Comparative Studies of the AHP Histidine-containing Phosphotransmitters Implicated in His-to-Asp Phosphorelay in *Arabidopsis thaliana*

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**Note**

The evolutionarily-conserved histidine to aspartate (His-to-Asp) phosphorelay signal transduction is common in both prokaryotes and eukaryotes. Such a phosphorelay system is generally made up of ‘a histidine (His)-kinase’, ‘a histidine-containing phosphotransmitter (HPt)’, and ‘a phospho-accepting response regulator (RR)’. In general, an HPt factor acts as an intermediate in a given multistep His-to-Asp phosphorelay. In *Arabidopsis thaliana*, this model higher plant has five genes (named *AHP1* to *AHP5*), each of which seems to encode an HPt factor. Recent studies suggested that the His-to-Asp phosphorelay involving the AHP factors is at least partly implicated in signal transduction in response to cytokinin (a plant hormone). Nevertheless, the properties of AHPs have not yet been fully clarified. Here we did comparative studies of all the AHP factors, in terms of (i) expression profiles in plants, (ii) intracellular localization, (iii) their ability to acquire a phosphoryl group in *vitro*, and (iv) ability to interact with the downstream components, ARRs (*Arabidopsis* response regulators). The results of this study provided us with a comprehensive view at the molecular level for understanding the functions of the AHP phosphotransmitters in the His-to-Asp phosphorelay.

**Key words:** *Arabidopsis thaliana*; His-to-Asp phosphorelay; HPt signal transducers; response regulators

In *Arabidopsis thaliana*, results of recent intensive studies suggested that Histidine-to-Aspartate (His→Asp) phosphorelays are involved in signal transduction for propagation of certain stimuli, such as plant hormones (e.g., cytokinin). Indeed, this model plant has 11 members of the histidine-kinases (generally termed as HK) family, of which AHK4 (also known as CRE1 or WOL), together with its homologs (AHK2 and AHK3), was recently demonstrated to serve as a cytokinin receptor. Furthermore, this higher plant has five genes (*AHP*-series) each encoding a histidine-containing phosphotransfer factor (generally termed HPt), and 22 genes (*ARR*-series) each encoding a response regulator (generally termed RR).

In general, a given HPt factor serves as an intermediate that links HK and RR through a phosphorelay. *Arabidopsis thaliana* has 5 HPt factors (*AHP1* to *AHP5*) each consisting of about 150 amino acids, and their entire sequences are very similar to each other (as schematically shown in Fig. 1A). Some of these AHPs (*AHP1*, *AHP2*, and *AHP3*) have previously been characterized to some extent. Although these results are still fragmentary, one can assume that these AHP factors are implicated in an AHK→AHP→ARR phosphorelay that is involved in the cytokinin signaling in this higher plant. In this respect, we recently cloned two new genes (*AHP4* and *AHP5*), and some preliminary studies were done to compile these AHP factors in *Arabidopsis thaliana*. Nonetheless, clarification of the biological functions of AHPs is at a very early stage. In this study, we did comparative studies of all the AHP factors, in terms of (i) their expression profiles in plants, (ii) intracellular localization, (iii) their ability to acquire a phosphoryl group in *vitro*, and (iv) their ability to interact with ARRs.

To gain an overview of the expression profiles of the AHP family genes in plants, Northern blot hybridization analyses were done with RNA samples prepared from various tissues and/or organs, including light-grown young seedlings, dark-grown etiolated seedlings, roots, leaves, stems, flowers, and siliques (Fig. 1B). Note that the amino acid sequences of AHPs are highly similar to each other (Fig. 1A). In this Northern blot hybridization, we thus used the highly divergent 3' non-coding sequences of the AHP genes as the probes in order to specifically detect each transcript. The transcripts of *AHP2*, *AHP3*, and *AHP5* were detected in every organ tested, more or less (Fig. 1B). The amino acid sequences of these AHPs are relatively similar to each other than others (Fig. 1A). This suggests that *AHP2*, *AHP3*, and *AHP5* might play certain redundant roles in plants. The transcript of *AHP1* was predominantly detected in roots. By contrast, the transcript of *AHP4* was mainly detected in aerial parts of plants, and it was hardly detected in roots. Interestingly, the apparent size of *AHP4* varied...
considerably from organ to organ (see each transcript detected in leaves, stems, and flowers), whereas a single transcript was detected for each of the others in every organ. This particular event as to AHP4 might be due to a difference of RNA processing, such as alternative splicing and/or alternative polyadenylation. This intriguing event remains to be elucidated. In any case, these results suggested that the expressions of AHPs in the organs of plants are not necessarily redundant, and that certain AHPs (in particular, AHP1 and AHP4) might each play a specific function in certain organs.

In the His-to-Asp phosphorelay in Arabidopsis thaliana, the upstream components (e.g., AHK4 cytokinin receptor) are assumed to be in the membrane, whereas the downstream components (e.g., type-B ARR transcription factors) are known to be located predominantly in the nucleus. Thus, it is of interest to see where AHPs are located in cells (Fig. 1C). To address this issue, we constructed the following recombinant GFP-fusion genes (encoding green fluorescent protein): GFP (an appropriate reference), GFP-ARR7 (another appropriate reference), and GFP-AHP1 to GFP-AHP5, all of which we constructed so as to be expressed under the CaMV 35S promoter in plant cells. The GFP-ARR7 fusion gene was previously used to demonstrate that this response regulator is capable of entering the nucleus. These recombinant genes were introduced into onion epidermal cells by particle-mediated DNA delivery procedures (Fig. 1C), as described previously. The microscopic observations supported the view that the localization of AHPs is not necessarily confined to the nucleus; rather they are predominately located in the cytoplasmic space, if not exclusively. Although the results of this experiment did not necessarily provided us with insights into the dynamics of intracellular localization of AHPs, it was at least suggested that no significant difference is observed amongst five AHPs with regard to the intracellular localization.

In general, the most fundamental biochemical nature of a given HPt factor is its ability to acquire a phosphoryl group at a histidine residue. To critically examine this nature for AHPs, each AHP was overexpressed in Escherichia coli (Fig. 2, uppermost panel). Each AHP coding sequence (i.e., cDNA) was adequately integrated into a versatile E. coli expression vector (pET-system). In every case, the resulting E. coli lysates contained a large amount of each corresponding AHP polypeptide. These proteins were purified according to the conventional purification procedures, mainly with a FPLC, as described previously. In every case, a highly homogenous protein sample was obtained (Fig. 2, middle panel). These purified AHP proteins were incubated with the purified E. coli cytoplasmic membranes containing the overexpressed ArcB (anaerobic sensor) His-kinase, in the presence of radioactive \( \gamma^{32}P \) [ATP]. This procedure has previously been developed to demonstrate that AHP1 and AHP2 are capable of acquiring a phosphoryl group from the E. coli His-kinase. When incubated with the E. coli membranes, in every case, the AHP polypeptides were rapidly and radioactively phosphorylated (Fig. 2, lowermost panel). It was found that all the AHP factors have the ability to

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** *Arabidopsis thaliana* Has 5 Genes Encoding the AHP Family of HPt Factors, with Expression Profiles in Plants and Cells.

(A) A schematic representation of the common structural design of the AHP factors, each consisting of about 150 amino acids. Each of these AHPs contains a crucial phosphorylation site (H, histidine). For these homologous AHP factors, a non-rooted neighbor-joining phylogenetic tree was constructed. (B) An overview as to the expression profiles of the AHP family transcripts. RNA samples were prepared from various organs and/or tissues. They are: (1) young seedlings (10-day-old in light), (2) etiolated seedlings (10-day-old in dark), (3) roots (from 30-day-old plants), (4) leaves (from 30-day-old plants), (5) stems (from 30-day-old plants), (6) flowers comprising of floral meristems and organs (from 40-day-old plants), and (7) siliques containing seeds (from 50-day-old plants). Northern blot hybridization analyses were done with each specific probe (see Text), as indicated, according to the procedures described previously. The expression of UBQ10 was also analyzed as a loading reference. The hybridized bands were detected with a phosphoimage analyzer (BAS-2500, FujiXerox, Japan). (C) Intracellular localization of the AHP family factors. A set of recombinant genes was constructed, each of which encodes the indicated types of GFP-fusion proteins (e.g., GFP-AHP1). These genes are under the control of the CaMV 35S promoter. The DNA fragments encompassing each recombinant gene were used to bombard onion epidermal cells, as described previously. As a reference, the same samples were observed under the same microscope equipped with Nomarski optics (e.g., for the GFP-AHP5 sample, and others are not shown).
undergo phosphorylation. The in vitro result supported the view that each AHP factor is most likely integrated into an in vivo His-to-Asp phosphorelay in its own right.

If these AHP factors are indeed integrated into a His-to-Asp phosphorelay, as proposed above, they should also have the ability to interact with the downstream components (i.e., response regulators). As mentioned above, the response regulators of *Arabidopsis thaliana* have been intensively characterized, and they are referred to as the ARR-series. To examine the possible interaction between AHPs and ARRs, here we adopted the yeast two-hybrid assay with ARR1 and ARR2 as preys, as described previously. These are the well-characterized representatives of the type-B ARR family of transcription factors.

In 1998, before the entire genome sequencing of *Arabidopsis thaliana* was completed, we for the first time identified three AHP genes (named *AHP1*, *AHP2*, and *AHP3*), each of which encodes a protein the amino acid sequence of which considerably resembles the HPt domain of the *E. coli* ArcB His-kinase. We then demonstrated that the in vitro natures of these plant gene products fulfill the diagnostic criteria of HPt factors. Then, several lines of in vivo evidence supported the view that these AHP factors are involved in the His-to-Asp phosphorelay signaling in response to cytokinin. When the entire genome sequencing has been completed in 2000, however, it was revealed that this higher plant has two more genes, each of which most likely encodes an AHP homolog, as has previously been compiled. These newly discovered AHP factors (AHP4 and AHP5) have not yet been characterized in comparison with others. To clarify a whole network of interactions between AHPs and ARRs, we adopted the yeast two-hybrid assay with ARR1 and ARR2 as preys, as described previously. These are the well-characterized representatives of the type-B ARR family of transcription factors. We constructed a set of yeast recombinant plasmids, into which we integrated each entire AHP coding sequence so as to serve as baits. The results of such intensive yeast two-hybrid assays clearly showed that all the AHP factors have the ability to physically associated with both ARR1 and ARR2 (Fig. 3). The type-B ARR family consists of 11 members. We do not know whether AHPs are capable of interacting equally well with these ARRs other than ARR1 and ARR2. We would not be surprised, even if other ARRs interacted with all the AHP factors, because these ARRs each contain a highly homologous phospho-accepting receiver domain.
His-to-Asp phosphorelays, we needed to comprehensively characterize the natures of all these AHP factors. For this reason, a comparative study was done here with reference to all of the AHP factors. The results provided us with insights into the comprehensive properties of AHPs, as demonstrated in this study.

First of all, all the AHP factors showed the biochemical ability to acquire a phosphoryl group (Fig. 2), and they also showed the ability to interact with the downstream ARR components (Fig. 3), demonstrating that not only the previously characterized AHPs (AHP1, AHP2, and AHP3), but also newly characterized ones (AHP4 and AHP5) are capable of serving as an HPt factor involved in a multistep His-to-Asp phosphorelay. It was also demonstrated that these AHP factors are located mainly in the cytoplasmic space, whereas it was previously shown that the downstream ARR components are predominately located in the nucleus (Fig. 1C). The question then arose as to where they meet in the cells. The AHP factors might have an ability to enter the nucleus under certain conditions. This issue remains to be addressed. In any case, all the AHP factors showed essentially the same biochemical natures when compared with each other. However, it was revealed that the expression profiles of their transcripts in plants are considerably different from each other (Fig. 1B). These findings of this study must be taken into consideration for better understanding of the role of each AHP factor in His-to-Asp phosphorelays in plants. Thus, we are now in the position to start conducting a series of genetic studies, which should include the characterization of the null-mutants for each of the AHP genes. These are under way in our laboratory.

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References