Identification of Amino Acid Substitutions that Render the *Arabidopsis* Cytokinin Receptor Histidine Kinase AHK4 Constitutively Active

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In *Arabidopsis*, three genes (*AHK2, AHK3* and *AHK4/CRE1*) encode histidine kinases (His-kinases), which serve as cytokinin receptors. To understand how the external cytokinin signal activates the His-kinase across the cell membrane, we exploited the power of microbial genetics to isolate several *AHK4* mutants that function independently of cytokinin in both prokaryotic and eukaryotic assay systems. In each mutant, a single amino acid substitution within the second membrane-spanning segment, or within the region around the phosphorylation His site, renders the His-kinase constitutively active. These mutant receptors appear to have a ‘locked-on’ conformation, even in the absence of stimulus. We discuss the implications of these data for the structure and function of the cytokinin receptor His-kinases in plants.

**Keywords:** Cytokinin assay system with microbial cells — Cytokinin receptor — His–Asp phosphorelay signaling — His-kinase — Structure and function.

Abbreviations: His-kinase, histidine kinase; iP, isopentenyl adenine; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

Cytokinins are a class of plant hormones that are important for the regulation of cell division and differentiation at almost every developmental stage in higher plants (Mok and Mok 2001). In *Arabidopsis thaliana*, the *AHK4* gene (also known as *CRE1* or *WOL*) encodes a histidine kinase (His-kinase) which acts as a cytokinin receptor (Yamada et al. 2001). Together with the products of the homologous *AHK2* and *AHK3* genes, this His-kinase is involved in the His-to-Asp phosphorelay signal transduction system (Higuchi et al. 2004, Nishimura et al. 2004, Kim et al. 2006, Riefler et al. 2006). In the last half-decade, new findings have begun to shed light on the molecular mechanisms underlying the cytokinin-mediated signal transduction network in higher plants (for recent reviews, see Hwang et al. 2002, Heyl and Schmulling 2003, Kakimoto 2003, Mizuno 2004, Ferreira and Kieber 2005). Nevertheless, progress towards a thorough understanding of the molecular functions of the cytokinin receptor His-kinases is at an early stage. To address this issue, we attempted to isolate *AHK* mutant genes, the products of which function constitutively and independently of cytokinin. To this end, we adopted sophisticated microbial genetics techniques.

First, we took advantage of an *Escherichia coli* two-component signal transduction system that was previously developed for assaying *AHK4* His-kinase activity (Suzuki et al. 2001, Yamada et al. 2001). As shown in Fig. 1A, the *AHK4* His-kinase can serve as a cytokinin-responsive sensor in *E. coli* cells transformed with an expression vector carrying the *AHK4* gene (pINΔEH-AHK4). In this system, *AHK4* His-kinase activity can be measured both quantitatively and qualitatively, as illustrated in Fig. 1A. The *E. coli* host strain is KMI001 [ΔrcsC::Km, wza::lacZ Δ (cps operon)], which carries a wza promoter fused to the lacZ (β-galactosidase) gene, but lacks the rcsC His-kinase gene (Yamada et al. 2001). In this background, the *AHK4* gene product is capable of propagating the cytokinin signal through the downstream YojN (HPt factor)–RcsB (response regulator) phosphorelay pathway, even in the absence of the authentic RcsC His-kinase, resulting in the activation of the wza::lacZ target gene in a cytokinin-independent manner (Fig. 1A; for more detail, see Suzuki et al. 2001). Using this system, we attempted to isolate a set of *AHK4* mutant genes, the products of which can function constitutively (i.e. independently of cytokinin).

We first considered the protein structure of *AHK4*. As illustrated schematically in Fig. 1B and C, the *AHK4* His-kinase has at least two transmembrane segments (denoted TM1 and TM2) in the N-terminal region, followed by the catalytic His-kinase domain, which is followed by the receiver domain containing a phospho-accepting aspartate (D) residue. The phosphorylated domain containing the substrate His site lies in front of the His-kinase domain. The N-terminal region of about 270 amino acids (indicated by red lines in Fig. 1B and C), flanked at each end by TM1 and TM2, is most probably the cytokinin-binding domain and predicted to be extracellular (Fig. 1C). The amino acid sequence of the cytokinin-binding domain is highly

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Constitutive-active cytokinin receptor mutants

Through the first round of screening, we identified three independent AHK4 mutant genes, each of which were found upon nucleotide sequencing to encode a single amino acid substitution: G435C (Gly to Cys substitution at amino acid position 435), F436S (Phe to Ser) and V471A (Val to Ala) (Fig. 1B). The E. coli cells expressing these AHK4 mutant genes exhibited high levels of β-galactosidase activity even in the absence of cytokinin (schematically illustrated in the inset pictures in Fig. 1A). Based on this logic, random base substitution mutations were introduced into the region extending from the cytokinin-binding domain to the N-terminal end of the catalytic domain and including the phosphorylated His site (corresponding to the 1.2 kb HindIII–HindIII fragment in pINΔEH-AHK4, see Fig. 1B). This was achieved by means of conventional PCR-assisted in vitro mutagenesis (Kato et al. 1996). Escherichia coli cells were then transformed with the mutagenized plasmid library, spread on agar plates without cytokinin, and blue transformants were selected.

Through a second round of screening, we found five constitutive AHK4 mutant genes whose products function in a cytokinin-independent manner in E. coli. (A) Assays of β-galactosidase activity in E. coli KM001 cells harboring the wild-type AHK4 gene on pINΔEH-AHK4. Cells were grown overnight at 25°C in Luria broth containing 50 mM sodium phosphate buffer and 40 mM glucose, supplemented with the indicated concentrations of trans-zeatin (IZ). Several independent experiments were conducted with essentially the same results (for instance, see Fig. 2A). (B) Schematic representation of the structure of AHK4, which has two membrane-spanning segments at its N-terminal end (green rectangles denoted by TM1 and TM2), followed by the His-kinase domain (green shaded bar), which is followed by the receiver domain containing a phospho-accepting aspartate (D) residue. Note that the phosphorylated His site (boxed site) lies in front of the kinase domain. The N-terminal portion of about 270 amino acids (indicated by the red line) is assumed to be the cytokinin-binding domain. PCR-assisted and localized random mutagenesis of the pINΔEH-AHK4 plasmid was carried out in vitro, by introducing base substitutions into the HindIII–HindIII segment within the AHK4 cDNA, as indicated. Part of the amino acid sequence of this mutagenized region is shown, along with those of AHK2 and AHK3 (note that an N-terminal part was not shown for clarity of the figure). In this study, five constitutive AHK4 mutant genes were identified, as mapped on the sequence. (C) The five mutations identified in this study are mapped onto a hypothetical schematic structure of AHK4. The β-galactosidase activities of KM001 cells harboring these constitutive AHK4 mutant genes were examined through replicated examinations with essentially the consistent results (for instance, see the results of Experiment-1 and Experiment-2).
we isolated two further $AHK4$ mutants, M447T (Met to Thr) and M494L (Met to Leu) (Fig. 1B). A number of other candidates were discarded because they contained two or more amino acid substitutions. We next confirmed that all five mutants reproducibly exhibited high levels of $\beta$-galactosidase activity in the presence or absence of cytokinin (Fig. 1C, Experiment-2). Notably, all the mutants had amino acid substitutions within a narrow region extending from TM2 to just downstream of the phosphorylated His site, as schematically depicted in Fig. 1C (note that the structure illustrated is solely hypothetical).

Interestingly, all five constitutive $AHK4$ mutants also retained a limited ability to respond to a higher concentration of cytokinin, providing our first indication that the mutant proteins might still be able to bind cytokinin. This was explored later in our studies (see Fig. 3B). In summary, we succeeded in identifying a set of $AHK4$ mutants with constitutive His-kinase activity in the $E. coli$ assay system.

As mentioned earlier, Arabidopsis has two $AHK4$ homologs, $AHK2$ and $AHK3$. If the substituted amino acid residues in our five $AHK4$ mutants were indeed fundamental to the structure and function of the cytokinin receptors in general, the analogous amino acid substitutions would be expected to render the homologous $AHK2$ and $AHK3$ mutants constitutively active. To test this, we constructed the $AHK2$-I586A and $AHK3$-V449A mutants, respectively, by means of site-directed mutagenesis with a versatile $E. coli$ expression vector ($pSTV28$-$AHK2$ and $pSTV28$-$AHK3$, respectively, see Fig. 2B). It should be pointed out that $AHK2$ and $AHK3$ also have the conservative hydrophobic amino acid residues at the mutagenized positions (Ile586 and Val449, respectively, see Fig. 1B). To provide a negative reference for the assay, we employed $AHK4$ mutant T301I (Thr301 to Ile). This corresponds to the well-documented $wol$ (wooden legs) mutation, located within the extracellular cytokinin-binding domain (see Fig. 1B, C) (Mahonen et al. 2000). The $wol$ product has been characterized as a loss-of-function mutant with regard to its cytokinin-binding and cytokinin-induced His-kinase activity (Yamada et al. 2001). We also constructed the analogous $AHK2$-T418I and $AHK3$-T281I mutants. As shown in Fig. 2, the $AHK2$-I586A, $AHK3$-V449A and $AHK4$-V471A mutants were all constitutively active in the $E. coli$ assay system even in the absence of cytokinin. In contrast, $AHK2$-T418I, $AHK3$-T281I and $AHK4$-T301I ($wol$) could not be activated even in the

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**Fig. 2**  Construction of constitutively active mutants of $AHK2$ and $AHK3$. (A and B) Based on the observation that $AHK4$-$V471A$ is a constitutive active mutant (A), analogous mutations were created by site-directed mutagenesis of the $AHK2$ and $AHK3$ genes in the $E. coli$ versatile expression vector, to yield $AHK2$-I586A and $AHK3$-V449A (B). (C and D) The $AHK2$-$T418I$ and $AHK3$-$T281I$ mutant genes were also constructed and introduced into KMI001 cells, as appropriate negative references corresponding to $AHK4$-$T301I$ ($wol$) (see Fig. 1B). These mutant genes were introduced into $E. coli$ KMI001 cells, and $\beta$-galactosidase activity was measured, as described in the legend to Fig. 1. These experiments (A, C and D) were conducted with duplicate or triplicate samples, and essentially the same results were obtained.
presence of cytokinin. These results further strengthen the conclusion that the amino acid residues in question are important for the commonly conserved structure and function of the AHK2/AHK3/AHK4 cytokinin receptors.

We next turned to a eukaryotic host, the fission yeast Schizosaccharomyces pombe, in which AHK4 is also capable of functioning as a cytokinin receptor (Suzuki et al. 2001). Schizosaccharomyces pombe has its own intrinsic phosphor-phytin system consisting of Phk1, Phk2 and Phk3 (Histinases), Spy1 (HPt factor) and Mcs4 (response regulator). This system negatively regulates the progression of the cell cycle [specifically, the transition from G2 to the mitotic (M) phase] in response to environmental stresses. Accordingly, an S. pombe Δphk1/2/3 mutant, lacking all three Histinases, tends to enter into the M phase precociously, thereby giving rise to shorter cell lengths (about 10 μm) at cell division, compared with the wild-type cells (about 13 μm; see the quantitative data at the left hand side of Fig. 3A). As demonstrated previously (Suzuki et al. 2001), this mutational lesion in phk1/2/3-deficient S. pombe cells was complemented by the wild-type AHK4 gene in a cytokinin-dependent manner, but not by the AHK4-T301I (wol) mutant gene (Fig. 3A). We then introduced the five AHK4 mutant genes identified in our E. coli screen into the S. pombe cells using an appropriate S. pombe expression vector (see Materials and Methods). As expected, all five mutants were able to complement the mutational lesion of the S. pombe mutant even in the absence of cytokinin (Fig. 3A).

More critically, we combined one of the five amino acid substitutions, F436S, with the T301I (wol) mutation, to construct the AHK4-F436S-T301I (wol) double mutant. This double mutant was fully able to compete the S. pombe mutational lesion in the absence of cytokinin (Fig. 3A). In other words, the T301I (wol) lesion was fully masked by the F436S gain-of-function mutation. Considering that AHK4-T301I (wol) has no ability to bind cytokinin (Yamada et al. 2001), this result strongly supports the view that the mutant AHK4-F436S His-kinase is capable of taking the active conformation without binding cytokinin.

To provide further evidence in support of this conclusion, we prepared membrane fractions from the S. pombe cells expressing the AHK4 (wild type), AHK4-G435C, AHK4-V471A, AHK4-F436S, AHK4-T301I (wol) and AHK4-F436S-T301I (wol) proteins. The samples were analyzed by SDS–PAGE, showing that they contained a large amount of proteins that were likely to be AHK4-related polypeptides (Fig. 3B, see the inset picture of gel). Using these isolated membranes, we carried out cytokinin-binding assays with [3H]labeled isopentenyl adenosine (iP; Fig. 3B), as described previously (Yamada et al. 2001). AHK4 (wild type), AHK4-G435C, AHK4-F436S and AHK4-V471A were able to bind [3H]iP, while AHK4-T301I (wol) was not. Critically, the double mutant AHK4-F436S-T301I (wol) did not bind [3H]iP (Fig. 3B), even though this mutant was fully active in the complementation assay in the absence of cytokinin (Fig. 3A). Together,
this demonstrated conclusively that AHK4-F436S is constitutively active in the strict sense that it can function without binding to cytokinin. We consider this likely to be the case with the other four constitutive mutants as well.

In this study we identified five amino acid substitutions that render the AHK His-kinases constitutively active (Fig. 1). Furthermore, we demonstrated that the mutants are capable of functioning without binding to cytokinin (Fig. 3). The amino acid substitutions are located within the second membrane-spanning segment or within the region around the phosphorylated His site (Fig. 1C). We previously proposed that cytokinins bind directly to the extracellular domain, which is flanked by the two membrane-spanning segments (Yamada et al. 2001). The bound cytokinin may cause a conformational change in the membrane-spanning segments and/or the region around the His site. This would result in propagation of the cytokinin signal across the membrane and, consequently, modulate the cytoplasmic His-kinase activity. In light of this model, the results of this study are best explained by assuming that certain amino acid alterations in the second membrane-spanning segment and/or the flanking region cause a conformational change that is analogous to that caused by cytokinin binding. Hence, these His-kinases most probably have a ‘locked-on’ conformation, capable of propagating the cytokinin signal without stimulus. Importantly, this model appears to be applicable to AHK2 and AHK3 too, as judged by the fact that we successfully created such ‘locked-on’ and ‘kinase-negative’ wol versions of each homolog (Fig. 2).

The main aim of this study was to gain new insights into the structure and function of the plant cytokinin receptor His-kinases at the molecular level. As discussed above, this has been accomplished at least in part. The results provide us with an important basis for investigating the function of cytokinin receptors in plants. For instance, it would be of great interest to observe the phenotype of these cytokinin-independent AHK mutant genes in transgenic Arabidopsis plants. We have already initiated this experiment by introducing an AHK4 mutant gene into wild-type plants (Columbia ecotype), downstream of the natural AHK4 promoter sequence. However, we have not yet succeeded in establishing a stable transgenic line, perhaps due to a harmful effect of the constitutively active cytokinin receptor (data not shown). We may need to adopt a more sophisticated approach to isolate stable transgenic lines, for instance by employing an inducible promoter (or a tissue-specific promoter) and a more suitable host plant (e.g. lacking the endogenous AHK genes). These lines of investigation are currently underway. It will also be of interest to apply the same genetic manipulations to other useful higher plants, such as rice and members of the family Leguminosae. It was recently shown that the cytokinin-responsive signal transduction pathways, involving highly conserved cytokinin receptors, play important roles in characteristic developmental processes. These include the regulation of the number of panicle branches in rice, Oryza sativa (Ashikari et al. 2005, Kurakawa et al. 2007), and the regulation of nodulation in the legume Medicago truncatula (Gonzalez-Rizzo et al. 2006).

In conclusion, we have characterized the Arabidopsis cytokinin receptor His-kinases with reference to their structure and function, by adopting unique E. coli and S. pombe genetic systems. As a result, we succeeded in creating constitutively active (gain-of-function or ‘locked-on’) mutants of the Arabidopsis cytokinin receptors. It would be rather difficult to isolate such mutants through conventional plant genetics. This study is a good example of the power of microbial genetics leading to general and fundamental knowledge about molecular and applied plant biology.

**Materials and Methods**

The E. coli K-12 strain used in this study was KMI001 [ArcrC::Km’, wzc::lacZ Δ (cps operon)], which is a derivative of SRC122 (Takeda et al. 2001). The versatile E. coli plasmids pIN-III and pSTV28 were used in this study (see Figs. 1A, 2B). The Arabidopsis AHK4 cDNA was cloned downstream of the lpp-lac promoter of pIN-III to yield pINΔEH-AHK4. Similarly, the AHK2 and AHK3 cDNAs were cloned onto pSTV28 to yield pSTV28-AHK2 and pSTV28-AHK3, respectively. In pSTV28-AHK2 and pSTV28-AHK3, it should be noted that the AHK2 and AHK3 coding sequences are in the reverse orientation relative to the lacUV5 promoter. For assays of β-galactosidase activity, KMI001 cells were grown at 25°C in Luria broth with or without cytokinin, as described previously (Suzuki et al. 2001).

The 1.2 kbp HindIII–HindIII fragment, encoding the N-terminal portion of AHK4 in pINΔEH-AHK4 was isolated and purified. It was subjected to PCR-assisted mutagenesis in the presence of 0.3 mM MnCl2 with an appropriate pair of primers, as described by Kato et al. (1996). The amplified PCR products were digested with HindIII and the fragments were purified, and used to replace the corresponding original portion in pINΔEH-AHK4. The resulting plasmids were introduced into KMI001 cells and blue transformants were selected on agar plates containing 40 μg ml⁻¹ X-Gal as described in the text.

The S. pombe strain used in this study was KI011 (phkh1::ura4’+, phkh2::ura4’+, phkh3::ura4’+), which is a derivative of JY741 [h’− leu1-32 ade6-M216 ura4-D18] (Aoyama et al. 2001). A set of Arabidopsis mutant AHK4 genes was cloned downstream of the rmt1’ promoter on the versatile S. pombe plasmid pREP1 (Maundrell 1993). Analyses of the G2/M cell cycle progression of S. pombe cells were carried out as described previously (Suzuki et al. 2001).

The in vitro cytokinin binding assays of the S. pombe membranes containing the Arabidopsis AHK4 proteins were carried out according to the method described previously (Yamada et al. 2001).
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