicated by min), samples were immediately analyzed by SDS/PAGE, followed by autoradiography. Note that ARR3-H6 was rapidly phosphorylated and dephosphorylated. Appropriate control experiments are described in the text.

ARR Exhibits *in Vivo* Activity Expected for His-Asp Signaling in *E. coli*. The above finding led to the idea that these ARRs might be able to function as His-Asp signal transducers in *E. coli*. Thus, a set of plasmids was constructed (pIN-ARR series), by which the presumed receiver domain of each ARR can be expressed in *E. coli* (Fig. 4*A*). By using these plasmids, the *in vivo* nature of ARRs was assessed on the basis of the following experimental rationale. We employed the plasmid (pIMA005) that specifies a mutant ArcB protein (named ArcB*) containing His-717, but lacking both His-292 (His to Leu substitution) and Asp-576 (Asp to Gln substitution) (see

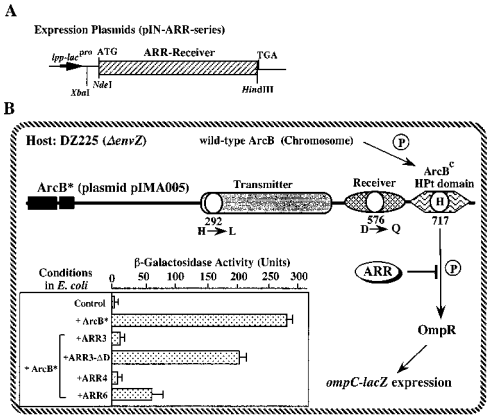

FIG. 4. *In vivo* interaction between an *E. coli* HPt domain and the ARR receivers. (*A*) The structure of plasmids (pIN-ARR-series) by which the ARR receiver domains were moderately expressed in *E. coli* by using the *lpp-lac* promoter. (*B*) An experimental design and the determined β-galactosidase activity in *E. coli*, showing a possible *in vivo* interaction between the *E. coli* HPt domain and the ARR receiver domains. The *E. coli* strain DZ225 (Δ*envZ*, *arcB*$^+$) carrying an *ompC-lacZ* fusion was transformed with plasmid pIMA005. This plasmid carries a mutated *arcB* gene, to express a mutant ArcB sensor kinase (ArcB*). The ArcB* protein has only a functional HPt domain. In *E. coli*, one can observe a typical His-Asp multistep phosphotransfer signaling pathway, namely, ArcB (transmitter-receiver) to ArcB* (HPt domain) to OmpR (receiver). This particular signal transduction can be monitored by measuring *ompC-lacZ* expression (β-galactosidase activity) (7, 9). The pIN-ARR series of plasmids were further introduced into these cells and β-galactosidase activities were determined, as indicated in the inset graph. Other details are given in the text.

Fig. 4*B*). The mutant ArcB* protein has a functional HPt domain (ArcB^c), but its transmitter and receiver domains are presumably inactive. Provided that ArcB* is expressed through a multicopy plasmid, it is capable of phosphorylating the OmpR response regulator (7, 39, 40). The resultant phospho-OmpR activates an *ompC-lacZ* fusion gene in strain DZ225 (Δ*envZ*), as shown schematically in Fig. 4*B*. The *ompC* gene is a target of the OmpR transcriptional activator (40). This particular *in vivo* event can be used as a hallmark of the typical His-Asp signaling between the HPt domain and the receiver domain in *E. coli*, as demonstrated (7, 9). As shown in Fig. 4*B* (see *Inset*), *E. coli* strain DZ225 carrying an *ompC-lacZ* fusion gene exhibited a Lac$^-$ phenotype (control). When the strain was transformed with pIMA005 carrying the *arcB** gene, the β-galactosidase activity of the transformant increased substantially (+ArcB*), as explained above. Importantly, when plasmids of the pIN-ARR-series, each carrying an ARR gene, were further introduced into the cells, the level of β-galactosidase activity dramatically decreased (+ARRs). These *in vivo* results are interpreted to mean that the ARR receivers, expressed in *E. coli*, compete with the OmpR receiver by titrating the phosphoryl group from the HPt domain of ArcB. This interpretation is consistent with the *in vitro* results shown in Fig. 3. More importantly, when the presumed phosphoaccepting aspartate residue in the ARR3 receiver was changed to an asparagine residue (Asp-94 to Asn, see Fig. 1*A*), the mutated ARR3 gene lost the ability to interfere with activation of the fusion (+ARR3-ΔD). This result indicates that the presumed phosphoaccepting Asp-94 in the ARR3 receiver indeed plays a crucial role in the His-Asp phosphotransfer.

From these *in vitro* and *in vivo* results (Figs. 3 and 4), we conclude that the family of ARR genes in *Arabidopsis* encode response regulators, for which the receiver domains are capable of functioning as phosphoacceptors.

DISCUSSION

In *Arabidopsis*, three sensor kinases have so far been discovered, namely, ETR1, ERS, and CKI1, all of which contain a transmitter domain (14, 18, 20). The ETR1 and CKI1 sensors also contain a receiver domain at their relatively C-terminal ends. The ETR1 and ERS kinases function as ethylene receptors, while the putative CKI1 kinase is implicated in a cytokinin signal transduction pathway. One can reasonably suppose that His-Asp phosphotransfer signaling systems operate in *Arabidopsis*. If so, it should also have other signal transducers that function as either response regulators or HPt domains (see Introduction). In this study, we demonstrated that *Arabidopsis* indeed possesses a group of response regulators, each of which contains a phosphoaccepting receiver domain (Fig. 1).

Five distinct ARR genes were found to be expressed in *Arabidopsis* (Fig. 2), and the corresponding cDNA clones were obtained. Each ARR gene encodes a protein containing a stretch of amino acids similar to those of bacterial receivers. In particular, they possess all of the amino acid residues characteristic of bacterial receivers at appropriate positions (Fig. 1). The deduced ARR receivers have relatively short extensions at both their N and C termini. Besides these unassigned sequences, they contain neither a transmitter nor a HPt domain.

To prove that ARRs function as phosphoaccepting receivers in a His-Asp signaling pathway, we obtained *in vivo* and *in vitro* evidence that ARRs are capable of functioning as typical response regulators, by employing a well-characterized *E. coli* His-Asp phosphotransfer system. They exhibit phosphoaccepting receiver activity, and a certain aspartate residue was shown to be crucial for the phosphotransfer reaction (Figs. 3 and 4). The *in vitro* results showed further that the observed phosphorylation of the ARR polypeptides was transient (Fig. 3). Based on the current concept of bacterial receiver domains, this particular event is not surprising, but rather consistent with the concept. In general, a receiver domain is capable of accepting a phosphoryl group from a phosphohistidine on a transmitter or HPt domain (3). This phosphotransfer reaction appears to be catalyzed by the receiver itself. In this sense, receivers are enzymes capable of undergoing "autophosphorylation," by using a phosphohistidine residue as substrate. The phosphorylated receiver is relatively unstable, and is readily converted to its unphosphorylated form, because receiver dephosphorylation also appears to be autocatalytic (3). For example, the observed *in vitro* half-life of phospho-CheY is only a few seconds. In this sense, receivers are enzymes capable of undergoing "dephosphorylation," and this apparent autophosphatase activity confers a characteristic half-life on a given receiver. The results in Fig. 3*C* for ARRs revealed both of these functional properties, which are characteristic of classical bacterial receivers. We conclude that ARRs of *Arabidopsis* are indeed capable of functioning as typical receivers involved in the His-Asp phosphotransfer signaling.

Although we mainly intended to demonstrate the presence of functional response regulators in plants, it is of interest to discuss the possible function of ARRs in *Arabidopsis*. Like the *E. coli* CheY receiver that regulates flagella rotation (37, 38), these small ARR response regulators may function as an on-off molecular switch at an intermediate step of a His-Asp phosphotransfer system in *Arabidopsis*. In this sense, some ARRs may operate in a downstream step of the presumed His-Asp phosphotransfer pathway initiated by the ETR1 sensor, or others may be doing so in another pathway mediated by the CKI1 sensor. Is a signal transducer containing the HPt domain involved in the His-Asp phosphotransfer pathway in *Arabidopsis*? We demonstrated that the *Arabidopsis* ARR receivers can acquire a phosphoryl group from a typical *E. coli* HPt domain (Fig. 3). Together with this fact, one can suspect the existence of another type of signal mediator (or HPt domain) operating between the sensors and the response regulators in *Arabidopsis*. In this respect, we recently identified four distinct *Arabidopsis* cDNA clones (named AHP1 to AHP4), each encoding a polypeptide containing a putative HPt domain (unpublished result).

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