Note

Biochemical Characterization of a Putative Cytokinin-Responsive His-kinase, CK11, from *Arabidopsis thaliana*

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His-Asp phosphorelays are evolutionary-conserved powerful biological tactics for intracellular signal transduction. Such a phosphorelay is generally made up of “sensor histidine (His)-kinases”, “response regulators”, and “histidine-containing (HPI) phospho-transmitters”. Results from recent intensive studies suggested that, in the higher plant *Arabidopsis thaliana*, His-Asp phosphorelays may be widely used for propagating environmental stimuli, such as phytohormones (e.g., ethylene and cytokinins). In this study, we characterized, *in vitro*, the putative cytokinin-responsive CK11 His-kinase, in terms of His-Asp phosphorelays. It was demonstrated for the first time that the receiver domain in this sensor exhibits a strong phosphohistidine phosphatase activity toward some *Arabidopsis* HPI phospho-transmitters (AHP1 and AHP2), suggesting the functional importance of the receiver domain for a presumed interaction of the sensor His-kinase with other His-Asp phosphorelay components.

Key words: *Arabidopsis thaliana*; His-Asp phosphorelay; signal transduction; sensor His-kinase; receiver

Widespread bacterial signal transduction circuits are sometimes referred to as “two-component systems” or “histidine to aspartate (His-Asp) phosphorelays”, and they regulate a large variety of cellular responses, including bacterial chemotaxis, osmoregulation, and microbiopathogenesis. Such a phosphorelay signaling system is generally made up of two or more multidomain signal transducers that are generally referred to as “sensors” and “response regulators”, each of which contains one or more phosphotransfer signaling domains. Namely, they are histidine (His)-kinases, receivers, and histidine-containing phosphotransmitters (HPT). The His-Asp phosphorelay mechanism appears to be an evolutionary-conserved powerful biological tactic for intracellular signal transduction. In a given bacterial species, like *Escherichia coli*, these His-Asp phosphorelay systems involving a number of signal transducers are tuned to operate over a wide range of stimuli.

In higher plants, the first discovery of ethylene (a phytohormone) receptors immediately implied that the bacterial His-Asp phosphorelay mechanism may operate in *Arabidopsis*, because the structural designs of their primary amino acid sequences turned out to be very similar to those of bacterial sensor His-kinases. Such an intriguing assumption was further strengthened by the subsequent finding that a similar His-kinase is also implicated in a cytokinin-mediated signal transduction pathway in this higher plant (see Fig. 1A). These findings may not be surprising, because many instances of such His-Asp phosphorelay pathways have been discovered in diverse eukaryotic species, including yeasts, fungi, and slime molds, suggesting that this particular signal transduction mechanism is not restricted to prokaryotes. In fact, our inspection of the current *Arabidopsis* databases found that this plant has at least 11 sensor His-kinases. Five (ETR1, ETR2, ERS1, ERS2, and EIN4) have been demonstrated to be ethylene receptors, and two (CK11 and CK12) were proposed to be implicated in a cytokinin (another phytohormone) responsiveness. One (ATHK1) was shown to be a putative osmosensor (K. Shinozaki, personal communication), and the occurrence of the other three was predicted in the *Arabidopsis* genomic sequences (note that we recently cloned the corresponding cDNAs, and confirmed their existence). Furthermore, it was recently demonstrated that this higher plant has a set of response regulators, each of which contains a typical receiver domain. A set of HPI phospho-transmitters was finally uncovered very recently. One can thus envisage that these uncovered lineups of His-Asp phosphorelay signal transducers must be important through their abilities to propagate environmental signals in *Arabidopsis*. Nonetheless, in higher plants, clarification of the His-Asp phosphotransfer signal transduction mechanism is at a very early stage.

As mentioned above, one of our co-authors recently discovered, through an extensive genetic study, that a bacterial-type of sensor His-kinase appears to be implicated in a cytokinin-mediated signal transduction in *Arabidopsis*. This putative cytokinin receptor (named CK11) has a typical His-kinase structure at its N-terminal part (Fig. 1A). Interestingly, it also has a receiver domain at its C-terminal end. To gain insight into the biochemical abilities of these presumed His-Asp phosphorelay signaling domains, here we attempted to purify each domain by using *E. coli* recombinant DNA tech-
Fig. 1. Schematic Representation of Structure of the CKII Sensor His-kinase of Arabidopsis, and Expression of Each Domain in *E. coli*.

(A) The structure of the *Arabidopsis* CKII sensor His-kinase is schematically shown, below which each region expressed in *E. coli* is also schematically shown. (B) Three types of *E. coli* expression plasmids (pET-CKII-N, pET-CKII-H, pET-CKII-R), constructed in this study, were transferred into *E. coli* BL21 cells. The cells were grown in the presence (+) and absence (−) of IPTG. Total cellular proteins were prepared from the harvested cells by sonication, and then they were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, followed by staining with Coomasie Brilliant Blue.

Fig. 2. Purification of the Receiver Domain of CKII, and Characterization of its *in vitro* His-Asp Phosphorelay with the Purified Hpt Phosphotransmitters.

(A) Sodium dodecyl sulfate (SDS) polyacrylamide gel showing the purified polypeptides, CKII-R, AHP1, and AHP2, used in this study. (B) *In vitro* analyses of phosphorelay between CKII-R and AHP1. Radioactively phosphorylated AHP1 was purified, as described previously (denoted by P-AHP1, lane 0).† To this sample, CKII-R was added under the conditions described previously.† At times (min), the samples were analyzed by SDS polyacrylamide gel electrophoresis, followed by autoradiography. (C) Essentially the same experiments as those in (B) were done for AHP2. In this case, however, in addition to CKII-R, its mutant derivatives (CKII-R-DQ and CKII-R-DE) were also examined, as indicated (see the text). Note that several radioactive bands were detected at the position of AHPs. Smaller bands are most likely degradation products. (D) The *in vitro* phosphorelay reactions, observed in (B) and (C), are inferred to be as schematically shown.

Techniques. As schematically shown in Fig. 1A, we tried to express the following three distinct CKII polypeptides in *E. coli* cells, namely, the region extending from Met-1 to Met-656 (named CKII-N), the region extending from Met-383 to Lys-681 (named CKII-H), and the region extending from Thr-949 to Arg-1121 (named CKII-R). The CKII-N region covers a presumed signal-input domain as well as the His-kinase domain, while the CKII-R region encompasses only the His-kinase domain. The CKII-R region was designed so as to contain only the C-terminal receiver domain. Each corresponding CKII coding sequence was appropriately cloned into an *E. coli* expression vector, pET22b(+) (Novagen, USA), so as to be placed under the T7 phage promoter. *E. coli* BL21 cells carrying these resultant plasmids, respectively, were grown in an appropriate medium with and without IPTG (an inducer of the T7 promoter). Each cell lysate was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (Fig. 1B). We did not succeed in overproducing CKII-N, but both CKII-H and CKII-R were expressed in large amounts. However, it was found that CKII-H was produced in an insoluble form, while CKII-R was in a soluble form (data not shown). Thus, in this study we focused our attention on the soluble one, CKII-R, and characterized its biochemical ability, particularly, in terms of His-Asp phosphorelay.

The CKII-R polypeptide with a histidine tag was purified in a highly homogenous soluble form by a Ni-column procedure (Novagen, USA) (Fig. 2A). As mentioned above, we have recently identified a set of *Arabidopsis* Hpt phosphotransmitters (another common His-Asp phosphorelay signal transducer, see in-
troducory section), and demonstrated that the purified AHP1 and AHP2 polypeptides can be phosphorylated at a certain histidine residue in an E. coli in vitro system. To examine the presumed His-Asp phosphotransfer interaction between CKI1-R and AHPs, we also purified AHP1 and AHP2, as described previously (Fig. 2A). Then, the radioactively phosphorylated AHP1 and AHP2 polypeptides were prepared, as described previously (see lanes 0 in Figs. 2B an 2C, and the structure in Fig. 2D). These in vitro phosphorylated AHP polypeptides are known to be very stable in a buffer. In this study, however, we found that when the radiolabeled AHP1 polypeptide was incubated with the purified CKI1-R polypeptide, the radioactive phosphoryl group on AHP1 very rapidly disappeared, within a minute (Fig. 2B). The same event happened on between phospho-AHP2 and CKI1-R (Fig. 2C, the panel on the left side).

According to the current concept as to the function of the receivers, a given receiver principally has one or more of the following types of phosphotransfer activities: phosphoacceptor from histidine, phosphodonor for histidine, and phosphatase toward phosphohistidine. In these activities, a certain aspartate residue, invariably found at the center of the receiver domain, is known to be crucial (see Fig. 2D, Asp-1050 in the case of CKI1-R). Considering these, to examine further the nature of the reaction observed between CKI1-R and AHPs, we purified mutant CKI1-R polypeptides, in which the crucial aspartate residue was changed either to glutamine or glutamate by site-directed mutagenesis of pET-CKI1-R (see the legend to Fig. 1) (they were referred to as CKI1-R-DQ and CKI1-R-DE, respectively). These polypeptides were also purified as each soluble and stable form. When the radioactively phosphorylated AHP2 was challenged by these mutant receiver domains, nothing happened on AHP2 even after prolonged incubation (Fig. 2B). From these results, we conclude that the receiver domain of the CKI1 sensor has an in vitro ability to exert its strong phosphohistidine phosphatase activity toward AHP1 and AHP2. This particular reaction can be inferred, as schematically shown in Fig. 2D, in which it is assumed that CKI1-R would acquire a phosphoryl group from AHPs, and then would release it very rapidly.

A number of sensor His-kinases have recently been uncovered for the higher plant, Arabidopsis. One can assume that they are most likely involved in presumed His-Asp phosphorelay signaling pathways, yet their biochemical natures are entirely elusive. An in vitro autophosphorylation activity has been demonstrated so far only for the ETR1 etylene receptor. Note also that most of the Arabidopsis sensors, like CK11, have their own receiver domains at their C-terminal ends, but their biochemical nature was never assessed. Here we demonstrated for the first time that the receiver domain of CK11 has a strong phosphohistidine phosphatase activity toward a set of phosphohistidine-containing phosphoamidated (AHPs) that were also recently demonstrated to be implicated in His-Asp phosphorelay. Supposing that this in vitro activity of the CK11 receiver domain reflects the one in its intact form, it is tempting to speculate that the CK11 receiver domain may have an ability to negatively modulate its own signaling pathway by removing a phosphoryl group from a previously phosphorylated AHP(s). In such a manner, the CK11 sensor may function in concert with some AHPs through the presumed phosphorylation and dephosphorylation via the receiver domain. In any event, our in vitro results in this study should provide us with a clue for understanding the complex mechanisms underlying the presumed His-Asp phosphorelay signaling in higher plants. In this respect, the His-kinase domain of CK11, overexpressed in this study, also remains to be examined.

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