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# An *Arabidopsis* protein that interacts with the cytokinin-inducible response regulator, ARR4, implicated in the His-Asp phosphorylay signal transduction

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**Abstract Previously, *Arabidopsis thaliana* was shown to possess a set of response regulators (ARR-series), which are implicated in the prokaryotic type of signal transduction mechanism, generally referred to as the His-Asp phosphorylay. Among them, ARR4 is a typical phospho-accepting response regulator, whose expression was recently demonstrated to be rapidly induced by a cytokinin-treatment of the plant. To gain insight into the presumed His-Asp phosphotransfer signaling mechanism as well as the role of ARR4 in this higher plant, in this study we adopt the widely used yeast two-hybrid system, and report the identification of an *Arabidopsis* protein that has an ability to interact physically with the cytokinin-inducible ARR4 response regulator.**



*Key words:* Response regulator; Two-hybrid analysis; His-Asp phosphorylay; Signal transduction; *Arabidopsis thaliana*

## 1. Introduction

The widespread His-Asp phosphorylay signaling systems are characterized by three types of common signal transducers: a sensor His-kinase, a histidine-containing phosphotransmitter (HPt), a response regulator [1–6]. Such signal transduction systems were once thought to be restricted to prokaryotes. However, many instances were recently discovered in diverse eukaryotic species including yeasts [7–9], fungi [10], slime molds [11–13], and higher plants [14,15]. The best characterized is the osmo-responsive signal transduction pathway in the budding yeast, which involves three components, namely, Sln1p (sensor His-kinase), Ypd1p (HPt), and Ssk1p (response regulator) [7–9]. Interestingly, this signaling pathway is directly linked to a classical MAP (mitogen-activated protein) kinase signaling cascade [16]. This instance suggests that the prokaryotic type of His-Asp phosphorylay mechanism and the eukaryotic type of protein phosphorylation cascade are fully compatible in a given eukaryotic cell to perform in concert a task of intracellular signal transduction.

In the higher plant, *Arabidopsis thaliana*, several sensor His-kinases have been reported (e.g. ETR1 and CKI1) [14,15,17,18]. The ETR1 sensor functions as an ethylene receptor, while the CKI1 sensor is presumably involved in a cytokinin-mediated signaling pathway. Furthermore, we and others recently demonstrated that this plant possesses a group of response regulators (ARR-series) that contain a functional phospho-accepting receiver domain [19,20]. In the light of the well-established yeast scenario mentioned above, it is tempting to speculate that some response regulators may operate at a downstream of the presumed His-Asp phosphorylay initiated by either the ethylene-responsive sensors or the cytokinin-responsive sensors. In fact, Taniguchi et al. [21], and Brandstatter and Kieber [22] demonstrated recently that certain response regulators, ARR4/IBC7 and ARR5/IBC6, are markedly and rapidly induced by a cytokinin-treatment of *Arabidopsis*, supporting the view that these particular response regulators may be implicated in the presumed cytokinin-responsive signal transduction. However, examination of such a newly emerging signaling pathway in this higher plant is at a very early stage. To clarify such a singling network, one of possible approaches would be to search proteins that interact physically with the response regulators (ARRs). To this end, here we adopt the yeast two-hybrid system, and report the identification of *Arabidopsis* protein that has an ability to interact with the cytokinin-inducible ARR4 response regulator.

## 2. Materials and methods

### 2.1. Yeast two-hybrid system

A kit for two-hybrid analysis (MATCHMAKER, Clontech) was obtained through TOYOBO Co. 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 Clontech, namely, *Arabidopsis thaliana* MATCHMAKER cDNA Library. cDNAs were prepared from mRNA of 3 weeks old green vegetative tissue of *Arabidopsis* (cv. Columbia).

### 2.2. Plasmid construction

The following plasmids were constructed from pGBT9 (see above), namely, pGBT9-ARR3, pGBT9-ARR4, and pGBT9-ARR4-ΔC. Each coding sequence was prepared by polymerase chain reaction (PCR) with an appropriate pair of primers: ARR3, 5′-CGGTCGACTTTC-TTGTCTACGAAGGTCG and 5′-CACTGCAGTCTCTAAGCTA-ATCCGGGAC; ARR4, 5′-CGGTCGACTTTCTTGTTTACGAA-GGTCG and 5′-ATACTGCAGCTAATCTAATCCGGGACTC; ARR-ΔC, 5′-TTGGATCCATATGAGCGTCGGTGGTATC and 5′-TAGGATCCTTCCGTTTGTTTCCGTTG. Each amplified DNA segment was ligated into the corresponding restriction sites in pGBT9.

### 2.3. Protein expression in Escherichia coli and purification

For a series of in vitro experiments, commercially available *E. coli* expression systems were used: one is the pET His-tag system (plasmid pET-22b+, Novagen), and the other is the GST gene fusion system (plasmid pGEX-4T-1, Novagen). To construct pET-ARR4-F, the almost entire ARR4 coding sequence was prepared by PCR with primers (5′-GGGGATCCATATGAGCGTCGGTGGTATCGGAG-GA and 5′-CACGGATCCAATCCGGGACTCCTCATCTTCTGC).

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pET-ARR4-ΔC was constructed previously (formerly named pET-ARR4) [19]. To construct pGEX4T-1-AtDBP-ΔN, pGEX-4T-1, which was derived from the cDNA-expression bank (see above), was digested with *Eco*RI digestion. This *Eco*RI fragment was ligated into the corresponding sites in pGEX40T-1. To isolate His-tagged polypeptides, the rapid affinity purification pET His-Tag system was used (Novagen), whereas, to isolate GST-fusions, Bulk and RediPack GST Purification Modules was used (Pharmacia Biotech). Other details were those recommended by each supplier.

## 3. Results

### 3.1. Experimental rationale and design

As reported previously [19], the *Arabidopsis* response regulators, named ARR3 (231 amino acids) and ARR4 (259 amino acids) have a common receiver domain (about 140 amino acids each). In both the proteins, however, each receiver domain is followed by each unique C-terminal extension (see Fig. 2). The amino acid sequences of both the receiver domains are almost identical (90% identical and 7% similar), yet, those of the C-terminal extensions are quite divergent from each other (i.e. they exhibit virtually no homology, but, both the sequences are rich in proline, serine, glutamate, and aspartate residues). In the hope of searching *Arabidopsis* proteins that interact with these response regulators, the yeast two-hybrid system was adopted. The *ARR3* coding sequence extending from Ser-8 to the C-terminal Ala-231 was connected to the GAL4 DNA-binding domain in pGBT9, to yield a bait vector (named pGBT9-ARR3). Similarly, the *ARR4* coding sequence extending from Ser-8 to the C-terminal Asp-259 was appropriately placed in the vector, to yield another bait (named pGBT9-ARR4). An *Arabidopsis* cDNA-expression library was obtained, in which approximately $3\times10^6$ independent cDNA clones were connected to the GAL4 activation domain in the yeast vector pGAD10. An extensive two-hybrid screening was carried out with the yeast strain HF7c carrying both the *GAL1-HIS3* and (*GAL4* 17-mers)$_3$-*CYC1-lacZ* fusions on the genome, on the bases of the histidine-autotrophy as well as the LacZ-expression. The putative candidates (pGAD10-series), thus scored, were further selected in another strain SFY526 carrying the *GAL1-lacZ* fusion. Among $10^7$ yeast transformants screened, no positive clone was obtained for pGBT9-ARR3. For GBT9-ARR4, however, we succeeded in isolating 13 positive clones, each of which was assumed to carry an *Arabidopsis* cDNA, whose translational product might interact with ARR4 in the yeast cells.

### 3.2. Characterization of the Arabidopsis cDNA clones

The thirteen *Arabidopsis* cDNA clones were examined by restriction endonuclease digestion. The results showed that these cDNA inserts were classified into three distinct groups (three in Clone-A, six in Clone-B, and four in Clone-C). The members of each group were assumed to have each identical cDNA insert. In any case, all of them were analyzed by nucleotide sequencing. As the results, we identified three cDNA clones, which are distinctive from, but clearly related to, each other, as shown in Fig. 1. The cDNA insert of Clone-A corresponded to the almost entire coding sequence of the previously characterized *Arabidopsis* gene, called *AtDBP* (*Arabidopsis thaliana* DNA-binding protein) [23]. The cDNA insert of Clone-B appears to be derived from the same *AtDBP* gene, although its 5′-portion corresponding to the N-terminal 54 amino acids is missing. Interestingly, the deduced amino acid sequence for Clone-C is highly homologous to that of AtDBP. This cDNA also appears to lack its 5′-portion, but note that its deduced amino acid sequence is 77% identical to that of AtDBP. Henceforth, the previously characterized AtDBP was designated as AtDBP1 (Clone-A and -B), whereas the newly identified homologue was designated as AtDBP2 (Clone-C). In any case, these results are convincing enough for us to believe that these fishes are not false positives, and that the identified AtDBPs may exhibit an ability to interact with ARR4.

### 3.3. Interaction and specificity

Thus, three representatives were characterized to demonstrate that they are indeed positive clones as far as the yeast two-hybrid analyses are concerned (Fig. 2A). The yeast strain HF7c carrying pGBT9-ARR4 and each one of the identified clones was streaked on appropriate agar-plates to examine the histidine-autotrophy, and the same cells were assayed for the β-galactosidase activity. The results clearly gave us a positive sign. We further analyzed the specificity of the presumed interaction between ARR4 and AtDBP1, by utilizing one of the clones (Clone-B, named pGAD10-AtDBP1-ΔN) (Fig. 2B). To this end, we examined three different baits, pGBP9-ARR4, pGBP9-ARR4-ΔC, and pGBP9-ARR3, as schematically indicated in Fig. 2B. In contrast to the case of ARR4, neither the

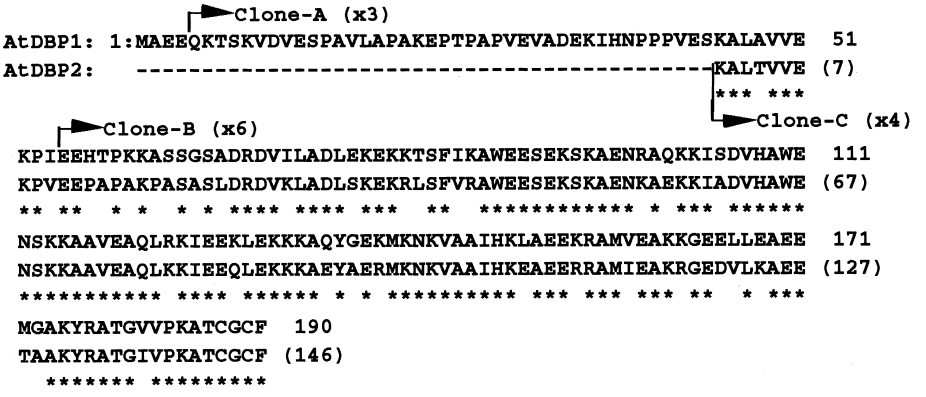


Fig. 1. Identification of proteins that may interact with ARR4. A: Three types of positive clones were identified (13 clones were classified into A, B, C, as indicated), their deduced amino acid sequences are shown. Clone-A and -B were found to correspond to the previously characterized *Arabidopsis* DNA-binding protein (AtDBP1), whereas Clone-C was found to specify a homologue of AtDBP1 (thus named AtDBP2).

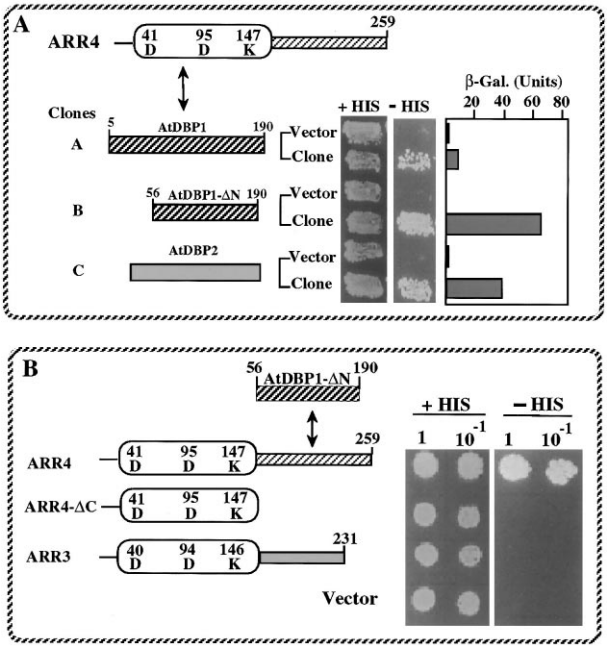


Fig. 2. Two-hybrid analyses. Two-hybrid analyses were carried out, as schematically shown. The results were scored with special reference to the histidine-autotrophy on appropriate agar-plates (+His and −His, respectively) (A and B) and the LacZ-expression (A).

intact ARR3 nor the truncated ARR4-ΔC lacking the C-terminal region were not functional, as judged by the histidine-autotrophy. These results suggested that the common (or highly conserved) receiver domain is not sufficient for the presumed ARR4-AtDBP1 interaction, and that the unique C-terminal region of ARR4 is responsible for the interaction (it is not known whether or not the C-terminal domain is sufficient). The N-terminal region of AtDBP1 may exhibit an inhibitory effect on the interaction, because Clone-A showed a poor ability, as conapred with Clone-B (see Fig. 2A).

### 3.4. In vitro physical interaction between ARR4 and AtDBPs

It was critical to examine the presumed interaction between ARR4 and AtDBP1 by using an in vitro purified system. The entire coding sequence of ARR4 was introduced into an appropriate *E. coli* His-tag expression vector, whereas the coding sequence of AtDBP1-ΔN was C-terminally fused to the glutathione S-transferase (GST) coding sequence in an appropriate GST expression vector. The His-tagged ARR4 polypeptide was purified (Fig. 3A, lane 1). Similarly, the His-tagged ARR4-ΔC polypeptide lacking the C-terminal regions was also purified (lane 9). The GST-AtDBP1-ΔN fusion was expressed in *E. coli*, and then it was recovered in a crude soluble fraction of the *E. coli* lysate (lane 2). This crude lysate containing the GST-AtDBP1-ΔN fusion was first mixed with glutathione Sepharose 4B, in order to specifically retain the fusion on the resin. Then, the resin containing the GST-AtDBP1-ΔN fusion was incubated in the absence and presence of the purified ARR4 polypeptide (lanes 3 and 4, respectively). Finally, the GST-AtDBP1-ΔN fusion was specifically eluded from the resin with a buffer containing glutathione, in order to see if the ARR4 polypeptide was concomitantly released. The ARR4 polypeptide was identified by immunoblotting with an anti-ARR4 antiserum (Fig. 3B). The results showed that ARR4 was clearly recovered with GST-AtDBP1-ΔN (lane 4). Also conducted were critical control experiments, which included the following: (a) incubation of the ARR4 polypeptide with the plain GST polypeptide (lane 7), (b) incubation of ARR4 alone without the GST-AtDBP1-ΔN fusion (lane 8), and (c) incubation of the ARR4-ΔC polypeptide with the GST-AtDBP1-ΔN fusion (lane 10). Taking all these results together, it was concluded that the ARR4 and AtDBP1 polypeptides are capable of interacting physically and specifically with each other under the certain in vitro conditions.

### 3.5. Inspection of the AtDBP sequence

The *Arabidopsis* gene encoding AtDBP1 was characterized almost a decade ago [23]. Since then, no further study dealing

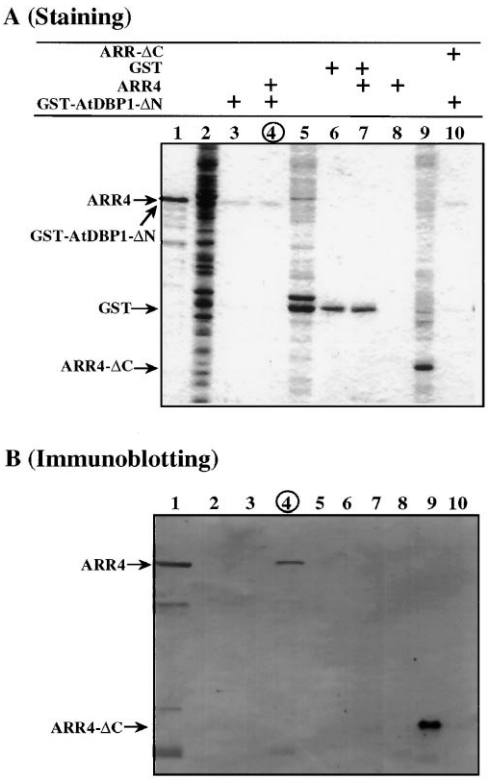


Fig. 3. In vitro binding assay showing a specific interaction between ARR4 and AtDBP1. A: The following polypeptides were prepared: lane 1, ARR4; lane 2, GST-AtDBP1; lane 5, GST; lane 9, ARR-ΔC. By using these polypeptides, a series of in vitro binding assays were carried out (lanes 3, 4, 6, 7, 8, and 10), as follows. In a tube containing glutathione Sepharose 4B, first, either GST-AtDBP1-ΔN or GST were added to be retained on the resin (note that the amount of GST-AtDBP1-ΔN used was 20 times of that applied in lane 2), and then either ARR4 or ARR-ΔC were further added (note that the amounts of these proteins added were 10 times of those applied on lanes 1 and 9, respectively), in each combination indicated. After washing extensively with a phosphate buffer, the resin was treated with a buffer containing glutathione to specifically elute GST-AtDBP1. The eluted samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, followed by Coomassie Brilliant Blue. B: The SDS-polyacrylamide gel was subjected to immunoblotting analysis with an anti-ARR4 polyclonal antiserum (the gel was prepared essentially with the same procedures as those for the gel-A, except that the amount of ARR4 in lane 1 was 1/10 of that in the gel-A). Note that the most critical lanes are highlighted by circles. Other details are given in the text.

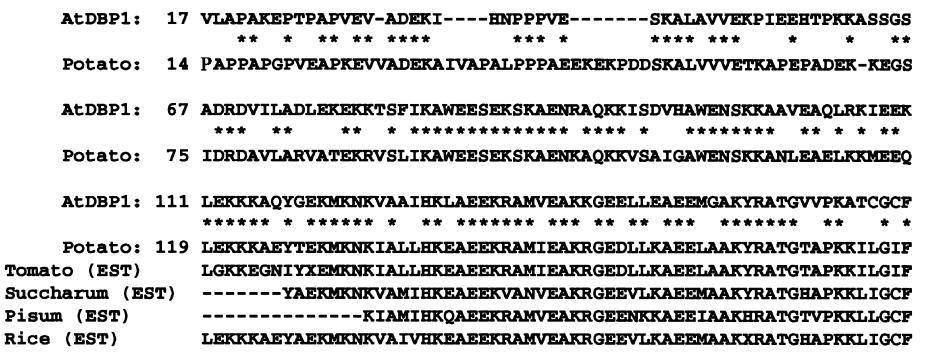


Fig. 4. Alignments of amino acid sequences of a set of AtDBP1 homologues in higher plants. The amino acid sequences of AtDBP1 and a potato protein (known as Remorin) were aligned in their entire regions (each short N-terminal region was deleted for clarity of this figure). Several amino acid sequences, deduced from the current plant EST databases, were also aligned (GenBank accession numbers: tomato, AA824852; succharum, AA842790; pisum, AA430932; rice, AA772757). Note that only their presumed C-terminal sequences, consisting of 40–60 amino acids, are available in the EST databases.

with this particular gene has appeared. To gain insight into AtDBP1, therefore, it would be worth inspecting the AtDBP1 sequence on the bases of the currently available databases. Such an inspection of the GenBank database with the BLAST programs revealed the existence of a few more coding sequences, whose predicted amino acid sequences exhibit significant similarities to that of AtDBP1 (e.g. GenBank accession numbers gb:AB00524, gp:AF058919_21, gp:ATTS1209_1, and gp:AC002339_3). Nevertheless, they are not identical to the newly identified AtDBP2. This inspection suggested that this plant has several AtDBP homologues. The same inspection revealed a more intriguing fact that a potato protein, whose amino acid sequence is very similar to AtDBP1, has been reported previously (gb:STU72489) (Fig. 4). This highly homologous potato protein was named 'Remorin', and has been characterized biochemically [24]. A further inspection of the current plant expression sequence tag (EST) databases revealed that several homologues occur in other higher plants, including tomato, saccharum, pisum, and rice, suggesting that the AtDBP homologues seem to be ubiquitous in higher plants (Fig. 4). It should be noted, however, that no homologous sequence was found in spices other than plants.

## 4. Discussion

In *Arabidopsis*, the discovery of the ethylene receptors [14] and the presumed cytokinin receptors [15] immediately suggested that the well-documented bacterial His-Asp phosphorylay mechanism may operate widely in this higher plant, because the uncovered structural designs of their amino acid sequences turned out to be very similar to those of classical bacterial sensor His-kinases. This assumption was further strengthened by the recent findings that this higher plant possesses also a group of response regulators (ARR-series) [19,20]. It is thus tempting to speculate that some ARRs may operate at a downstream of the His-kinases that would be activated by either ethylene or cytokinin. In this context, ARR4 is particularly interesting, since its expression was demonstrated to be rapidly induced by a cytokinin-treatment of *Arabidopsis* under certain conditions [21,22], supporting the view that ARR4 may function at an intermediate step in the presumed cytokinin-responsive signaling pathway. The results in this study revealed that ARR4 has an ability to interact physically with the known protein, AtDBP1, and its homologue, AtDBP2.

AtDBP1 was originally reported as a lysine-rich, non-specific DNA-binding protein, almost a decade ago [23]. The authors emphasized that AtDBP1 has a molecular weight and an amino acid composition resembling histone H1. The amino acid sequence appears to be highly hydrophilic, and does not show features indicative of transmembrane domain. The authors also demonstrated that the expression of AtDBP1 in the shoot apex could be induced by exogenous auxin, although its induction appears to be part of the indirect, long-term auxin response in the plant tissue. It will be of interest to see if the expression of AtDBP1 is affected by a cytokinin-treatment in *Arabidopsis*. In any event, the function of AtDBP1 is virtually unknown, so far, because no relevant paper appeared since then. However, a hint with regard to the property of AtDBP1 has come from the study on another higher plant, potato [24]. The potato protein, named Remorin, is highly homologous to AtDBP1 (Fig. 4). Remorin was reported as a plasma membrane-associated, uranide-binding phosphoprotein. Namely, this potato protein is copurified tightly with plasma membranes, and is phosphorylated in vitro and in vivo, and furthermore, it can bind in vitro polyanionic ligands including oligogalacturonides and DNA [24,25]. These respective facets, reported independently for AtDBP1 and Remorin, are apparently puzzling. To be on the safe side, it may be only worth emphasizing without any speculation that the homologues of these proteins occur ubiquitously in higher plants (Fig. 4). In any case, our results provided a new insight into the function of this interesting protein of higher plants.

What is the biological implication of the results in this study? As generally accepted in the prokaryotic scenario [1–6], the *Arabidopsis* small ARR response regulators most likely function as an on-off molecular switch, whose activity is modulated by phosphorylation, at an intermediate step of a presumed His-Asp phosphotransfer signaling pathway. AtDBP1 may be a downstream target, whose activity is directly regulated through the interaction with ARR4. Alternatively, AtDBP1 may be an upstream regulator, which affects the function of ARR4 by indirectly modulating a signaling interaction between ARR4 and its yet unknown upstream and/or downstream target(s). Or, AtDBP1 may exert a purely

structural role, which allows ARR4 to be localized at a certain intracellular compartment, where certain steps of signal transduction take place (e.g. on the surface of plasma membranes). In any case, identification of the specific interaction between ARR4 and AtDBP1 should shed light on the His-Asp phosphorylay in this higher plant.

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