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Rapid Paper

The Arabidopsis Sensor His-kinase, AHK4, Can Respond to Cytokinins

Tomomi Suzuki [1], Kumiko Miwa [1], Kuniko Ishikawa, Hisami Yamada, Hirofumi Aiba and Takeshi Mizuno[2]

Laboratory of Molecular Microbiology, School of Agriculture, Nagoya University, Chikusa-ku, Nagoya, 464-8601 Japan

His-to-Asp (His→Asp) phosphorelay mechanisms are presumably involved in propagation of certain environmental stimuli, including phytohormones, in *Arabidopsis thaliana*. In addition to the previously characterized His-kinases, namely, the ETR1 family of ethylene receptors, CKI1 cytokinin-sensor, and ATHK1 osomo-sensor, this higher plant has three more His-kinases (named AHK2, AHK3, and AHK4). By employing the well-known His→Asp phosphorelay systems in both the fission yeast and *Escherichia coli*, evidence is presented showing that the AHK4 His-kinase has an ability to serve as a cytokinin-responsive environmental sensor. Taking advantage of this AHK4-dependent His→Asp phosphorelay system in *E. coli*, a phosphorelay interaction between the Arabidopsis His-kinase and histidine-containing phosphotransmitters (AHPs) was also demonstrated for the first time.

Key words: Arabidopsis — His-kinase — Phosphorelay — Phytohormones — Cytokinins.

Abbreviations: AHK, Arabidopsis His-kinases; AHP, Arabidopsis HPt factors.

Introduction

Common prokaryotic type of intracellular signal transduction mechanisms are generally referred to as "histidine-to-aspartate (His→Asp) phosphorelay systems" (or "two-component regulatory systems") (Appleby et al. 1996). Such a His→Asp phosphorelay involves two or more common signal transducers, a sensor exhibiting histidine (His)-kinase activity, a response regulator containing a phospho-accepting receiver domain, and a histidine-containing phosphotransmitter (HPt) (Parkinson and Kofoid 1992, Mizuno 1998, and references therein). To date, numerous instances of such His→Asp phosphorelay systems, involved in a wide variety of adaptive responses to environmental stimuli, have been uncovered for not only many prokaryotic species, but also certain eukaryotic species (Mizuno 1997, Wurgler-Murphy and Saito 1997, Chang and Stewart 1998). In the higher plant, *Arabidopsis thaliana*, results from recent intensive studies suggest that His→Asp phosphorelay mechanisms are involved in propagation of certain environmental stimuli, such as phytohormones (e.g. ethylene and cytokinin) (Chang and Stewart 1998, D'Agostino and Kieber 1999, and references therein). An inspection of the Arabidopsis databases revealed that this model plant has at least 11 sensor His-kinases (The Arabidopsis Genome Initiative 2000). Five of them (ETR1, ETR2, ERS1, ERS2, and EIN4) have been demonstrated to be ethylene receptors (Chang et al. 1993, Hua et al. 1995, Hua and Meyerowitz 1998, Sakai et al. 1998a). Two (CKI1 and CKI2) were claimed to be cytokinin-sensors (Kakimoto 1996), and one (AtHK1) was proposed to be a putative osmo-sensor (Urao et al. 1999). The other three (named AHK2, AHK3, AHK4) were recently predicted in the Arabidopsis genome sequence databases, and the occurrence of each cDNA was confirmed by cloning and sequencing, as noted previously (Imamura et al. 1999, see the discussion therein). Furthermore, this higher plant possesses a number of response regulators (ARR-series), each of which contains a typical phospho-accepting receiver domain (Brandstatter and Kieber 1998, Imamura et al. 1998, Sakai et al. 1998b, Urao et al. 1999). These at least 20 members of the ARR family are classified into two distinct subtypes (type-A and type-B), as judged from their structural designs and expression profiles (Taniguchi et al. 1998, Imamura et al. 1998, Imamura et al. 1999, D'Agostino and Kieber 1999). In addition, Arabidopsis has at least five genes each encoding a typical HPt phosphotransmitter (Miyata et al. 1998, Suzuki et al. 1998). These facts strongly suggest that His→Asp phosphorelay signal transduction mechanisms are very common in this higher plant, thus, they presumably play crucial biological roles (for a comprehensive review, see Schaller 2000). Nevertheless, little is known about their biology and physiology.

We have been extensively studying the His→Asp phosphorelay system in *A. thaliana* (Imamura et al. 1998, Imamura et al. 1999, Suzuki et al. 1998, Suzuki et al. 2000, Suzuki et al. 2001). With regard to the sensor His-kinases, particularly, we have been characterizing three new ones (named AHK2, AHK3, and AHK4). They were predicted to occur in the recently released Arabidopsis genome sequence database (AHK2, accession number gp:AB011485_11 in chromosome

[1] These two authors equally contributed to this work.
[2] Corresponding author: E-mail, tmizuno@agr.nagoya-u.ac.jp; Fax, +81-52-789-4089.

V; AHK3, accession number gp:AC004557_11 in chromosome I; AHK4, accession number gp:AC007069-2 in chromosome II). Their corresponding cDNAs were each cloned and characterized. The results showed that their inferred protein structures, including their N-terminal domains, are very similar to each other. Moreover, they belong to the so-called hybrid sensor family of His-kinases that contains both a His-kinase domain and a C-terminal receiver domain (Fig. 1, details will be described elsewhere). With regard to any His-kinases, in general, one of the most critical and tough questions is "what is the primary signal that is perceived by this particular sensor?" Except for the ETR1-family of ethylene sensors, in fact, no primary signal has yet been determined convincingly for any other Arabidopsis His-kinases, even for CKI1. Here we attempted to clarify the biological function of AHKs by employing the fission yeast and *E. coli* His→Asp phosphorelay systems, and will provide evidence that AHK4 is mostly likely a cytokinin-responsive sensor.

Materials and Methods

Yeast strains and plasmids

Schizosaccharomyces pombe was grown mainly at 30°C in YES-medium (yeast extract medium) or EMM-medium (synthetic minimal medium) (Moreno et al. 1991). Strains used are as follows (Aoyama et al. 2000). A wild-type strain used is JY741 *h*$^-$ *leu1-32 ade6-M216 ura4-D18*, and others are its derivative *phk1::ura4*$^+$, *phk2::ura4*$^+$, and *phk3::ura4*$^+$ triple-mutant (designated as ΔPhk1/2/3, details will be described elsewhere), its *mcs4::ura4*$^+$ derivative (named Gp14) (Cottarel 1997), and its *spy1::ura4*$^+$ derivative (named KI001) (Aoyama et al. 2000). An *S. pombe* versatile plasmid, pREP1, was used. This plasmid carries the high-strength *nmt1*$^+$ promoter (Maundrell 1993). The Arabidopsis cDNA that encodes the entire amino acid sequence of AHK4 was cloned downstream of the *nmt1*$^+$ promoter on pREP1 (at the unique *Bam*HI site). Standard yeast genetics for *S. pombe* and sporulation analyses were adopted, all according to those described previously (Moreno et al. 1991). To analyze the G_2/M cell cycle progression, cells were grown at 30°C in EMM medium, supplemented with and without *trans*-zeatin [6-(4-hydoxy-3-methylbut-2-enylamino)purine; *t*-zeatin] or benzyladenine (100 μM each), and at the exponential growth phase, they were observed under a microscope and photographed. The average cell size of septated cells, which were considered to be onset of the cell division, was measured for 20 individuals. An *S. pombe* membrane farction was prepared and fractionated according to the methods described previously (Yamada et al. 1994, Panaretou and Piper 1996).

E. coli strains and plasmids

E. coli K-12 strains, used in this study, are all derivatives of CSH26 [*thi ara Δ(pro-lac)*] (Ishige et al. 1994). They are ST001 (*cps*::*lacZ*), ST261 (Δ*yojN*, *cps*::*lacZ*), and SRC122 (Δ*rcsC*, *cps*::*lacZ*). All these derivatives were constructed, according to the conventional method of *E. coli* genetics, as described previously (Yamashino et al. 1994). These cells were mainly grown at 37°C with Luria-broth, unless otherwise noted. Plasmid-bearing cells were grown with appropriate antibiotics such as ampicillin (50 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}). An *E. coli* versatile plasmid, pIN-III, was mainly used, which carries the high-strength *lpp-lac* promoter (Masui et al. 1983). The Arabidopsis cDNA that encodes the entire amino acid sequence of AHK4 was cloned downstream of the *lpp-lac* promoter, followed by a prokaryotic ribosome-binding site, on pIN-III (at the unique *Bam*HI site). As another *E. coli* versatile plasmid, pACYC184 was also used. To conduct an assay of β-galactosidase activity, it should be noted that cells were grown at 25°C (or room temperature) on Luria-agar plates, supplemented with and without *t*-zeatin or benzyladenine (200 μM to 0.1 μM each). The plates contained also X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), which is an indicator of β-galactosidase activity.

Arabidopsis and related materials

The Columbia ecotype of *Arabidopsis thaliana* (L.) Heynh. was examined. Plants were grown with 16 h-light/8 h-dark fluorescent illumination at 22°C on soil, or on agar plates containing MS salts and 2% sucrose (Imamura et al. 1998, Imamura et al. 1999).

Recombinant DNA techniques

The conditions used for DNA manipulation enzymes were those recommended by the enzyme suppliers (Takara Shuzo or Toyobo, Kyoto, Japan). Other recombinant DNA techniques were all carried out, according to the standard protocols (Sambrook et al. 1989). When required, sequencing of double-stranded DNA on plasmids was performed by using an automated DNA sequencer (Model 373A) (Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacture's instructions with the recommended sequencing kits. Using appropriate pairs of primers, if necessary, polymerase chain reaction (PCR) was carried out to prepare DNA segments. The standard conditions were primarily 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min, 25 cycles with Thermal Cycler 480 (Takara Shuzo, Kyoto, Japan). A PCR kit was used according to the supplier's instructions. Note that all of the critical constructs on plasmids, used in this study, were confirmed by DNA sequencing.

Results and Discussion

One of the Arabidopsis His-kinases can function as a cytokinin-responsive sensor in the fission yeast, S. pombe

In the eukaryotic fission yeast, *S. pombe*, a multistep His→Asp phosphorelay system was documented previously (Shieh et al. 1997, Shiozaki et al. 1997, Aoyama et al. 2000). This system consists of the Phk1/2/3 (three His-kinases) →Spy1 (HPt factor) →Mcs4 (response regulator) phosphorelay-steps. It plays an important role in regulation of the G_2 to mitotic (G_2/M) cell cycle progression in the manner schematically explained in the Fig. 1 (for the mitotic cell cycle control in the fission yeast, see Nurse 1975, Dunphy 1994, Millar 1999). In *S. pombe*, it is well known that certain mutations (e.g. *cdc25*) that are delayed in the timing of the G_2/M transition divide at a cell length longer than the wild-type cells (Millar and Russell 1992). On the other hand, mutants (e.g. *wee1*) that result in a precocious progression of the G_2/M transition divide at a shorter cell length (Russell and Nurse 1987). Accordingly, an *S. pombe* mutant that lacks all of the three His-kinases (Phk1, Phk2, Phk3) is precocious in the entry to M-phase, thereby exhibiting an ovoid and shorter cell morphology (Fig. 1, panel c). As shown, the wild-type cells were measured to be 12.9±0.6 μm in length at cell division, whereas the mutant cells were 10.3±0.6 μm (with regard to the ΔPhk1/2/3 mutant, details will be described elsewhere) (Fig. 1a, c). Here the Ara-

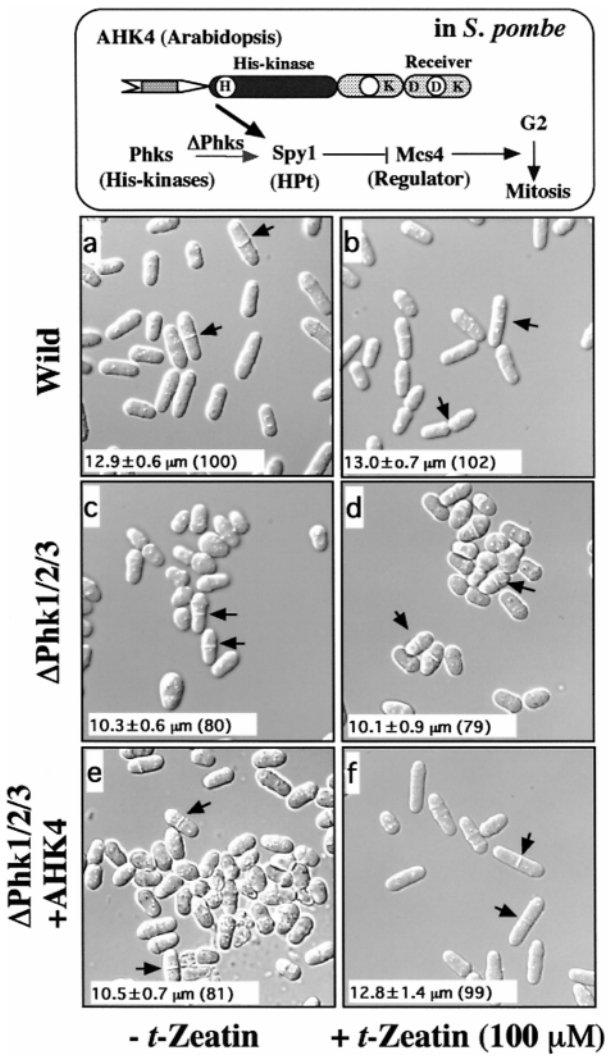
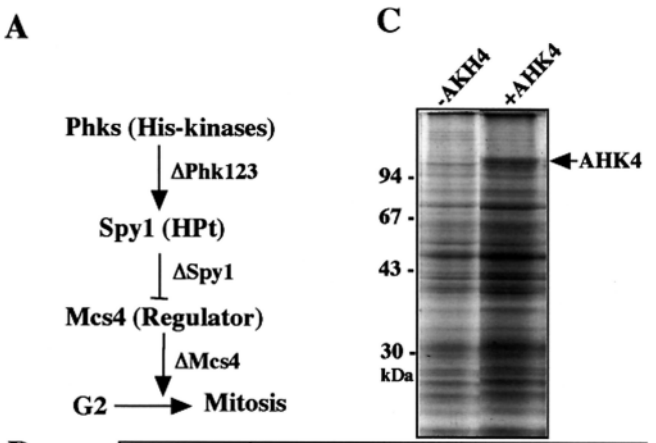


Fig. 1 The Arabidopsis AHK4 His-kinase functions as a cytokinin-responsive sensor in the fission yeast, *S. pombe*. The upper panel shows the schematic structure of AHK4, which consists of a putative N-terminal signal input domain, followed by a His-kinase domain, an atypical receiver domain, and a C-terminal classical receiver domain. The well-known His→Asp phosphorelay signaling scheme in *S. pombe* is also shown, in which the Phk1/2/3→Spy1→Mcs4→phosphorelay system play a crucial role in regulation of the G_2/M cell cycle progression. Note that phospho-Spy1 functions in a negative manner toward Mcs4 through phosphorylation, and phospho-Mcs4 functions in a positive fashion toward the G_2/M transition (Aoyama et al. 2000). AHK4 was introduced into *S. pombe* mutant cells lacking Phk1/2/3. The cells were grown in EMM medium at 30°C (Moreno et al. 1991), supplemented with and without *t*-zeatin (100 μM), and at the exponential growth phase, they were observed under a microscope and photographed. The average cell size of septated cells (see arrows), which are onset of the cell division, was measured for 20 individuals. Note that the ΔPhk1/2/3 cells are significantly shorter (80%) than the wild-type cells.

bidopsis AHK4 gene was introduced into this His-kinase-deficient (ΔPhk1/2/3) mutant by mean of an *S. pombe* versatile expression vector (pREP1) (Maundrell 1993). In the plasmid, the entire AHK4-cDNA was cloned under the *S. pombe nmt1*$^+$ promoter. This plasmid carrying the functional AHK4 gene was introduced into ΔPhk1/2/3 cells, and they were grown in a conventional liquid medium (Moreno et al. 1991, Aoyama et al. 2000). When the cells at the exponential growth phase were observed under a microscope, it was found that the defect of ΔPhk1/2/3 cells in the G_2/M progression was completely suppressed by introducing the Arabidopsis AHK4 gene, as judged by their lengths at cell division. In other words, the complemented cells entered into M phase at a normal cell size (Fig. 1f). However, it should be emphasized that this was observed only when a cytokinin, *t*-zeatin (final 100 μM), was added in the growth medium (Fig. 1, compare panels e and f). Note that the another cytokinin, benzyladenine (final 100 μM), also caused the same event, as demonstrated below (see Fig. 2). It should also be noted that a very low concentration of these cytokinins (0.1 μM) was sufficient to cause the effect (data not shown).

To explain this intriguing observation further, we needed to conduct several critical experiments (Fig. 2). According to the *S. pombe* His→Asp phosphorelay signaling scenario (panel A), the function of the Phk-sensors is dependent upon the downstream signal transducers, Spy1 (HPt factor) and Mcs4 (response regulator) (Cottarel 1997, Aoyama et al. 2000). If AHK4 did indeed exert its function in *S. pombe* through its activity of His-kinase, the suppression phenomenon should be dependent upon the downstream Spy1→Mcs4 phosphorelay. The results form simple epistatic analyses, employing either ΔSpy1 or ΔMcs4 mutant, indicated that this is the case, as follows (panel B). Both ΔPhk1/2/3 and ΔSpy1 cells showed the same phenotype of the precocious progression of the G_2/M transition (a shorter morphology), as expected, whereas ΔMcs4 cells showed a delayed progression (an elongated morphology) (Aoyama et al. 2000). It was found that the cytokinin-dependent suppression by AHK4 was not observed in both the ΔSpy1 and ΔMcs4 cells. This indicates that the function of AHK4 in *S. pombe* is dependent of both Spy1 and Mcs4. It would also be worth mentioning that when these *S. pombe* cells expressing AHK4 were fractionated into subcellular components by a sucrose-density gradient centrifugation, a major band(s) was detected in a certain membrane fraction, when analyzed by SDS-PAGE (panel C) (Laemmli 1970). The apparent molecular mass (ca. 115 kDa) of this band is coincident with the calculated molecular weight of the full length of AHK4 (120,729). These results collectively supported the view that the Arabidopsis His-kinase, AHK4, is capable of functioning as a cytokinin-responsive sensor in *S. pombe* through the presumed AHK4 (His→Asp) →Spy1 (His) →Mcs4(Asp) phosphorelay, thereby being able to regulate the *S. pombe* cell cycle in response to the external plant hormone.



B

	-AKH4 (Vector)	+AHK4 Cell-length onset of Cell-division [μm (%)]		
	+ None	+ None	+ BA	+ t-Zeatin
Wild	13.0±0.8 (100)	12.9±0.6 (100)	13.6±0.6 (105)	13.1±0.7 (102)
ΔPhk123	10.30±0.6 (80)	10.5±0.7 (81)	12.7±1.3 (99)	12.8±1.4 (99)
ΔSpy1	10.5±0.6 (81)	10.3±0.6 (80)	10.7±0.6 (83)	10.9±0.7 (84)
ΔMcs4	17.4±1.0 (135)	15.5±1.4 (120)	15.0±1.2 (116)	15.2±0.8 (117)

Fig. 2 The Arabidopsis AHK4 His-kinase functions as a cytokinin-responsive sensor in a manner dependent on a multistep His→Asp phosphorelay in the fission yeast. (A) The multistep Phk1/2/3→Spy1→Mcs4 phosphorelay is schematically represented, in which three types of *S. pombe* mutants used in this study (ΔPhk1/2/3, ΔSpy1, and ΔMcs4) are also shown. (B) The AHK4 gene was introduced into these mutant cells. They were grown and analyzed with special reference to the G_2/M cell cycle progression, as described (see Fig. 1). Each of final 100 μM benzyladenine (BA) and *t*-zeatin in DMSO was added. Note that DMSO alone was added to the control culture. (C) The ΔPhk1/2/3 cells, with and without the AHK4 gene, were harvested, and they were further fractionated into soluble and membrane fractions. The membrane (or insoluble) fraction was loaded onto a sucrose-density gradient, and then centrifuged. A certain membrane fraction from this sucrose-density gradient was comparably analyzed by means of SDS-PAGE. The denoted major band, found in the +AHK4 cells, is most likely represents the AHK4 polypeptide expressed in *S. pombe*.

AHK4 can function as a cytokinin-responsive sensor also in the bacterium, E. coli

To verify the presumed function of AHK4 further, it was then demonstrated that AHK4 is capable of serving as a cytokinin-responsive sensor even in the prokaryotic eubacteirum, *E. coli*, as shown below. In *E. coli*, as many as 30 distinct His→Asp phosphorelay signaling pathways operate in response to a wide variety of environmental stimuli (Mizuno 1997). Among them, the Rcs-phosphorelay system is unique in that this system involves three components, RcsC (hybrid sensor similar to AHK4 in its structural design), YojN (HPt factor), and RcsB (response regulator), as schematically shown in Fig. 3 (Stout and Gottesman 1990, Stout 1994, Stout 1996, Mizuno 1997, Takeda, S. and Mizuno, T. unpublished data). This particular His→Asp phosphorelay is involved in regulation of an extracellular polysaccharide synthesis by activating the *c*apsular *p*olysaccharide *s*ynthesis (*cps*) operon (Gottesman et al. 1985, Gottesman and Stout 1991, Roberts 1996). The RcsC→YojN→RcsB signaling pathway can easily be monitored by measuring β-galactosidase activity of *E. coli* cells carrying a *cps::lacZ* fusion gene at the λ*att* site on the chromosome. In any event, the important aspect is that the signaling design of this RcsC→YojN→RcsB system seemingly resembles the fission yeast Phks→SypY→Mcs4 system (and perhaps the presumed Arabidopsis ones), in that they all consist of three components. We thus chose this particular *E. coli* His→Asp phosphorelay system, in order to further examine the biological activity of Arabidopsis AHKs. To this end, firstly, an *E. coli* strain having the Δ*rcsC* and *cps::lacZ* genetic backgrounds was constructed by conventional molecular genetics (details will be described elsewhere). Secondly, the entire AHK4 gene was appropriately cloned onto an *E. coli* versatile expression vector (pIN-III) that provides the bacterial promoter (transcription) and ribosome-binding (translation) sites for any heterologous gene (Masui et al. 1983). Similarly, the entire AHK2 and AHK3 genes were also placed on this *E. coli* plasmid, respectively. Finally, these Arabidopsis AHK genes were each introduced into the sensor-negative (ΔRcsC) strain carrying the *cps::lacZ* gene. The cells were grown at 25°C for an appropriate period on a conventional agar-plates (Luria-broth) containing X-Gal, supplemented with and without the plant hormones (*t*-zeatin and benzyladenine, respectively, final 200 μM) (Fig. 3). When they were grown on the plates containing the cytokinins, the *E. coli* cells expressing AHK4 formed dark-blue colonies. In the absence of the plant hormones, the cells showed white colonies (sometime pale-blue colonies, see also Fig. 4). The results of such a blue/white test were best interpreted by assuming that AHK4 can function as a cytokinin-responsive sensor through activating the *E. coli* YojN→RcsB→*cps::lacZ* pathway, thereby giving rise to blue colonies, provided that the cytokinins were present in the agar plates. It should be noted that the blue colonies on the plates produced a large amount of extracellular polysaccharides, thereby exhibiting a mucous and glittering morphology (see also Fig. 4). This is because the endogenous *cps* operon itself was also induced by AHK4 under the conditions. This event further supports the above notion. It may also be worth mentioning that such an AHK4 and cytokinin-dependent expression of *cps::lacZ* was not observed in the ΔRcsC/ΔYojN double-mutant background (data not shown). Furthermore, when a truncated form of AHK4 that lacks the C-terminal receiver domain was examined, AHK4 lost its ability to induce *cps::lacZ* (Fig. 3). Note also that neither AHK2 nor AHK3

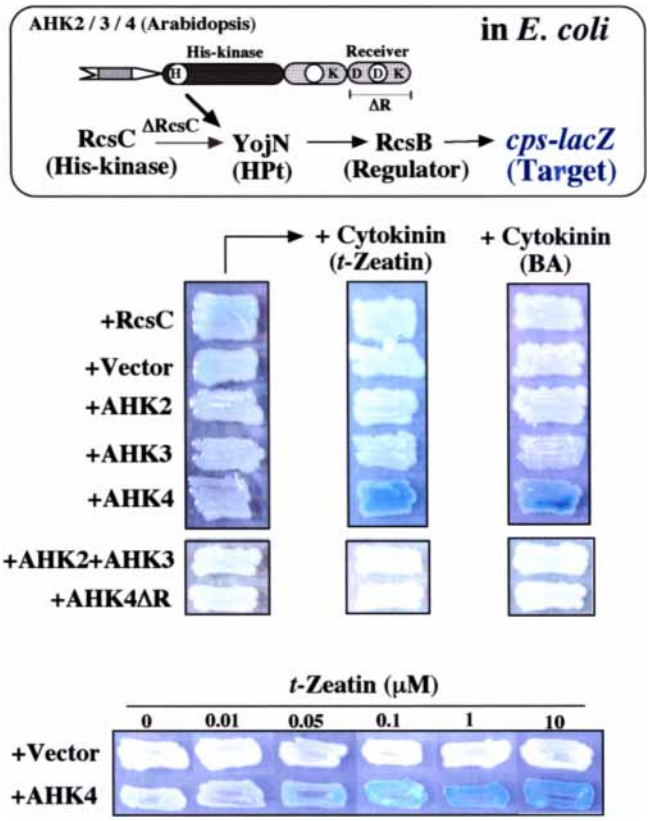


Fig. 3 The Arabidopsis AHK4 His-kinase functions as a cytokinin-responsive sensor in the eubacterium, *E. coli*. The upper panel shows the schematic structure of AHK4. In this study, a truncated AHK4 gene encoding a polypeptide without the C-terminal receiver domain was also constructed and used, as schematically shown (designated as AHK4ΔR). The well-established His→Asp phosphorelay signaling scheme in *E. coli* is also shown, in which the RcsC→YojN→RcsB→ phosphorelay system play a crucial role in regulation of the *cps* operon (here the *cps* promoter was fused with the *lacZ* gene on the chromosome). Note that phospho-RcsB functions in a positive fashion toward the *cps* transcription (Stout 1994). Either AHK2, AHK3, AHK4, or AHK4ΔR was introduced into *E. coli* mutant (ΔRcsC) cells lacking RcsC. Both AHK2 and AHK3 were also co-introduced (+AHK2+AHK3). It should be noted that the cells were grown at 25°C on Luria-agar plates, supplemented with and without *t*-zeatin or benzyladenine (200 μM each). The plates also contained X-Gal. After 40 h incubation, the plates were photographed. It should be also noted that the RcsC His-kinase did not respond to cytokinins, as expected, unless it is activated by a true stimulus (an osmotic shock, Stout 1994). In the bottom panel, varied concentrations of *t*-zeatin in medium were tested in terms of their abilities to activate the AHK4 His-kinase in *E. coli*.

showed such ability (event when both the genes were co-introduced into *E. coli*) (Fig. 3).

Cytokinins include a variety of related chemicals. In addition to *t*-zeatin and benzyladenine, kinetin, isopenteny adenine (IP) and isopentenyl adenosine (IPA) were also able to activate the AHK4 His-kinase in *E. coli* (data not shown). In the above experiments in *E. coli*, we used relatively high concentrations

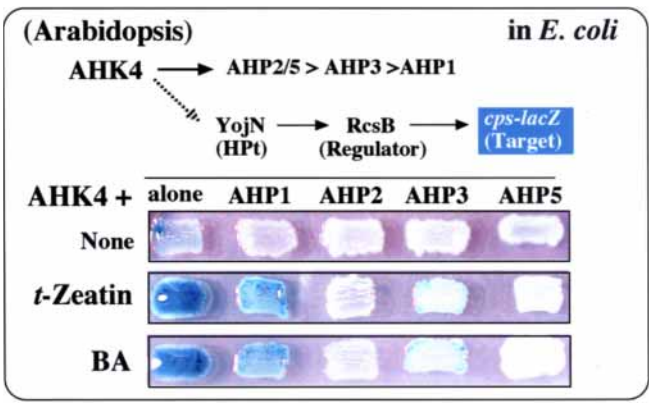


Fig. 4 The Arabidopsis AHK4 His-kinase shows an ability to interact with some Arabidopsis HPt factors, AHPs. AHK4 was introduced into *E. coli* mutant cells lacking RcsC. Also co-introduced were AHP1, AHP2, AHP3, AHP5, respectively. Other details are the same as those described in Fig. 3.

of cytokinins in medium (100 μM or 200 μM). We then needed to determine a minimum-effective concentration of cytokinins. The results showed that the minimum concentration was as low as 0.1 μM (Fig. 3), the value of which is as comparable as that effective toward plants in general. Collectively, it was thus possible to reconstitute an artificial and multistep AHK4 (His→Asp) →YojN (His) →RcsB(Asp) phosphorelay in *E. coli*, in which the Arabidopsis AHK4 sensor is capable of propagating an external cytokinin-signal to induce the *E. coli cps* operon.

AHK4 has an ability to interact with AHPs through phosphorelay

Finally, the *E. coli* system for monitoring the specific function of AHK4 allowed us to address a longstanding issue with regard to the Arabidopsis His→Asp phosphorelay, as follows. As mentioned above, Arabidopsis has at least five AHP genes each encoding an HPt phosphotransmitter (Suzuki et al. 2000). Nevertheless, so far neither in vivo nor in vitro evidence has been provided for that these AHPs indeed have an ability to interact with certain Arabidopsis His-kinases in any way (Suzuki et al. 2001). Here the AHP1, AHP2, AHP3, and AHP5 genes were each cloned onto an *E. coli* plasmid so as to be expressed in *E. coli* (note that these pACYC184 derivatives are compatible with the pBR322 derivative that carries the AHK4 gene in *E. coli* cells). Each of these AHP genes was co-introduced with the AHK4 gene into the ΔRcsC cells to see the cytokinin-dependent response (Fig. 4). The results showed that when either AHP2 or AHP5 gene was co-expressed with the AHK4 gene, AHK4 no longer showed the ability to signal the downstream YojN→RcsB pathway, resulting in white colonies. This event can be explained by assuming that AHK4 interacts with AHP2 (or AHP5) in a manner that a phosphoryl

group on AHK4 is more preferentially acquired by these cognate Arabidopsis HPt factors (rather than by *E. coli* YojN). Such an event (generally referred to as "signal titration") is indicative of that AHK4 interacts with AHP2/5 through an intimate phosphorelay reaction. Interestingly, such events were slightly less evident in the case of the AHP3 gene, and significantly less evident in the case of AHP1. Thus, the order of such effectiveness of signal titration seems to be AHP2/5>AHP3>AHP1. This may suggest a specificity of the presumed AHK4–AHPs phosphorelay interactions, assuming that these AHPs were expressed in *E. coli* at a similar level relative to each other. In fact, AHP2/3/5 are more closely related to each other than to AHP1, as judged by a phylogenetic tree, constructed for the AHP family members (Suzuki et al. 2000). In any case, the *E. coli* two-hybrid assay, developed here, is highly sensitive, and the results showed for the first time that AHK4 is capable of interacting with certain AHPs through phosphorelay.

Implication

Except for the ETR1-family of ethylene sensors, no external stimulus has yet been assigned convincingly for any other Arabidopsis His-kinases. All of our results in this study can be straightforwardly explained by assuming that AHK4 is a cytokinin-responsive sensor. AHK4 can respond to cytokinins quite effectively in *E. coli* (at a concentration of sub-μM). The results also suggested that the presumed binding of cytokinin to AHK4 causes an activation of its His-kinase activity. This is in contrast to the case of ETR1, in that the ethylene binding to the receptor is assumed to result in inactivation of its kinase activity (Hua and Meyerowitz 1998). It was also demonstrated that AHK4 is capable of interacting with AHPs through phosphorelay.

Considering the fact that the inferred protein structures of AHK2, AHK3 and AHK4, including their N-terminal domain, are very similar to each other, it is tempting to speculate that AHK2 and AHK3 are also cytokinin sensors. It is then puzzling that neither AHK2 nor AHK3 could response to cytokinins in our *S. pombe* and *E. coli* systems. A trivial explanation is that they were not expressed properly in the heterologous systems. Rather, AHK2 and AHK3 may function as a cytokinin sensor only when they form a heterodimer with AHK4. Alternatively, they may respond to another type of cytokinin, such as diphenylurea derivatives, which we did not test in this study. It would be of interest to examine these possibilities.

Finally, it should be emphasized that since we employed the heterologous *S. pombe* and *E. coli* His→Asp phosphorelay systems, no direct insight was gained into the physiological function of AHK4 in Arabidopsis. However, the experimental systems developed in this study will provide a new avenue, through which one can take unique experimental approaches, as follows. One can express the entire (or intact) form of AHK4 in a membrane fraction of *S. pombe* (Fig. 2). This result supports the assumption that AHK4 is most likely located in a certain membrane of the plant cell. This is consistent with the fact that AHK has hydrophobic stretches of amino acids at its N-terminal region. In any case, the isolated *S. pombe* membrane fraction containing AHK4 will allow us to examine the AHK4 function, in terms of not only its in vitro activity of phosphorelay, but also its in vitro ability to bind cytokinins directly. Although we have not yet succeeded in doing so, these lines of in vitro experiments are underway. On the other hand, by employing a simple blue/white screening in *E. coli*, it may be possible to isolate certain AHK4 mutants that show altered properties, such as "cytokinin-hypersensitive" and "cytokinin-independent". These results will provide us with insight into the structure and function of AHK4 at the molecular level. Once isolated, more interestingly, such presumably dominant mutants of the AHK4 gene may be introduced into the original plant to see the biological effect with special reference to cytokinin responses of Arabidopsis. These approaches are also underway in our laboratory.

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