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Short Communication

The Arabidopsis AHK4 Histidine Kinase is a Cytokinin-Binding Receptor that Transduces Cytokinin Signals Across the Membrane

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Common histidine-to-aspartate (His→Asp) phosphorelay is a paradigm of signal transduction in both prokaryotes and eukaryotes for the propagation of certain environmental stimuli, in which histidine (His)-kinases play central roles as sensors for environmental signals. For the higher plant, *Arabidopsis thaliana*, it was recently suggested that the His-kinase (AHK4 / CRE1 / WOL) is a sensor for cytokinins, which are a class of plant hormones important for the regulation of cell division and differentiation. Interestingly, AHK4 is capable of functioning as a cytokinin sensor in the eubacterium, *Escherichia coli* (Suzuki et al. 2001, *Plant Cell Physiol.* 42: 107). Here we further show that AHK4 is a primary receptor that directly binds a variety of natural and synthetic cytokinins (e.g. not only N^6-substituted aminopurines such as isopentenyl-adenine, *trans*-zeatin, benzyl-adenine, but also diphenylurea derivatives such as thidiazuron), in a highly specific manner (K_d = 4.55±0.48×10^{-9} M). AHK4 has a presumed extracellular domain, within which a single amino acid substitution (Thr-301 to Ile) was shown to result in loss of its ability to bind cytokinins. This particular mutation corresponds to the previously reported *wol* allele (*wooden leg*) that causes a striking phenotype defective in vascular morphogenesis. Collectively, evidence is presented that AHK4 and its homologues (AHK3 and possibly AHK2) are receptor kinases that can transduce cytokinin signals across the plasma membrane of *A. thaliana*.

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

Abbreviations: AHK4, Arabiodpsis histidine kinase 4; IP, isopentenyl-adenine; IPA, isopentenyl-adenosine, BA, benzyl-adenine; DAP, dimethyl-allyl-diphosphate.

Cytokinins are a class of plant hormones important for the regulation of cell division and differentiation (for overviews as to cytokinins, see text books, Mok and Mok 1994, Taiz and Zeiger 1998). They include a wide variety of related chemicals, some of which have been isolated from plants, and others have been chemically synthesized (Fig. 1A). Nearly all compounds active as cytokinins are N^6-substituted aminopurines and their ribosides, which are represented by *trans*-zeatin and isopentenyl-adenosine (IPA), respectively. Curious exceptions are certain diphenylurea derivatives, represented by thidiazuron, whose chemical structure is quite different from that of aminopurine derivatives (Fig. 1A). It has thus long been puzzling how plants recognize these different types of chemicals, and how these compounds exert the same effect on fundamental plant physiology as hormones. During the last three decades, intensive efforts have been made to find a cytokinin-binding protein that plays a crucial role in cytokinin-mediated signal transduction. Until recently, nonetheless, no such plausible candidate has been uncovered, except for the Arabidopsis receptor-like CKI1 histidine (His)-kinase that seemed to be involved in cytokinin signaling (Kakimoto 1996). Then, two recent papers independently suggested that the Arabidopsis AHK4 (or CRE1) His-kinase functions as a sensor for cytokinins (Inoue et al. 2001, Suzuki et al. 2001). Nevertheless, the results did not necessarily prove that AHK4 is the primary receptor that directly binds the plant hormone. Here we examine whether or not this is the case.

As reported previously (Suzuki et al. 2001), the plant AHK4 His-kinase can serve as a cytokinin-responsive sensor in the eubaterium, *Escherichia coli*. When the *AHK4* gene was introduced into an appropriate *E. coli* mutant strain (Δ*rcsC*, *cps*::*lacZ*), the gene product could propagate the endogenous YojN (HPt factor)→RcsB (response regulator)→*cps*::*lacZ* (target) signaling pathway in the absence of the authentic His-kinase, RcsC (Fig. 1A) (Takeda et al. 2001). In this assay, *E. coli* colonies become blue on agar-plates, containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), in response to cytokinins in medium. To gain insight into the nature of AHK4, here we further adopted this *E. coli* assay system, as follows. With regard to the chemical structures of cytokinins, there are three major sub-classes, as mentioned above. These three representatives (*trans*-zeatin, IPA, and thidiazuron) were examined by means of such spot assays on agar-plates, and also by directly measuring β-galactosidase activity (Fig. 1B). These

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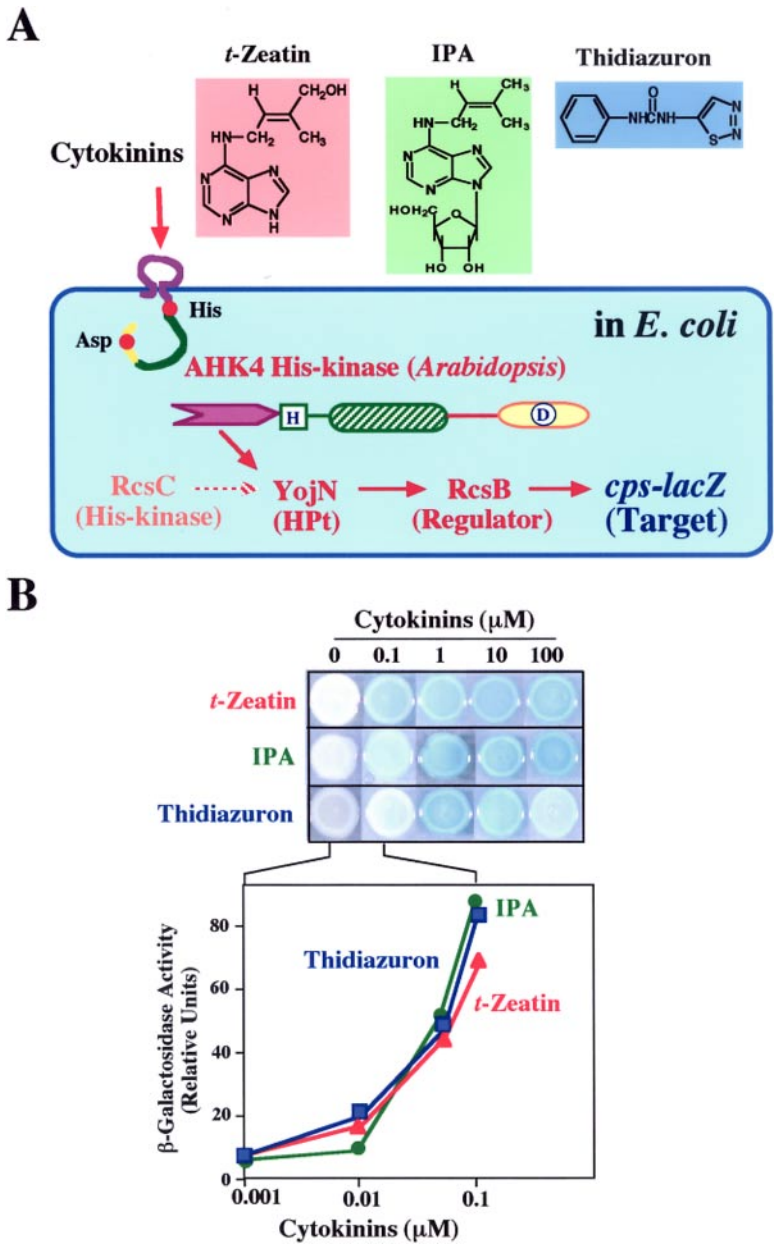

Fig. 1 The Arabidopsis AHK4 His-kinase functions as a cytokinin sensor in *E. coli*. (A) Structures of cytokinins, and conceptual views that explain how the Arabidopsis AHK4 protein functions as a sensor for cytokinins in *E. coli* (Suzuki et al. 2001). Three representatives of cytokinins are shown, including *trans*-zeatin, isopentenyl-adenosine (IPA), and thidiazuron. As illustrated, the *E. coli* RcsC→YojN→RcsB multistep phosphorelay is responsible for activation of the *cps* operon. The *E. coli* mutant (Δ*rcsC*) used in this study carries a *cps*::*lacZ* fusion gene on the chromosome (Takeda et al. 2001). When the Arabidopsis *AHK4* gene is expressed in this particular mutant, AHK4 is capable of signaling the downstream YojN→RcsB→*csp*::*lacZ* pathway in response to external cytokinins. Consequently, the target *csp*::*lacZ* gene is activated. (B) Based on these experimental rationales, *E. coli* cells carrying the plasmid that encompasses the *AHK4* gene were grown on agar plates, supplemented with 5-bromo-4-chloro-3-indoylyl-β-D-galactoside (X-Gal) (spot assay). Note that the cells were grown at 25°C on Luria-agar plates, supplemented with and without each indicated cytokinin. After 40 h incubation, the plates were photographed. Also, β-galactosidase activities were assayed with *E. coli* cells, grown in liquid medium containing varied concentrations of cytokinins (β-galactosidase assay). Such experiments were repeated several times, and representative data are shown.

chemicals were equally effective in inducing β-galactosidase at a concentration of approximately 5×10^{-8} M. The results supported the view that AHK4 is a sensor that perceives sensitively both types of cytokinins, i.e. N^6-substituted aminopurine and diphenylurea derivatives.

To determine whether or not AHK4 is a cytokinin-binding

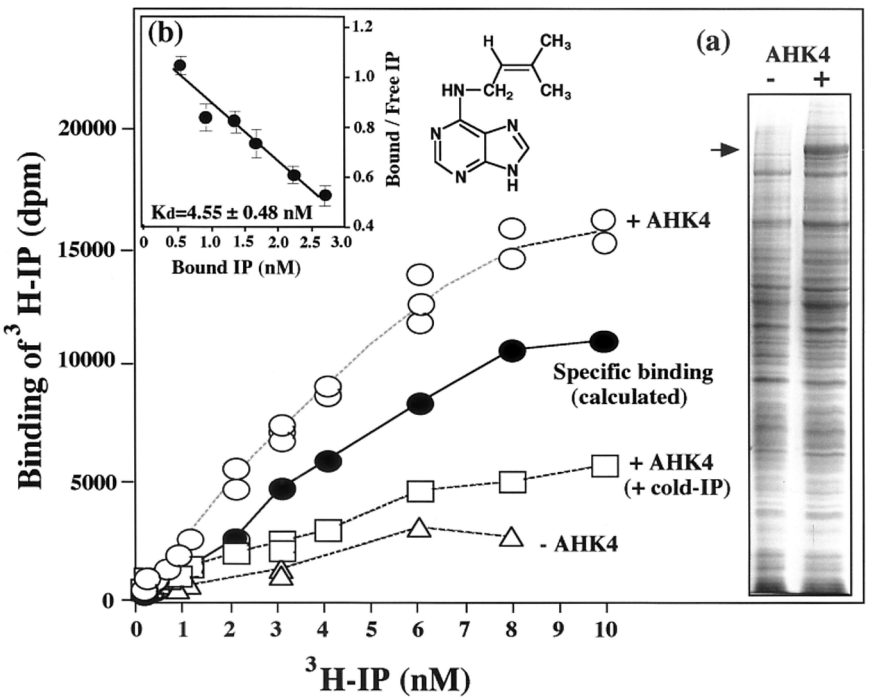

Fig. 2 In vitro binding assay of cytokinins with the AHK4 protein in the *S. pombe* membrane. The *AHK4* gene was introduced into a certain *S. pombe* strain (*phk1/2/3*Δ) with use of an appropriate *S. pombe* expression vector (Suzuki et al. 2001). The *AHK4* gene was cloned onto an *S. pombe* versatile expression vector (pREP1). In this plasmid, the entire *AHK4*-cDNA was cloned under the *S. pombe nmt1*$^+$ promoter. *S. pombe* cells were grown in EMM medium at 30°C. The *S. pombe* cells, with and without the *AHK4* gene, were harvested, and then they were fractionated into soluble and membrane fractions. The membrane fraction was loaded onto a sucrose-density gradient, and then centrifuged. A certain membrane fraction from this sucrose-density gradient was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (see inset-a). The major band, indicated by an arrow, was found exclusively in the AHK4-plus membrane. This membrane fraction was used as AHK4-containing *S. pombe* membranes for the following in vitro cytokinin-binding assay. For the binding assay, ^{3}H-labeled isopentenyl-adenine (^{3}H-IP) was synthesized as follows. First, ^{3}H-labeled isopentenyl-adenosine (^{3}H-IPA) was synthesized from ^{3}H-AMP and dimethyl-allyl-diphosphate (DAP) by incubating with an isopentenyl transferase. The product was treated with an alkaline phosphatase to remove the phosphoryl group at the position of C^5 of the adenosine moiety. The resulting ^{3}H-IPA was purified, and the yield was 44,276 dpm pmol^{-1}. To obtain ^{3}H-IP, the ^{3}H-IPA product was treated with 0.1 M HCl at 100°C. The yield of ^{3}H-IP was 39,393 dpm pmol^{-1}. This purified ^{3}H-IP in H_2O was stored at –25°C, and used for the following binding assay. More details will be described elsewhere (Takei, unpublished data). The AHK4-containg *S. pombe* membrane (50 μg) was incubated with varied concentrations of ^{3}H-IP in a buffer (100 μl), comprising 10 mM MES (pH 6.0), 1 mM $MgCl_2$, 0.05 $MnCl_2$, 0.1 mM $CaCl_2$, 10% glycerol, 0.1 mg bovine serum albumin. When necessary, an excess amount (200-fold) of non-radioactive (cold) IP was also added. The mixture was incubated for 30 min at 25°C. The bound and free ^{3}H-IP were separated by a filter-equipped mini-centrifuge tube (Ultrafree 0.1 μm filter, Millipore) by centrifugation. They are quantified by scintillation counter. Specific binding was determined by subtracting the binding in the presence of cold-IP from total binding. The binding assays were independently repeated three times, and all of these values are plotted. To estimate the binding affinity, the dissociation constant (K_d) was calculated by plotting the mean values according to the standard formula (Scatchard's plot), as shown in inset-b.

protein (or receptor), it was expressed in the fission yeast, *Schizosaccharomycess pombe*, as described previously (Suzuki et al. 2001) (Fig. 2, see inset-a). For the isolated *S. pombe* membrane, a band corresponding to AHK4 (approximately 130 kDa) was abundantly detected on sodium dodecyl sulfate polyacrylamide gel electrophoresis. This band was confirmed to specifically react with an anti-AHK4 antiserum. Such AHK4-containing membranes were used for an in vitro binding assay with ^{3}H-labeled isopentenyl-adenine (^{3}H-IP) (Fig. 2). The *S. pombe* membrane was incubated with ^{3}H-IP. Then, the membrane was collected by a membrane filter by centrifugation, in order to see if ^{3}H-IP was specifically retained on the membrane containing AHK4. Such filter-binding assays were repeatedly carried out in the presence and absence of an excess amount (200-fold) of a non-radioactive competitor (cold-IP). It was shown that the radiolabeled cytokinin associates specifically with the membrane containing AHK4, but not with the membrane lacking AHK4 (Fig. 2). The results were further treated to induce "a Scatchard plot" (see inset-b). The results of such intensive in vitro binding assays were best interpreted by assuming that AHK4 associates with IP in a highly specific manner. Its dissociation constant (K_d) was calculated to be $4.55 \pm 0.48 \times 10^{-9}$ M.

To prove this further, a competitive binding assay was also

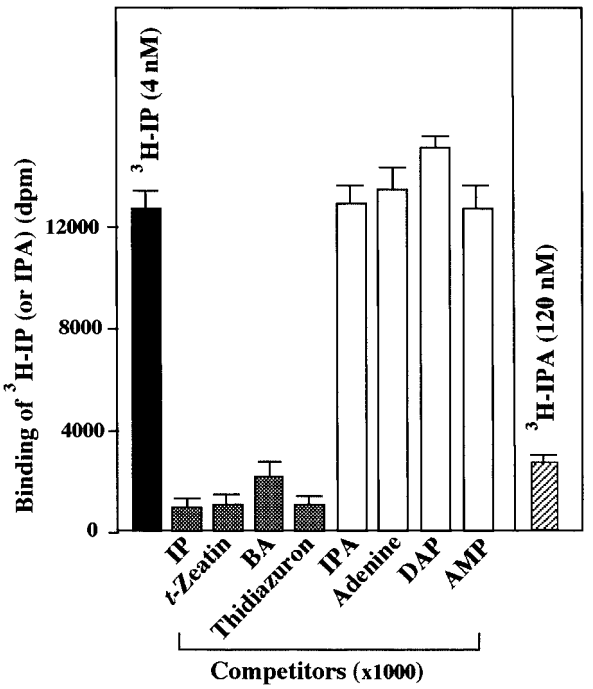

Fig. 3 Competitive cytokinin-binding assay. Binding assays with the AHK4-containing *S. pombe* membrane and ^{3}H-IP was carried out in the absence and presence of an excess amount (1,000-fold) of indicated cytokinin-related chemicals, as described in Fig. 2. Similarly, a binding assay was carried out with ^{3}H-IPA without competitor. The average of two experiments is shown.

carried out with use of *trans*-zeatin, benzyl-adenine (BA), thidiazuron, and IPA, as competitors (Fig. 3). Several cytokinin-related compounds, namely, adenine, dimethyl-allyl-diphosphate (DAP), and adenosine-monophosphate (AMP) were also tested, as negative references. The IP-binding to AHK4 was competed by *trans*-zeatin and BA. Interestingly, thidiazuron was also a strong competitor, but IPA was not. Together with the results for adenine, DAP, and AMP, which exhibited no ability to compete, it was concluded that AHK4 is the primary receptor for both the types of cytokinins (i.e. N^6-substituted aminopurine and diphenylurea derivatives). However, their riboside derivatives appear not to be such hormonal ligands for AHK4. This latter conclusion was further supported by the results of a direct binding assay with ^{3}H-labeled IPA that did not bind to the AHK4-enriched membrane (Fig. 3). As has been presumed previously (Mok and Mok 1994), therefore, riboside derivatives (e.g. IPA) appear to be active as cytokinins on their conversion to the respective bases (e.g. IP), as far as the AHK4 receptor is concerned. This seems to also be the case in the *E. coli* assay system (see Fig. 1B).

The structure of AHK4 is schematically shown (Fig. 4A). Its presumed phosphorylated histidine site is found in front of a His-kinase domain and a receiver domain. Furthermore, AHK4 has an amino-terminal extension, in which two stretches of hydrophobic amino acids (designated as TM1 and TM2) are located, together with an intervening sequence of about 270 amino acids. Judging from this, one can assume that the intervening sequence, flanked by the two putative membrane-spanning regions, most likely protrudes outside of the cells, thereby functioning as the ligand-binding domain. In this context, it is worth mentioning that, among eleven Arabidopsis His-kinases including five ethylene receptors (for a review, see Schaller 2000), two others (named AHK2 and AHK3) are highly homologous to AHK4 in their entire amino acid sequences, including the presumed extracellular domain (Fig. 4A). This suggests that AHK2 and AHK3 might also be cytokinin receptors. To examine this, AHK3 was expressed and examined in the *E. coli* strain, as done for AHK4. The results indicated that AHK3 is also capable of propagating external cytokinin signals, thereby inducing β-galactosidase in *E. coli* (Fig. 4B). It should be noted that the response is relatively low, as compared with in the case of AHK4 (see Fig. 4B).

It has previously been proposed that another His-kinase (named CKI1) appears to be involved in cytokinin signaling in Arabidopsis (Kakimoto 1996). CKI1 differs considerably from AHK4 in the amino acid sequences of their amino-terminal regions (data not shown). This raised the question of whether or not CKI1 is a cytokinin receptor. By adopting the *E. coli* assay system, here CKI1 was expressed in the same *E. coli* strain. CKI1 was active in inducing β-galactosidase in *E. coli* cells (Fig. 4C). Nevertheless, the level of β-galactosidase activity did not fluctuate at all in response to the exogenous cytokinins in the medium. In other words, CKI1 was constitutively active in *E. coli*. Furthermore, we isolated the *S. pombe* membrane containing CKI1, but this membrane did not show the in vitro ability to bind ^{3}H-IP (data not shown). It is thus unlikely that CKI1 is a cytokinin receptor.

The developmental ontogeny of the vascular system, consisting of the xylem, phloem and (pro)-cambium, is central in plant physiology. In this regard, an intriguing mutant of Arabidopsis has previously been isolated, named *wooden leg* (*wol* mutant), which results in a reduced cell number and exclusive xylem differentiation within the vascular tissue (Scheres et al. 1995). Recently, it was reported that the *WOL* locus is identical to *AHK4* (or *CRE1*) (Mahonen et al. 2000). It is thus tempting to speculate that there is a link between cytokinin signaling and vascular morphogenesis. However, no evidence was presented for that the *wol* phenotype is indeed relevant to the cytokinin signaling. The *wol* mutation was characterized as a single amino acid substitution at an amino acid position of 301 in AHK4 (Thr to Ile). This particular mutation is located in the presumed extracellular domain, which was assumed to serve as a cytokinin binding domain (see Fig. 4A). To address these two critical issues, it is of interest to determine the molecular nature of the AHK4-*wol* mutant His-kinase, particularly, in terms of its ability to respond to cytokinins. This was first performed with the *E. coli* assay system (Fig. 5A), and the result showed that the AHK4-*wol* mutant is inactive in the *E. coli* assay. In

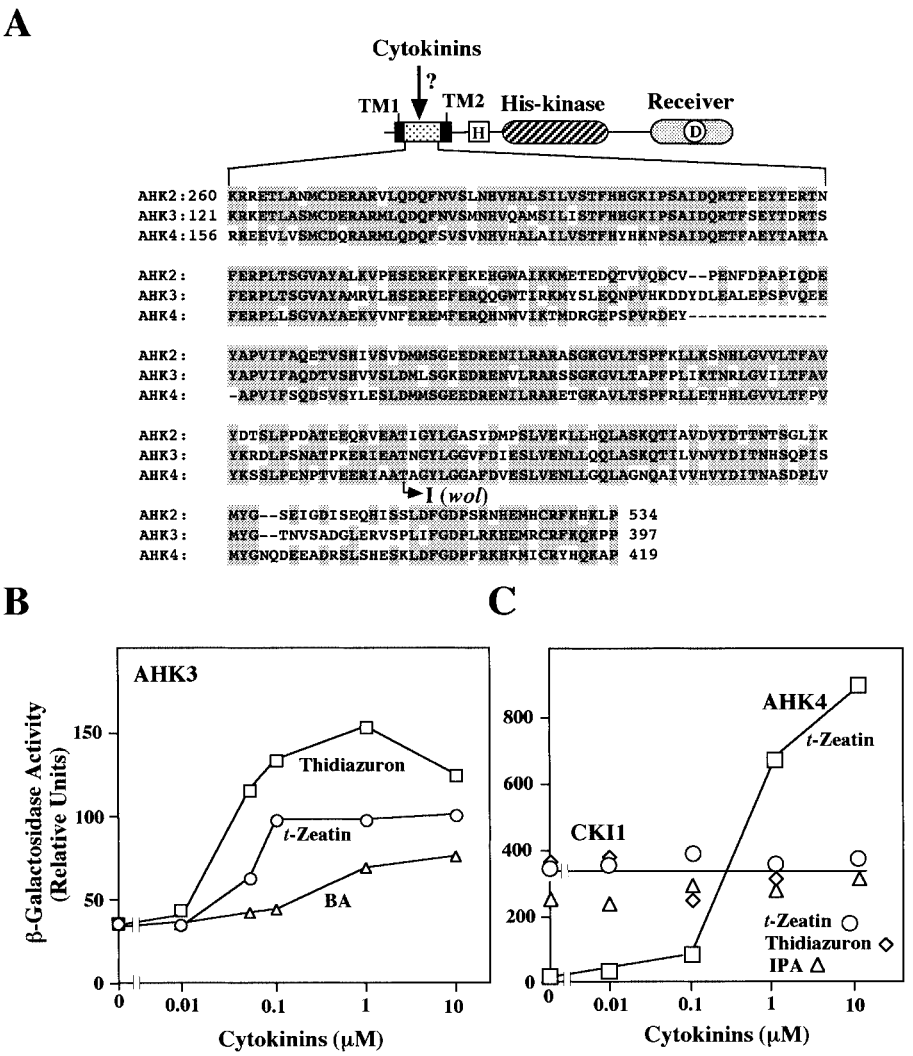

Fig. 4 (A) Structure of AHK4 and comparison with other His-kinases, AHK2 and AHK3. As reported previously (Ueguchi et al. 2001), Arabidopsis has three homologous His-kinases (AHK2, AHK3, AHK4), the entire amino acid sequences are highly similar to each other, as schematically shown. The similarities are seen not only in the common His-kinase and receiver domains, but also in their N-terminal extensions. Each N-terminal extension encompasses two putative membrane-spanning regions (TM1 and TM2) and an intervening sequence. Among AHK2, AHK3, and AHK4, the amino acid sequences of these intervening regions are aligned, in which conserved amino acids are shaded. Note that two possible initiation methionine residues can be inferred for AHK4 (Mahonen et al. 2000, Inoue et al. 2001, Suzuki et al. 2001). The upstream one was adopted here. The previously reported *wol* mutant has the single amino acid substitution at the position of 301 (Thr to Ile), as indicated (Mahonen et al. 2000). (B) AHK3 also functions as a cytokinin sensor in *E. coli*. The *AHK3* gene was cloned onto an *E. coli* versatile expression vector (pIN-III), similarly as in the case of the *AHK4* gene (Suzuki et al. 2001). The *AHK3* gene was introduced into the *E. coli* strain (Δ*rcsC cps*::*lacZ*) (see Fig. 1). The cells were grown in the presence of varied concentrations of cytokinins (*trans*-zeatin, BA, or thidiazuron), and then they were assayed for β-galctosidase activity. (C) CKI1 functions as a His-kinase in *E. coli*, but it does not respond to cytokinins. The *CKI1* gene was cloned onto an *E. coli* versatile expression vector (pBlue-Script). This is a kind gift from T. Kakimoto (1996). The *CKI1* gene was introduced into the same *E. coli* strain, and then characterized, as described above, with special reference to its responsiveness to cytokinins (*trans*-zeatin, IPA, or thidiazuron). The function of AHK4 was also monitored in this experiment, as an appropriate reference.

other words, it has no ability to respond to external *trans*-zeatin. The results of the in vitro binding assay with the *S. pombe* membrane containing the AHK4-*wol* mutatnt protein directly showed that it has no ability to bind cytokinins (Fig. 5B). Note that the AHK4-*wol* mutant protein was accumulated in the yeast membrane, as much as in the case of the wild-type protein (Fig. 5B, see inset). These findings revealed that the *AHK4-wol* mutation represents "a loss of function", with regard to cytokinin binding and signaling. Furthermore, these results support the view that the presumed extracellular domain is the region that directly associates with cytokinins. These suggest that the external cytokinin signal is most likely trans-

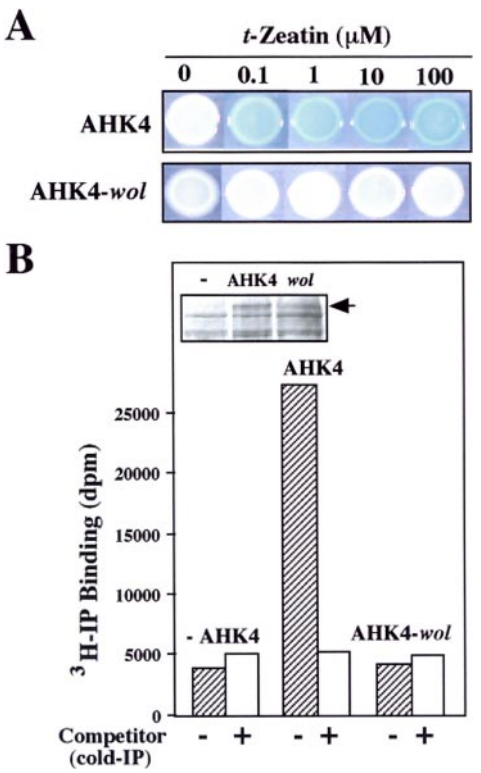

Fig. 5 Natures of a certain AHK4 mutant, named *wooden leg* (*wol*). The previously reported *wol* mutant of Arabidopsis is allelic to the *AHK4* gene (Mahonen et al. 2000). The mutant product is designated here as AHK4-*wol*, which has the Thr-301 to Ile amino acid substitution (see Fig. 4). The natures of this mutant protein was compared with the wild-type protein. (A) AHK-*wol* has no ability to propagate cytokinin-signals in the *E. coli* assay system. The *AHK4-wol* gene, encoding the mutant AHK4 protein with the Thr-301 to Ile substitution, was constructed on an *E. coli* plasmid vector by means of site-directed mutagenesis. A spot assay was carried out for *E. coli* cells carrying the mutant *AHK4-wol* gene, with varied concentrations of *trans*-zeatin in agar-plates, as described in Fig. 1. (B) AHK4-*wol* has no ability to bind cytokinins in the in vitro binding assay with the *S. pombe* membrane. The *AHK4-wol* mutant gene was cloned onto an *S. pombe* versatile expression vector, similarly as in the case of the wild-type *AHK4* gene. The *S. pombe* membrane fraction containing the AHK4-*wol* protein was examined for its ability to bind ^{3}H-IP in vitro, in the presence and absence (+ and –) of the competitor (IP) (200-fold), as described in Fig. 2. Such binding-assays were repeated three times, and representative data are shown. The *S. pombe* membranes with and without the wild-type AHK4 protein were also examined, as references. These *S. pombe* membranes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, in order to make sure that each contains a comparable amount of AHK4-polypeptide (see inset).

duced by AHK4 across the membrane.

The results of our study, together with those reported previously (Inoue et al. 2001, Suzuki et al. 2001), led us to conclude that AHK4 serves as a cytokinin-binding receptor that can directly perceive both aminopurine and diphenylurea derivatives, but not riboside derivatives. It was also suggested that Arabidopsis has two more homologous cytokinin receptors, AHK2 and AHK3. For the latter, its ability to propagate an external cytokinin signal was indeed demonstrated in this study. These cytokinin receptors presumably function as His-kinases, involved in a His→Asp phosphorelay signaling. One can assume that the direct binding of cytokinins to the extracellular domain of AHK4 triggers its intrinsic His-kinase activity, and consequently AHK4 propagates such hormonal signals across the membrane by itself. This event must be verified by means of an in vitro phosphorylation assay. In any case, according to the current knowledge concerning the His→Asp phosphorelay (Mizuno 1998), these receptor kinases act, most likely, in concert with downstream components, such as histidine-containing phosphotransfer (HPt) intermediates and response regulators. Indeed, Arabidopsis has five genes each encoding an HPt factor (AHP-series) (Suzuki et al. 2000), and 20 genes each encoding a response regulator (ARR-series) (Imamura et al. 1999). Thus, the AHK4-mediated multistep His→Asp phosphorelay pathway must be involved in a signaling network in response to cytokinins that are a class of plant hormones central to the regulation of cell division and differentiation, such as vascular morphogenesis, as indeed exemplified by the *wol* and *cre1* mutations.

Acknowledgements

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