Note

Compilation and Characterization of Histidine-Containing Phosphotransmitters Implicated in His-to-Asp Phosphorelay in Plants: AHP Signal Transducers of Arabidopsis thaliana

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Histidine (His)-to-Aspartate (Asp) phosphorelay signal transduction systems are generally made up of a “sensor histidine (His)-kinase”, a “response regulator”, and a “histidine-containing phosphotransmitter (HPt)”. In the higher plant, Arabidopsis thaliana, results from recent intensive studies suggested that the His-to-Asp phosphorelay mechanism is at least partly responsible for propagation of environmental stimuli, such as phytohormones (e.g. ethylene and cytokinin). Here we compiled the members of the HPt family of phosphotransmitters in Arabidopsis thaliana (AHP-series, Arabidopsis HPt phosphotransmitters), based on both database and experimental analyses, in order to provide a comprehensive basis at the molecular level for understanding the function of the AHP phosphotransmitters that are implicated in the His-to-Asp phosphorelay of higher plants.

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

The common prokaryotic type of intracellular signal transduction mechanisms is generally referred to as “histidine-to-aspartate (His-to-Asp) phosphorelay systems” (or “two-component regulatory systems”). Such a His-to-Asp phosphorelay involves two or more of common signal transducers, a sensor with histidine (His)-kinase activity, a response regulator containing a phospho-accepting receiver domain, and a histidine-containing phosphotransmitter (HPt). To date, numerous instances of His-to-Asp phosphorelay systems, involved in a wide variety of adaptive responses to environmental stimuli, have been reported for not only many prokaryotic species, but also certain eukaryotic species.

In the higher plant, Arabidopsis thaliana, results from recent intensive studies suggested that His-to-Asp phosphorelay mechanisms are involved presumably in propagation of environmental stimuli, such as phytohormones (e.g. ethylene and cytokinin). An inspection of the Arabidopsis databases found that this model plant has at least 11 sensor His-kinases. Five (ETR1, ETR2, ERS1, ERS2, and EIN4) have been demonstrated to be ethylene receptors, and two (CKI1 and CKI2) were assumed to be involved in a cytokinin responsiveness, and one (ATHK1) was proposed to be a putative osmosensor. Furthermore, it was recently demonstrated that Arabidopsis thaliana has a number of response regulators (ARR-series, Arabidopsis response regulators), each containing a typical phospho-accepting receiver domain. This plant has at least 16 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 and expression profiles. For example, the type-A family of response regulators is induced by cytokinin-treatment of plants at the level of transcription, but the type-B family of response regulators is not.

As mentioned above, certain multi-step His-to-Asp phosphorelay systems often involve another type of phosphorelay signal transducer, which are collectively termed “histidine-containing phosphotransmitters (HPt)”. Together with His-kinases and phospho-accepting receivers, a given HPt phosphotransmitter (or HPt domain) serves as a crucial intermediate in a His-to-Asp phosphorelay pathway by acquiring and transferring a phosphoryl group from and to a receiver, respectively. In this respect, it was recently reported that Arabidopsis has at least three genes each encoding a typical HPt phosphotransmitter. Each of these contains only a HPt domain consisting of about 150 amino acids, and their amino acid sequences are very similar to each other. The Arabidopsis genome sequencing has almost been completed, and most of the nucleotide sequence data are now available in public. When considered this fact, now is the time to compile the members of Arabidopsis HPt phosphotransmitters (AHP-series).

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in order to gain insight into the His-to-Asp phosphorelay network in *Arabidopsis thaliana*. In this note, based on experimental analyses, we attempted to compile the AHP family of signal transducers in this higher plant.

Not only our group, but also other group recently isolated three cDNA clones from *Arabidopsis thaliana*, each encoding AHP1 (or ATHP3), AHP2 (or ATHP1), and AHP3 (or ATHP2), respectively (Fig. 1).21) Here we extensively searched for new genes, each of which encodes an as yet uncharacterized AHP phosphotransmitters, in the currently available *Arabidopsis* databases. Such an inspection revealed the occurrence of three more genes in the GenBank nucleotide sequence databases (accession nos. AC001645, AC002560, AC009322), each of which most likely encodes a new AHP. Their deduced amino acid sequences were aligned with those of the known AHPs (Fig. 1(A), note that the first methionine of each sequence was not yet confirmed experimentally). At a first glance, their amino acid sequences and molecular masses are very similar to each other. Two of them were thus designated as AHP4 and AHP5, respectively. However, the last one was designated intentionally as APHP1, due to the reason argued below. Namely, although the predicted amino acid of APHP1 is very similar to other AHPs, it does not contain the crucial histidine residue that should serve as a phosphorylation site in phosphorelay.23) In any case, to gain an insight into the relationship among these AHP and APHP amino acid sequences, a phylogenetic tree was also constructed by the neighbor-joining method with the program of CLUSTAL-X (Fig. 1(B), each chromosome-location, I to V, is in parentheses).24) It was suggested that AHP2, AHP3, and AHP5 are very closely related to each other (note also that these set of AHPs showed an expression profile similar to each other, as mentioned below, see Fig. 2(B)).

It should be emphasized that the above result is just based on a simple inspection of the *Arabidopsis* genome sequences. For example, the AHP5 coding sequence was not predicted and annotated in the nucleotide sequence database (GenBank accession no. AC002560). And also, the unusual nature of the APHP1 amino acid sequence might be simply due to an error of nucleotide sequencing. Thus, we needed to isolate each corresponding cDNA clone, in order to confirm not only the nucleotide and amino acid sequences, but also their occurrences. Through polymerase chain reaction (PCR)-based screening, we succeeded in isolating all the cDNAs corresponding to AHP4, AHP5, and APHP1, respectively. Our results confirmed not only the cDNA sequences of AHP4 and AHP5 (GenBank accession nos. AB041766 and AB041767, respectively), but also the notion that the amino acid sequence predicted for APHP1 indeed lacks the crucial histidine site (with a His-to-Asn substitution). This particular hypothetical protein was thus designated as “Arabidopsis pseudo-HPt phosphotransmitter (APHP)”.

Based on these, the genomic (exon-intron) structure of each predicted gene was schematically analyzed and compared (Fig. 2(A)). In terms of the exon-intron arrangement, these genes have a structural design very similar to each other, suggesting that they most likely had evolved from a common ancestor (note that certain introns are missing in some case).

Standard Northern hybridization analyses were previously done for AHP1 to AHP3.21) The AHP1 transcript appeared to be expressed predominantly...
(but not exclusively) in roots, while the AHP2 and AHP3 transcripts were detected in all the organs tested, including leaves, stems, flowers, and siliques. To gain an insight into the expression of other AHP genes, RT (reverse transcriptase)-PCR analyses with appropriate primers specific for each gene were done with total RNA samples from roots and leaves (Fig. 2(B)). The results from RT-PCR analyses for AHP1, AHP2, and AHP3 were consistent with those from standard Northern hybridization analyses, as mentioned above. The AHP5 transcript was detected both in roots and leaves, as in the case of AHP2 and AHP3. The AHP4 transcript was hardly detected both in roots and leaves, whereas the relatively (or very) low level of the AHP1 transcript was detected in roots. In short, it was found that the AHP (and AHPH) genes appear to be expressed in plants in a manner different from each other, although their gene-products are very similar to each other in their amino acid sequences. In this connection, it would be worth mentioning that the type-A response regulators are induced at the level of transcription by cytokinin-treatment of plants.\textsuperscript{14,18,19,20} It was thus interesting to see whether or not the expression of some AHPs is also regulated in a similar manner by cytokinin. Such an examination, done for AHP1, AHP2, and AHP3, showed that it is not the case (data not shown). In sum, based on these experimental analyses, it was confirmed that \textit{Arabidopsis thaliana} has at least five genes each encoding a typical HPt phosphotransmitter (\textit{i.e.} AHP-series). Furthermore, this plant has a gene, named AHP1, which encodes an AHP-like protein lacking the phosphorylated histidine site. This one may play an as yet unknown unique role in a His-to-Asp phosphorelay network.

If such plant HPT phosphotransmitters, found in \textit{Arabidopsis thaliana}, play a fundamental biological role in a manner that is common (or general) amongst higher plants, one can then expect that there must be homologous (or orthologous) proteins in many other plants, if not all. A further inspection to this end revealed that this is indeed the case (Fig. 3). Using the currently available plant EST databases, a search was done for cDNA sequences each encoding a protein very similar to AHPs. As compiled in Fig. 3, a wide variety of plant species including both dicots (\textit{e.g.}, cotton and tomato) and monocots (\textit{e.g.}, maize and rice) appear to express certain transcripts, each of which specifies a protein strikingly similar to AHPs in their amino acid sequence. This fact is best interpreted by assuming that the HPT phosphotransmitters as a component of His-to-Asp phosphorelay play a fundamental role common amongst higher plants.

Finally, one should remember that \textit{Arabidopsis} has a number of genes for His-kinases (11 or more) as well as response regulators (16 or more). From a biological viewpoint, some of them most likely play a redundant role in a given biological process. In general, such a situation of genetic redundancy makes it very difficult to analyze their functions by means of a straight-forwards genetic approach, as indeed was experienced in the case of the ethylene-sensor His-kinases.\textsuperscript{19} In this context, this model plant appears to have a relatively limited number of genes for HPT phosphotransmitters, as compared with in

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\textbf{Fig. 2.} Schematic Representations of Structures of \textit{Arabidopsis} AHP Genes and Their Expression at the Transcriptional Level. (A) The genomic structure of the AHP genes are schematically shown in terms of their exon-intron arrangements. The rectangles represent "exon", and the lines represent "intron". The positions of the putative first methionine and phosphorylated histidine residues are indicated, respectively. For AHP1 to AHP3, each cDNA with a poly-A tail was isolated previously.\textsuperscript{21} For AHP4 and AHP5, the cDNAs corresponding to their presumed coding region were isolated in this study (thus, the structures of 3'-proximal non-coding regions are not known). (B) Expression profiles of the AHP genes were characterized by RT-PCR methods with each specific primer-set. Total RNA samples prepared from roots (R) and leaves (L), respectively, were analyzed.

\textbf{Fig. 3.} Alignment of Deduced Amino Acid Sequences of Putative Plant HPT Phosphotransmitters. The currently available plant EST databases were searched for nucleotide sequences, the deduced amino acid sequences from those are strikingly similar to those of the \textit{Arabidopsis} AHPs. The GenBank accession numbers, cited here are: Medicago (AW268037), cotton (AT129367), maize (AW219133), soybean (Glycine) (AW349155), iceplant (AW353454), maize (AI677390), and rice (D49138 and C25474). It should be noted that these are not entire amino acid sequences. Gaps (\textendash) were introduced for optimal alignment, in which the highly conserved amino acid residues are shaded.
the case of His-kinases and response regulators. By taking this advantage, systematic and post-genomic analyses of these Arabidopsis AHP genes should give us quick hints for understanding the His-to-Asp phosphorelay network that presumably involves other large families of 11 His-kinases and 16 response regulators. To this end, the data presented here provide a comprehensive basis at the molecular level for understanding the function of HPt phosphotransmitters in higher plants.

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