Note

Cellular Localization of the Signaling Components of Arabidopsis His-to-Asp Phosphorelay

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In the higher plant, Arabidopsis thaliana, histidine-to-aspartate (His-to-Asp) phosphorelay signal transduction systems play crucial roles in propagation of environmental stimuli, including plant hormones. This plant has 11 sensor His-kinases, 5 histidine-containing phosphotransfer (HPt) factors (AHPs), and 20 response regulators (ARRs). To gain new insight into the functions of these phosphorelay components, their intracellular localization was examined with use of GFP-fusion proteins, constructed for certain representatives of HPt factors (AHP2) and type-A and type-B ARRs (ARR6/ARR7 and ARR10, respectively). The results showed that AHP2 is mainly located in the cytoplasmic space, while both the types of ARRs have an ability to enter preferentially into the nuclei, if not exclusively. Together with the results from an in vitro phosphorelay assay with AHP2 and ARRs, these results are discussed, in terms of a general framework of the Arabidopsis His-to-Asp phosphorelay network.

Key words: Arabidopsis thaliana; His-to-Asp phosphorelay; response regulators; HPt factors; His-kinases

Common signal transduction mechanisms, generally referred to as histidine-to-aspartate (His-to-Asp) phosphorelays (or two-component regulatory systems), are involved in a wide variety of cellular responses to environmental stimuli.\(^{19}\) Such a His-to-Asp phosphorelay system generally consists of two or more common signal transducers; a sensor with histidine (His)-kinase activity, a histidine-containing phosphotransfer (HPt) factor, and a response regulator containing a phospho-accepting aspartate (Asp) in its receiver domain (see Fig. 1).\(^{20}\) To date, numerous instances of such His-to-Asp phosphorelays have been uncovered for not only many prokaryotic species,\(^{23}\) but also certain eukaryotic species.\(^{24}\) In the higher plant Arabidopsis thaliana, His-to-Asp phosphorelays are most likely involved in propagation of certain environmental stimuli, including plant hormones (e.g., ethylene and cytokinin) (Fig. 1).\(^{25,26}\) In fact, an inspection of the Arabidopsis entire genomic sequence revealed that this plant has a number of genes each encoding one of the phosphorelay components, which include 11 His-kinases, 5 HPt factors (AHPs), and 20 response regulators (ARRs).\(^{27}\) These members of the ARR-family are further classified into two distinct subtypes (10 members in type-A, and the other 10 members in type-B), as judged from their structural designs and the cytokinin-inducible expression profiles of their transcripts (Fig. 1).\(^{28,29}\) Among the His-kinases, the five ETR1-family members are known to function as ethylene-sensors.\(^{30,31}\) Recently, AHK4 (or CRE1) was demonstrated to be a cytokinin sensor.\(^{12,13}\) These facts strongly suggest that His-to-Asp phosphorelays play crucial biological roles in Arabidopsis. Nevertheless, clarification of the molecular mechanism underlying the presumed His-to-Asp phosphorelay network is at a very early stage.

We have been extensively studying the His-to-Asp phosphorelay in Arabidopsis.\(^{8,12,14-20}\) To extend these, as a next step, it is crucial to gain some idea about the cellular localization of each phosphorelay component. Most of the His-kinases are reasonably assumed to be located in the plasma membrane, because each contains one or more membrane-spanning hydrophobic domains.\(^{29}\) With regard to other phosphorelay components (namely, AHPs and ARRs), however, we have no idea about their cellular localization, although some type-B ARRs (ARR1, ARR2, ARR11) were recently shown to be located in the nuclei.\(^{21,22}\) In this study, therefore, we wanted to examine the cellular localization of AHPs as well as both the types of ARRs.

In this study, this was done with a conventional transient expression assay of GFP (green fluorescent protein) fusion proteins in onion skin epidermal cells.\(^{24}\) A set of GFP fusion recombinant genes was constructed. They were designed so as to encode GFP-alone (a control for the cytoplasmic localization), GFP-LIM13 (a control for the nuclear localization), GFP-AHP2 (a representative of HPt factors), and GFP-ARR10 (a representative of the type-B ARR family members). These genes were introduced

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into onion epidermal cells by using particle-mediated DNA delivery procedures.\textsuperscript{24,25} The resulting GFP-fusion proteins, expressed in onion epidermal cells, were examined under a fluorescence microscope (Fig. 2). In the case of GFP alone, its fluorescence was detected mainly in the cytoplasmic space, while in the case of GFP-LIM13, its fluorescence was detected as a dense glittering spot in the cells. This spot was confirmed to be coincident well with the position of the nucleus, which was DAPI-stained and observed under a microscope (data not shown). LIM13 is a meiosis-associated gene from \textit{Lilium longiflorum}, the product of which was previously demonstrated to be a nuclear-localizing protein.\textsuperscript{24} Based on these experimental rationales, it was found that a majority of GFP-AHP2 stays within the cytoplasmic space, but GFP-ARR10 is predominantly localized in the nuclei, if not exclusively (Fig. 2). It was thus suggested that the type-B ARR10 protein has a nuclear-localization signal(s) in its own amino acid sequence. These results led us to propose the general idea that HPr factors (or AHP2) most likely play a role mainly in the cytoplasm, but type-B ARRs (or ARR10) do so mainly in the nuclei. The latter idea is consistent with the previous reports that ARR1 and ARR2 (other representatives of type-B ARRs) function as DNA-binding transcription factors.\textsuperscript{22,23}

Fig. 2. Nuclear-localization of the GFP Fusion Derivatives of AHP2 and ARR10.

The recombinant genes each encoding GFP alone, GFP-LIM13, GFP-AHP2, and GFP-ARR10 were constructed. These genes are under the control of the plant 3SS promoter, as described previously.\textsuperscript{20} These DNA fragments encompassing each recombinant genes were respectively used to bombard into onion skin epidermal cells, as described previously.\textsuperscript{21} The expression and localization of each product was observed after 24 h of incubation by a fluorescence microscopy, as described previously.\textsuperscript{22} As schematically shown (Fig. 1), the type-B ARR family members have a large C-terminal extension, in which the commonly conserved domain, named B-motif, is found. Note also that the common receiver domain precedes each of these C-terminal domains. In contrast, the type-A ARR-family members are unique in that each of them apparently lacks such a large C-terminal extension. It is thus of interest to see where they are localized. To this end, a \textit{GFP-ARR6} fusion gene (a representative of type-A ARRs) was constructed, and then tested in onion epidermal cells (Fig. 3). The result showed that the localization of ARR6 is confined mainly in the nuclei. This was somewhat surprising for us, because ARR6 appears to consist of the receiver domain with a very short C-terminal extension of only about 30 amino acids (Fig. 3). To confirm this critical observation, we thus decided to construct a \textit{GFP-ARR7} gene (another representative of type-A ARRs). The results showed that ARR7 can also enter the nuclei. To further verify these intriguing views, we then constructed certain derivatives of GFP-ARR6 and GFP-ARR7. The resulting derivatives, GFP-ARR6-\textit{AC} and GFP-ARR7-\textit{AC}, lack each C-terminal extension (26 amino acids in ARR6, and 47 amino acids in ARR7, respectively) (Fig. 3). These C-terminally truncated type-A ARRs have lost their ability to enter the nuclei (Fig. 3), although they still have an \textit{in vitro} ability to undergo phosphorylation, as demonstrated previously.\textsuperscript{20} An inspection of the amino acid sequence of each C-terminal extension of ARR6 and ARR7 showed that ARR6 has a KRAK sequence, and ARR7 has a KRMR sequence, at each C-terminus. They may serve as a nuclear-localization signal (NLS). Note also that other eight members of the

![Image of Arabidopsis cells](https://example.com/fig1)

Fig. 1. Schematic Representations of the Structural Designs of the Signal Transducers Involved in the Presumed \textit{Arabidopsis} His-to-Asp Phosphorelay Network, and Their Cellular Localization.

The \textit{Arabidopsis} phosphorelay components include sensor His-kinases (H), HPr factors (AHPs), and response regulators (ARRs), each of which contains either phosphorylated histidine (H) or aspartate (D), as schematically shown. These are crucial for the presumed phosphorelay interaction between the signal transducers. Other details are given in the text.
type-A ARR family each has a short stretch of positively charged amino acids in each short C-terminal extension (e.g., KRK both in ARR3 and ARR4, data not shown). These results supported the general view that the type-A ARR-family members also have an ability to enter the nuclei, and their nuclear-localization signal (NLS) are most likely within their short C-terminal extensions, although verification of this view as to other members must await extensive experimentation.

To gain further insight from these results, described above, we needed to address the relevant issue as to the occurrence of biochemical and biophysical interactions between AHPs and ARRs. We previously demonstrated in vitro that ARR6 (type-A) is capable of interacting with AHP2 through a phosphorelay reaction.\(^8\) However, we did not succeed in demonstrating such a phosphorelay interaction between ARR10 (type-B) and AHP2.\(^9\) We thus wanted to re-investigate the possible phosphorelay interaction between AHP2 and ARR10. To this end, a set of recombinant polypeptides (AHP2, ARR6, ARR10-RB) were purified, as described previously (Fig. 4(A)).\(^8,16\) The purified ARR10-RB polypeptide encompasses the region extending from Met-1 to Ser-263, which contains both the receiver domain and B-motif. Having these purified polypeptides, first of all, the radioactive phospho-histidine-containing AHP2 was prepared in vitro, as described previously.\(^16\) This was done by incubating the purified AHP2 polypeptide with the *E. coli* cytoplasmic membrane in the presence of \(\gamma^{32}\)P ATP. This radioactive AHP2 polypeptide was then incubated either with ARR6 or ARR10-RB. They were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by autoradiography (Fig. 4(B)). The results showed that the radioactive phosphoryl group was transferred rapidly from AHP2 to ARR10-RB. In the case of ARR6, the radioactivity on AHP2 disappeared rapidly, as has been demonstrated previously.\(^8,14\) This is probably due to a strong phosphatase activity of ARR6 toward AHP2, as explained previously.\(^14\) In any event, it was demonstrated that not only type-A ARR6, but also type-B ARR10 have an ability to interact with AHP2 through a phosphorelay reaction (presumably a His-to-Asp phosphorelay).

The results in this study provided new insight into the mechanism underlying the His-to-Asp phosphorelay signal transduction in *Arabidopsis*, as finally discussed below. It is reasonably assumed that the sensor His-kinases are located at the cell surface, as mentioned above (Fig. 1). Some of them function as plant hormone receptors. Based on the recent results from others,\(^22,23\) it is also reasonably assumed that the type-B ARR family members function as DNA-binding transcription factors in the nuclei. The results of the GFP-fusion proteins in this study col-
lectively led us to envisage a general idea about the cellular localization of AHPs and both type-A and type-B ARRs, as summarized in Fig. 1. Nevertheless, these views leave us a puzzle. The in vitro results that AHPs can interact with ARRs through a phosphorelay reaction is seemingly inconsistent with the in vivo observation that AHP2 are localized in the cytoplasmic space, while both the types of ARRs are predominantly found in the nuclei. However, it should be emphasized here that the results from such steady-state analyses of cellular localization with GFP-fusion proteins do not necessarily provide us with a hint as to the dynamics of cellular localization of these proteins. Rather, our results collectively suggest that AHPs may interact with His-kinases and acquire a phosphoryl group, and then such a phosphorylated AHPs may enter into the nuclei. Alternatively, ARRs may interact with AHPs in the cytoplasmic space and acquire a phosphoryl group from AHPs, and ARRs may somehow migrate into the nucleus. We have so far no idea about such phosphorylation states of GFP-AHP2, GFP-ARR6, and GFP-ARR10, expressed in onion epidermal cells. Clarification of these issues must await further experiments. However, the proposed framework (Fig. 1), with regard to the cellular localization of the phosphorelay components, will provide an important basis for better understanding of the molecular mechanism underlying the His-to-Asp phosphorelay network, which is deeply involved in plant hormone responses in Arabidopsis.

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References

His-to-Asp Phosphorelay in *Arabidopsis thaliana*  


