RESEARCH ARTICLES

Histidine Kinase Homologs That Act as Cytokinin Receptors Possess Overlapping Functions in the Regulation of Shoot and Root Growth in Arabidopsis

Chika Nishimura,[a] Yoshi Ohashi,[a] Shusei Sato,[b] Tomohiko Kato,[b] Satoshi Tabata,[b] and Chiharu Ueguchi[a,1]

[a] Bioscience and Biotechnology Center, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan
[b] Kazusa DNA Research Institute, Kisarazu 292-0812, Japan

Cytokinins are plant hormones that may play essential and crucial roles in various aspects of plant growth and development. Although the functional significance of exogenous cytokinins as to the proliferation and differentiation of cells has been well documented, the biological roles of endogenous cytokinins have remained largely unknown. The recent discovery of the Arabidopsis Histidine Kinase 4 (AHK4)/CRE1/WOL cytokinin receptor in *Arabidopsis thaliana* strongly suggested that the cellular response to cytokinins involves a two-component signal transduction system. However, the lack of an apparent phenotype in the mutant, presumably because of genetic redundancy, prevented us from determining the in planta roles of the cytokinin receptor. To gain insight into the molecular functions of the three *AHK* genes *AHK2*, *AHK3*, and *AHK4* in this study, we identified mutational alleles of the *AHK2* and *AHK3* genes, both of which encode sensor histidine kinases closely related to AHK4, and constructed a set of multiple *ahk* mutants. Application of exogenous cytokinins to the resultant strains revealed that both AHK2 and AHK3 function as positive regulators for cytokinin signaling similar to AHK4. The *ahk2 ahk4* and *ahk3 ahk4* double mutants and the *ahk* single mutants grew normally, whereas the *ahk2 ahk3* double mutants exhibited a semidwarf phenotype as to shoots, such as a reduced leaf size and a reduced influorescence stem length. The growth and development of the *ahk2 ahk3 ahk4* triple mutant were markedly inhibited in various tissues and organs, including the roots and leaves in the vegetative growth phase and the influorescence meristem in the reproductive phase. We showed that the inhibition of growth is associated with reduced meristematic activity of cells. Expression analysis involving *AHK*:β-glucuronidase fusion genes suggested that the *AHK* genes are expressed ubiquitously in various tissues during postembryonic growth and development. Our results thus strongly suggest that the primary functions of *AHK* genes, and those of endogenous cytokinins, are triggering of the cell division and maintenance of the meristematic competence of cells to prevent subsequent differentiation until a sufficient number of cells has accumulated during organogenesis.

INTRODUCTION

Cytokinins are plant hormones that may play essential and crucial roles in various aspects of plant growth and development. Extensive studies involving exogenous application and elevated endogenous contents of cytokinins have revealed that they are involved in diverse processes, such as cell proliferation, shoot initiation, chloroplast biogenesis, apical dominance, and inhibition of leaf senescence (Mok and Mok, 2001). Although the functional significance of exogenous cytokinins has been well documented, the precise biological roles of endogenous cytokinins have remained largely unknown be-

cause of the lack of knowledge concerning cytokinin metabolism and signal transduction.

Recent genetic and molecular studies have begun to reveal the cytokinin signal transduction pathway in plant cells (Sheen, 2002; Heyl and Schmülling, 2003). The discovery of a cytokinin receptor strongly suggested that the cellular response to cytokinins involves a two-component signal transduction system. *Arabidopsis thaliana* plants carrying lesions in the *Arabidopsis Histidine Kinase 4* (*AHK4* or *CRE1/WOL*) gene, which encodes a sensor histidine kinase in the two-component system, exhibit a cytokinin-insensitive phenotype as to root explants and root growth (Inoue et al., 2001; Ueguchi et al., 2001b). The AHK4 protein expressed in heterologous systems, yeasts, and *Escherichia coli*, confers cytokinin responsiveness on cells (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001b). These results indicate that AHK4 positively regulates the cytokinin-signaling pathway as a direct receptor molecule. This was further confirmed by the finding in a biochemical experiment that AHK4 is able to bind to cytokinins in vitro (Yamada et al., 2001). In addition to AHK4, Arabidopsis ARR1, a response regulator functioning

downstream of sensor histidine kinases, has also been demonstrated genetically to function as a positive regulator in cytokinin signaling (Sakai et al., 2001). Furthermore, transient expression analysis involving Arabidopsis protoplasts demonstrated that all of the signaling components in the two-component system (i.e., sensor kinases, histidine-containing phosphotransfer domains, and response regulators) are involved in cytokinin signaling, and the simple picture has emerged that the signal transduction from the plasma membrane to the nucleus involves successive phosphorelay reactions between the signaling components (Hwang and Sheen, 2001). In addition to the genetic and molecular studies on signaling components, phenotypic analyses involving cytokinin-deficient transgenic plants to elucidate the biological functions of endogenous cytokinins were reported recently (Werner et al., 2001, 2003; Yang et al., 2003).

Although the *ahk4-1* mutant exhibited an insensitive phenotype as to externally added cytokinins, no apparent defective phenotype was observed as to its growth or development, and so the precise biological functions of the cytokinin receptor and endogenous cytokinins remained to be elucidated (Inoue et al., 2001; Ueguchi et al., 2001b). The lack of a particular phenotype in the *ahk4-1* mutant strongly suggested that a functional redundant gene(s) compensates for the loss of the *AHK4* gene product. The *AHK2* and *AHK3* genes are the most plausible counterparts for such a gene because they also encode sensor histidine kinases whose primary structures are closely related to that of AHK4 (Ueguchi et al., 2001a). In addition to structural similarity, AHK3 was previously shown to respond to cytokinins in the heterologous *E. coli* system (Suzuki et al., 2001). However, we have no direct evidence as to whether or not AHK2 and AHK3 act as cytokinin receptors in planta.

To gain an insight into the molecular functions of the AHK sensor histidine kinase family in this study, we conducted a series of phenotypic analyses involving several *ahk* mutants. We first isolated T-DNA insertional alleles of *AHK2* and *AHK3* and then constructed a set of mutant strains carrying the *ahk* mutations in various combinations. These strains were then subjected to a series of phenotypic analyses. The results obtained revealed that both AHK2 and AHK3 function as positive regulators for cytokinin signaling like AHK4. Furthermore, we concluded that the AHK cytokinin receptors play important roles in the postembryonic growth and development of several shoot and root organs through maintenance of cell proliferation activity.

RESULTS

Identification of T-DNA Insertional Mutations of the *AHK2* and *AHK3* Genes

The *ahk4-1* mutant is less sensitive to exogenous cytokinins than the wild type but exhibits no other obvious phenotype in the absence of exogenous cytokinins (Ueguchi et al., 2001b). This observation suggested that the *AHK2* (At5g35750) and *AHK3* (At1g27320) gene products might function as cytokinin receptors like AHK4 (At2g01830). To address this issue, we first screened T-DNA insertional alleles of the *AHK2* and *AHK3* genes using T-DNA insertion lines deposited in the Kazusa DNA Research Institute, Wisconsin University (Arabidopsis Knockout Facility),
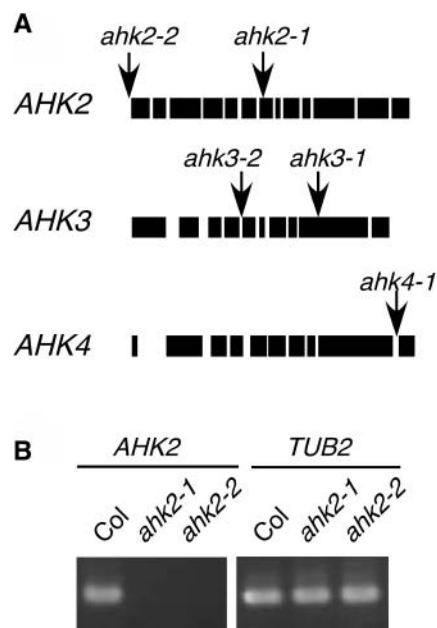
Figure 1. Structure of the *AHK* Genes and Expression of *AHK2* in *ahk2* Mutants.

(A) The structure of the chromosomal region encompassing each *AHK* gene is schematically presented. The closed rectangles represent the exons. The arrows above the genomic structures indicate the T-DNA insertion points in the *ahk* mutants.

(B) *AHK2* transcripts in the *ahk2* mutants. Total RNA was isolated from the indicated 2-week-old rosette plants and then subjected to RT-PCR analysis to amplify the cDNA region encoding the receiver domain as well as the *TUB2* transcript, as indicated above.

and the SALK Institute Genomic Analysis Laboratory (SIGnAL). As a result, two independent mutational alleles of the *AHK2* gene were obtained, which were named *ahk2-1* and *ahk2-2*. As shown in Figure 1A, the T-DNAs in *ahk2-1* and *ahk2-2* are inserted into the 7th exon, which encodes the third transmembrane segment just adjacent to the transmitter domain, and at 20 bp upstream of the initiation codon, respectively. We also identified two independent alleles of *AHK3* and named them *ahk3-1* and *ahk3-2*. The *ahk3-1* and *ahk3-2* alleles contain T-DNA insertions in the 9th exon, which encodes the transmitter domain, and in the 4th intron, which is adjacent to exons encoding the third transmembrane segment, respectively. Although the T-DNA in *ahk2-2* is inserted outside of the open reading frame, in RT-PCR analysis, to amplify the mRNA portion encompassing the receiver domain, the *AHK2* transcript was hardly detected in the *ahk2-2* mutant or in the *ahk2-1* mutant (Figure 1B).

Construction of a Series of Multiple *ahk* Mutants

None of the single *ahk* mutants (i.e., *ahk2-1*, *ahk3-1*, and *ahk4-1*) exhibited significant defective phenotype as to their growth, at least under the standard growth conditions. The *ahk2-1 ahk4-1* and *ahk3-1 ahk4-1* double mutants, constructed by means of

genetic crosses, also grew normally, whereas the aerial part of the *ahk2-1 ahk3-1* double mutant exhibited a semidwarf phenotype (see below). We further tried to construct a mutant strain lacking all of the *AHK* genes. The *ahk2-1 ahk4-1* double mutant was first crossed with the *ahk3-1 ahk4-1* double mutant, and then mutant strains carrying only one intact receptor gene per diploid (i.e., *ahk2-1/AHK2*; *ahk3-1/ahk3-1*; *ahk4-1/ahk4-1* and *ahk2-1/ahk2-1*; *ahk3-1/AHK3*; *ahk4-1/ahk4-1*) were obtained from the F2 populations. The resultant mutants grew normally and showed no apparent phenotype (data not shown). These strains were self-pollinated, and the growth of the progeny was scored on solid medium. The self-progeny included two types of seedlings, normal and markedly small ones. As shown in Table 1, the small seedling phenotype segregated as a single Mendelian recessive trait. Essentially the same results were obtained when the *ahk3-2* allele instead of the *ahk3-1* allele was used (Table 1). In each case, 24 representatives exhibiting a particular phenotype were randomly selected and subjected to PCR-based genotyping. The results revealed that the small seedlings are homozygous for the mutant alleles (i.e., *ahk2-1*, *ahk3-1*, and *ahk4-1*) at all three loci and that the normally growing plants carry at least one intact *AHK2* or *AHK3* gene per diploid (data not shown), indicating that the small seedling phenotype is tightly associated with the triple lesions of the *AHK* genes.

The materials thus obtained were used for the following detailed phenotypic analyses. To avoid tedious data presentation, we mainly present hereafter the results obtained with the *ahk2-1*, *ahk3-1*, and *ahk4-1* alleles because essentially the same results were obtained when we used the *ahk2-2* and *ahk3-2* alleles instead of the *ahk2-1* and *ahk3-1* alleles, respectively. It should be noted that all of the *ahk2* and *ahk3* mutants are derivatives of the Columbia (Col) ecotype, whereas the *ahk4-1* mutant has a Wassilewskija (Ws) background. Therefore, we used both Col and Ws as wild-type controls throughout the following experiments.

AHK2 and AHK3 Function as Positive Regulators in the Cytokinin-Signaling Pathway

To determine whether or not AHK2 and AHK3 function as signal transduction molecules in the cytokinin-signaling pathway, we examined the effects of exogenously supplied cytokinins on the greening and shoot induction of calli prepared from mutant

Table 1. Genetic Segregation of Seedling Phenotypes in the *ahk* Multiple Mutant

Parent	Number of Seedlings		
	Normal	Small	$\chi^{2\,a}$
ahk2-1/AHK2; *ahk3-1/ahk3-1*; *ahk4-1/ahk4-1*	347	126	0.677[b]
ahk2-1/ahk2-1; *ahk3-1/AHK3*; *ahk4-1/ahk4-1*	415	137	0.0097[b]
ahk2-1/AHK2; *ahk3-2/ahk3-2*; *ahk4-1/ahk4-1*	1110	330	3.33[b]
ahk2-1/ahk2-1; *ahk3-2/AHK3*; *ahk4-1/ahk4-1*	315	88	2.15[b]

[a] χ^2 was calculated based on an expected ratio of three normal to one small.
[b] $P > 0.05$.

hypocotyls. The calli of the *ahk2-1* mutant responded normally to *trans*-zeatin (*t*-zeatin), similar to those of the wild-type control (Col) (Figure 2A). However, the calli derived from the *ahk3-1* mutant exhibited a cytokinin-insensitive phenotype; that is, weak stimulation of cell proliferation. Greening and shoot production were only observed with higher concentrations of *t*-zeatin, although the magnitude of the defect was less than that observed for the *ahk4-1* calli (Figure 2A). This thus indicated that AHK3 also functions as a positive regulator in the cytokinin-signaling pathway. Although the calli of the *ahk2-1* single mutant showed a normal cytokinin response, the calli of the *ahk2-1 ahk3-1* double mutant exhibited a strong cytokinin-insensitive phenotype (Figure 2A). The fact that *ahk2-1* exhibited an additive effect when combined with *ahk3-1* indicated that AHK2 has the same molecular function as AHK3 (i.e., a positive regulator in the cytokinin-signaling pathway).

Although the same result (i.e., strong cytokinin insensitivity) was obtained with calli prepared from mature leaves instead of hypocotyls of the *ahk2-1 ahk3-1* mutant (data not shown), the calli prepared from root tissues was able to weakly respond to *t*-zeatin (Figure 2A). This result suggested that the lack of both *AHK2* and *AHK3* functions affects cytokinin sensitivity in a slightly different manner in each tissue. To investigate this further, we examined whether or not the root growth of each mutant is also insensitive to exogenously supplied cytokinins. As previously demonstrated (Inoue et al., 2001; Ueguchi et al., 2001b), the *ahk4-1* mutant exhibited cytokinin-insensitive root growth even with relatively higher concentrations of benzyl adenine (BA) (Figure 2B). However, the root growth of the *ahk2-1* and *ahk3-1* single mutants and the *ahk2-1 ahk3-1* double mutant was sensitive to BA in a similar manner as in the case of the wild-type control (Col) (Figure 2B), indicating that the roots of *ahk2-1* and *ahk3-1* mutants respond normally to cytokinins. This suggested that the responsiveness to exogenous cytokinins of roots is largely dependent on the AHK4 function.

To investigate the cytokinin response in the *ahk* mutants at the molecular level, we examined the effect of cytokinin treatment on the expression of *ARR5*, in which the transcription is rapidly induced by exogenously supplied cytokinins (Brandstatter and Kieber, 1998; Taniguchi et al., 1998). Two-week-old rosette plants were treated with *t*-zeatin, and then total RNA was isolated from the leaves and subjected to RT-PCR amplification. The *ARR5* transcript was rapidly induced by this treatment in the wild-type controls (Col and Ws) as well as in the *ahk2-1 ahk4-1* and *ahk3-1 ahk4-1* double mutants, whereas it was not induced in the *ahk2-1 ahk3-1* double mutant (Figure 2C). This demonstrated that the leaves of the *ahk2 ahk3* double mutant were unable to respond to exogenous cytokinins.
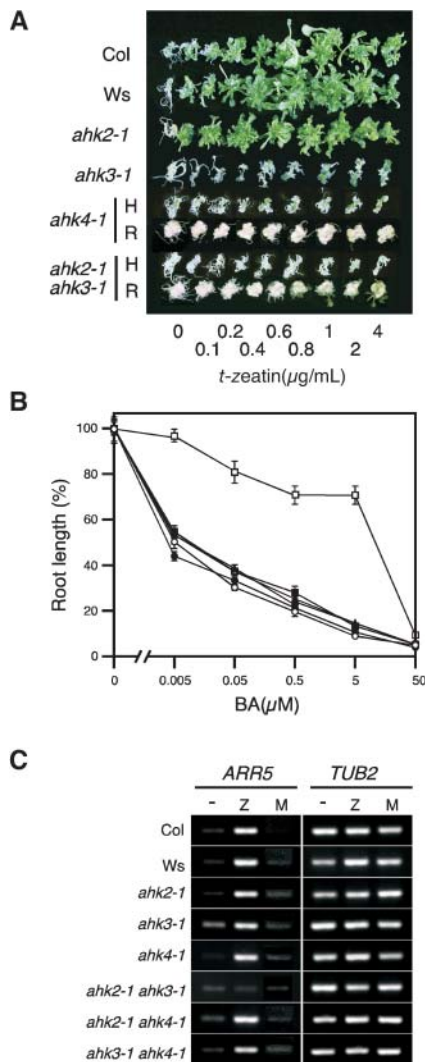
Seedling Phenotypes of the Multiple *ahk* Mutants

As mentioned above, the *ahk2-1 ahk3-1 ahk4-1* triple mutant exhibited a reduced seedling size with small cotyledons, slightly shortened hypocotyls, and significantly reduced root length (Figure 3A). The hypocotyl length of the triple mutant was ~65% of those of the wild-type strains (Figure 3B). This short hypocotyl phenotype was accounted for by reductions in both the number and length of the hypocotyl epidermal cells (Figures

Figure 2. In Vitro and in Vivo Responses to Exogenous Cytokinins.

(A) Effect of a cytokinin (*t*-zeatin) on rapid proliferation, greening, and shoot induction of calli. From the hypocotyl tissues of the indicated strains, calli were prepared. For the *ahk4-1* single mutant and *ahk2-1 ahk3-1* double mutants, root tissues (R) as well as hypocotyl tissues (H) were used. The resultant calli were incubated with continuous fluorescent illumination at 22°C for 10 d on shoot induction medium comprising MS medium supplemented with indolebutyric acid (0.2 µg/mL) and the indicated concentrations of *t*-zeatin.
(B) Root inhibition by an exogenous cytokinin. The wild types (Col and Ws, open and closed circles, respectively) and the *ahk2-1* (open triangles), *ahk3-1* (closed triangles), *ahk4-1* (open squares), and *ahk2-1 ahk3-1* (closed squares) mutants were grown on the surface of MS agar plates supplemented with the indicated concentrations of BA with continuous fluorescent illumination at 22°C for 10 d. The length of each main root was measured and expressed relative to the average value for the same strain on the medium without BA. Each value represents the average with standard deviation for 20 plants.
(C) Induction of *ARR5* transcription by a cytokinin. Two-week-old rosette plants of the indicated strains grown on MS agar plates were sprayed with 100 µM *t*-zeatin (Z) or 0.36% methanol (the solvent for *t*-zeatin; M). After 3 h, total RNA from the resultant plants as well as untreated ones (−)

3C and 3D). The hypocotyl length of the *ahk2-1 ahk3-1* double mutant was also slightly decreased, but this effect was largely because of reduction of the cell number, not of the cell size (Figures 3B to 3D). It should be noted that the *ahk2-1 ahk4-1* and *ahk3-1 ahk4-1* double mutants exhibited normal seedlings (Figure 3A). Although the magnitude of the defect in the hypocotyl epidermal cells was less significant (an ∼10% reduction in the cell number), the results suggested that loss of the cytokinin receptors moderately affects the process of embryogenesis because it is known that the number of hypocotyl epidermal cells is rigorously controlled during embryogenesis (Gendreau et al., 1997).

The Root Growth of the *ahk2 ahk3 ahk4* Triple Mutant Is Severely Impaired

In contrast with the moderate effect on embryogenesis, the postembryonic growth of roots was severely impaired in the *ahk2-1 ahk3-1 ahk4-1* triple mutant. Kinetic analysis demonstrated that the root elongation rate was drastically decreased in the triple mutant (∼20% of those of the wild-type controls, Col and Ws), whereas the root elongation was not affected in the *ahk* double mutants, including *ahk2-1 ahk3-1* (Figure 4A). In addition to the defective growth of the main root, lateral root formation was also severely impaired. At least during prolonged incubation on an agar plate for 2 weeks, no lateral root grew from the main root of the triple mutant (data not shown). In contrast with the lack of lateral roots, adventitious root formation at the junction of the hypocotyl and main root was rather enhanced in the triple mutant (Figure 4B).

To determine the cause of the defective root growth in the *ahk2-1 ahk3-1 ahk4-1* triple mutant, microscopic observations were performed. Longitudinal section images of root tips stained with propidium iodine revealed that, even in the triple mutant, the central region of the root apical meristem exhibited a typical regular structure and organization, in which quiescent center cells are surrounded by several initial cells, similar to in the wild-type strains (Col and Ws). However, the number of cell files in the vascular tissues was reduced, presumably because of the reduced number of vascular initial cells and reduced periclinal cell division, which resulted in narrow roots (Figure 4F). In addition, in the region adjacent to the apical part of the meristem, cells immediately became elongated in the triple mutant (Figure 4F). In the basal part of the wild-type root meristem, cells still divide and the cell volume starts to increase gradually (Beemster et al., 2003). In the triple mutant, cells leaving the apical part of the meristem seemed to directly enter the elongation zone, skipping the basal part of the meristem, so that the volume of the meristematic region was markedly decreased. This was further supported by the observation of root tips stained with 4',6-diamidino-2-phenylindole (DAPI) (Figure 4J). Therefore, the

was prepared, treated with DNase I, and then subjected to RT-PCR analysis to amplify *ARR5* or *TUB2* cDNAs. PCR products were separated by 2% agarose gel electrophoresis and then visualized with ethidium bromide.
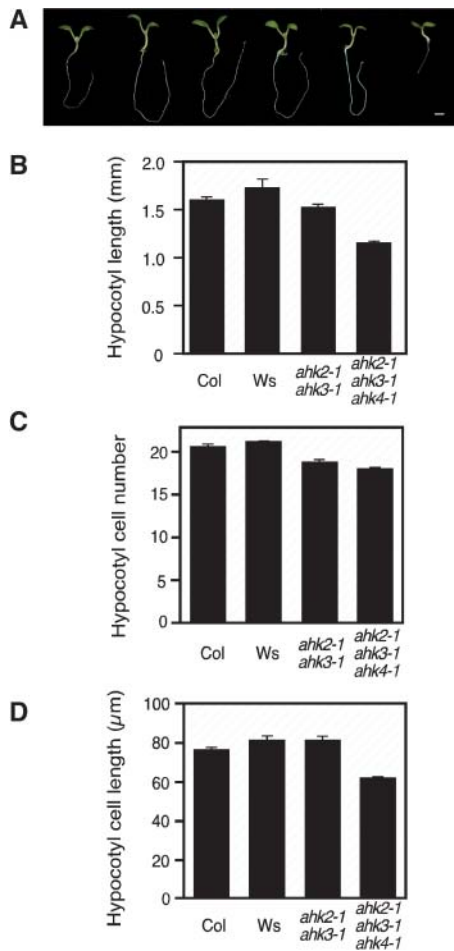
Figure 3. Seedling Phenotypes of the Multiple *ahk* Mutants.

Plants were grown on MS gellan gum plates with continuous fluorescent illumination at 22°C.

(A) Five-day-old seedlings. From left to right are Col, Ws, *ahk2-1 ahk4-1*, *ahk3-1 ahk4-1*, *ahk2-1 ahk3-1*, and *ahk2-1 ahk3-1 ahk4-1* seedlings. Bar = 1 mm.

(B), **(C)**, and **(D)** The hypocotyl length is shortened in the *ahk* multiple mutants. The length **(B)**, cell number **(C)**, and average cell length **(D)** of the hypocotyls of the 5-d-old seedlings were measured. Each value represents the average with standard deviation for 20 plants.

reduced growth rate in the triple mutant is associated with the reduced size of the cell division zone in the root tips.

Leaf Development Is Impaired in the Multiple *ahk* Mutants

A certain combination of the *ahk* mutations resulted in defective growth of shoots. The *ahk2-1 ahk4-1* and *ahk3-1 ahk4-1* double mutants exhibited normal sized rosette structures (Figures 5C and 5D), whereas the *ahk2-1 ahk3-1* double mutant, as well as the *ahk2-2 ahk3-2* double mutant, showed compact rosettes with shortened petioles and small leaf blades (Figures 5E and 5F). This particular phenotype was more prominent for the *ahk2-1 ahk3-1 ahk4-1* triple mutant (Figure 5G). The longitudinal length of leaf blades was affected much more than the lateral length, so

the leaf shape was slightly altered (Figures 5J and 5K). In addition to the reduced size of the leaves, the leaf vasculature of the double and triple mutants was impaired; that is, higher ordered veins were not formed, resulting in a decreased density of veins (Figures 5J and 5K). The development of stomatal guard cells and trichomes occurred normally even in the triple mutant (Figures 5N and 5O), suggesting that the differentiation processes in leaves were not affected in spite of the severe defect in the organ size.

The rate of leaf primordial formation was also affected, but only slightly. The numbers of leaves after 3 weeks of incubation were
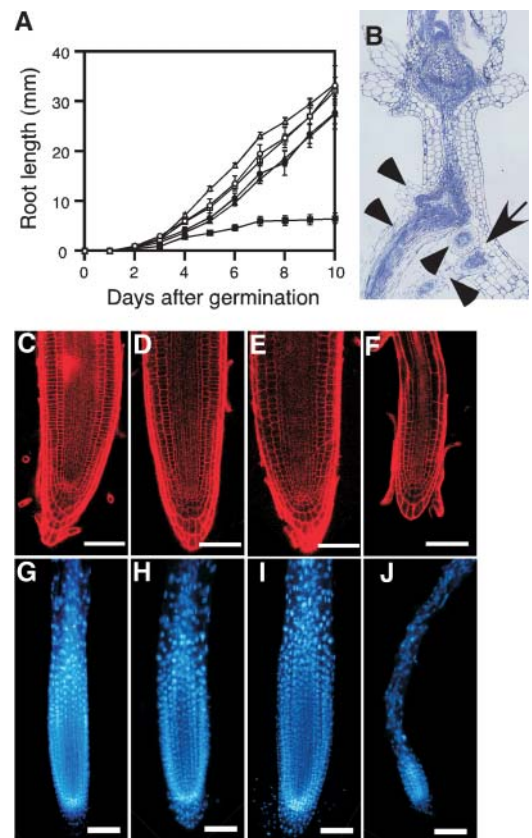

Figure 4. Root Growth Is Strongly Retarded in the *ahk2-1 ahk3-1 ahk4-1* Triple Mutant.

(A) The kinetics of root growth. Plants were germinated and grown on the surface of MS agar plates with continuous fluorescent illumination at 22°C. The length of main roots was measured every day. Each value represents the average with standard deviation for 20 plants. The strains used were as follows: Col (open circles), Ws (closed circles), *ahk2-1 ahk4-1* (open triangles), *ahk3-1 ahk4-1* (closed triangles), *ahk2-1 ahk3-1* (open squares), and *ahk2-1 ahk3-1 ahk4-1* (closed squares).

(B) Longitudinal section of the hypocotyl of the *ahk2-1 ahk3-1 ahk4-1* triple mutant (12-d-old seedling). The main root and adventitious roots are indicated by an arrow and arrowheads, respectively.

(C) to **(F)** Propidium iodine–stained root tips of 5-d-old seedlings of Col **(C)**, Ws **(D)**, *ahk2-1 ahk3-1* **(E)**, and *ahk2-1 ahk3-1 ahk4-1* **(F)** plants. Bars = 50 μm.

(G) to **(J)** DAPI-stained root tips of 5-d-old seedlings of Col **(G)**, Ws **(H)**, *ahk2-1 ahk3-1* **(I)**, and *ahk2-1 ahk3-1 ahk4-1* **(J)** plants. Bars = 50 μm.
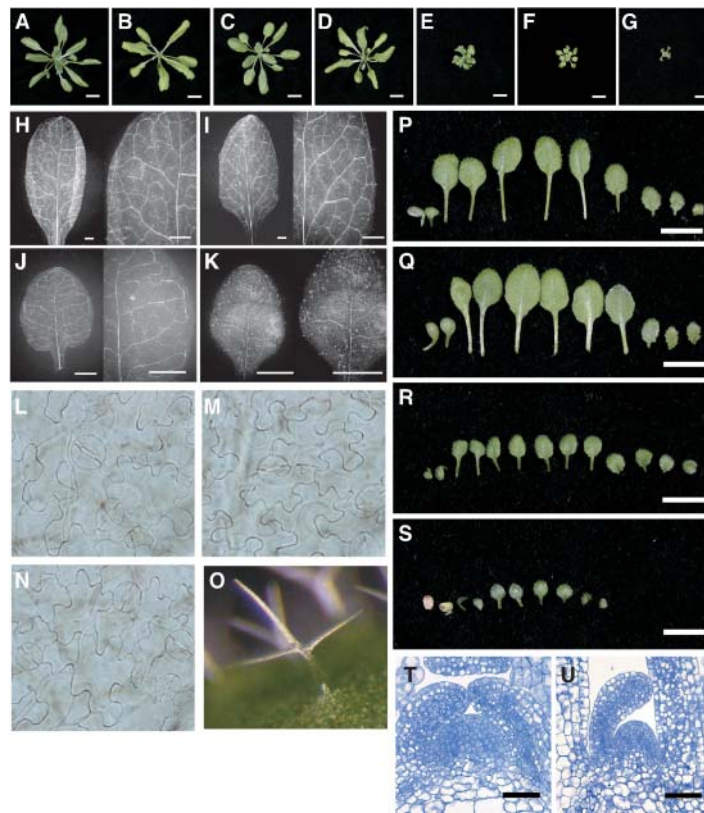
Figure 5. Rosette Phenotypes of the *ahk* Multiple Mutants.

Plants were grown on MS gellan gum plates with 16-h-light/8-h-dark fluorescent illumination at 22°C.

(A) to **(G)** Morphology of 3-week-old rosettes. The strains used were Col **(A)**, Ws **(B)**, *ahk2-1 ahk4-1* **(C)**, *ahk3-1 ahk4-1* **(D)**, *ahk2-1 ahk3-1* **(E)**, *ahk2-2 ahk3-2* **(F)**, and *ahk2-1 ahk3-1 ahk4-1* **(G)**. Bars = 1 cm.

(H) to **(K)** Vascular patterns of the fully expanded fourth mature leaves of 3-week-old Col **(H)**, Ws **(I)**, *ahk2-1 ahk3-1* **(J)**, and *ahk2-1 ahk3-1 ahk4-1* **(K)** plants. Leaves were fixed and cleared, followed by microscopic observation. The entire morphology (left) and a close-up view (right) are shown. Bars = 1 mm.

(L) to **(N)** Abaxial side of the fully expanded second mature leaves of 17-d-old Col **(L)**, Ws **(M)**, and *ahk2-1 ahk3-1 ahk4-1* **(N)** plants. Leaves were harvested, fixed, and cleared, followed by microscopic observation.

**(O)** Trichomes of the *ahk2-1 ahk3-1 ahk4-1* triple mutant.

(P) to **(S)** The number and morphology of leaves of 3-week-old Col **(P)**, Ws **(Q)**, *ahk2-1 ahk3-1* **(R)**, and *ahk2-1 ahk3-1 ahk4-1* **(S)** plants. Two cotyledons and mature leaves of each plant were collected and are presented according to age from left to right. Bars = 1 cm.

(T) and **(U)** Longitudinal sections of the SAM of Col **(T)** and *ahk2-1 ahk3-1 ahk4-1* **(U)** plants. Bars = 50 μm.

8.8 ± 0.63, 8.8 ± 0.79, 10.3 ± 0.92, and 7.5 ± 0.53 for Col, Ws, the *ahk2-1 ahk3-1* double mutant, and the *ahk2-1 ahk3-1 ahk4-1* triple mutant, respectively ($n = 10$ in all cases). The rate of leaf primordial formation was slightly decreased in the triple mutant, but the phyllotaxy seemed to be normal (Figures 5G and 5S). The slow leaf primordial formation suggested that the function of the shoot apical meristem (SAM) was moderately affected in the triple mutant. Thus, we next examined histologically the morphology of the SAM. The SAM region of the triple mutant at 12 d after germination (DAG) exhibited a typical regular structure and organization, but the size as well as the cell number was reduced (cf. Figure 5U with 5T). Therefore, the decreased production of leaf primordia is because of the decreased size and meristematic cell number in the SAM. The rather enhanced leaf production rate in the double mutant (Figure 5R) might suggest

a somewhat compensatory mechanism for the decreased sensitivity to endogenous cytokinins.

The reduction in leaf size should be caused by the reduced cell number and/or reduced cell size. To examine these possibilities, we performed kinematic analysis of leaf growth, with which it is possible to determine the cell division rate directly (De Veylder et al., 2001a). The second mature leaves were harvested from in vitro growing plants every day, and the leaf size as well as the number and size of the abaxial epidermal cells was determined. As shown in Figure 6A, the leaves of the *ahk2-1 ahk3-1* double mutant and *ahk2-1 ahk3-1 ahk4-1* triple mutants expanded slower than those of the wild-type controls. At 17 DAG, the areas of the leaves of the double and triple mutant were ∼55 and 20% of those of the controls (Col and Ws), respectively. The numbers of abaxial epidermal cells were also decreased in the
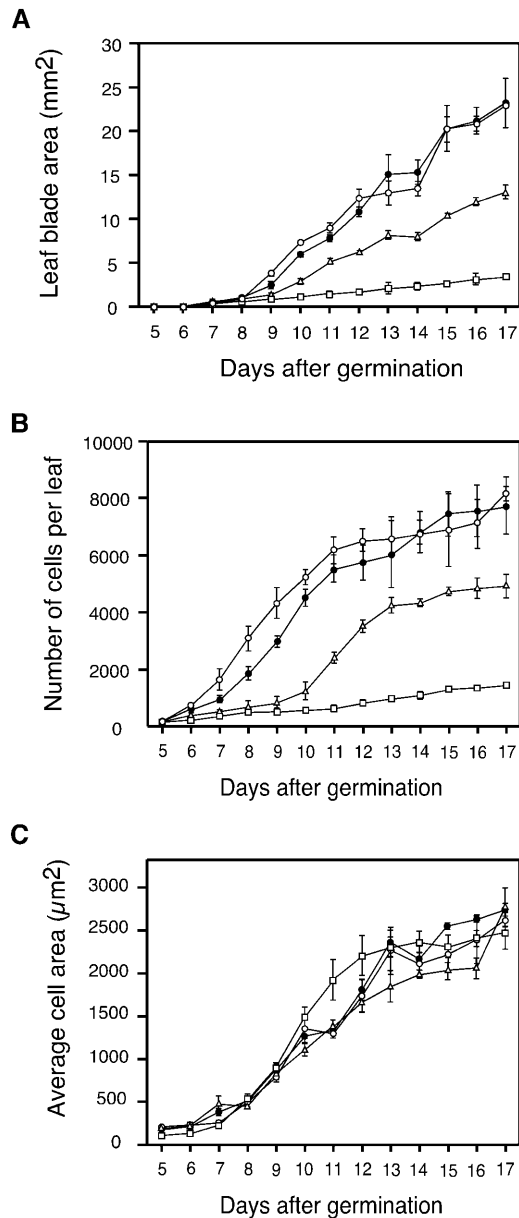
Figure 6. Kinematic Analysis of Leaf Development.

Plants were grown on MS gellan gum plates with continuous fluorescent illumination at 22°C. Second mature leaves were harvested every day and subjected to determination of leaf size and number and size of abaxial epidermal cells. The strains used were as follows: Col (open circles), Ws (closed circles), *ahk2-1 ahk3-1* (open triangles), and *ahk2-1 ahk3-1 ahk4-1* (open squares).

(A) Leaf blade area.

(B) Number of abaxial epidermal cells.

(C) Size of abaxial epidermal cells. Each value represents the average with standard deviation for six plants.

multiple mutants. The mature leaves of the double and triple mutants contained ∼50 and 20% cells compared with those of the wild-type plants (Col and Ws), respectively (Figure 6B). Despite the reduced cell number, the final size of the leaf cells was the same (Figure 6C). In the middle of leaf development (from 10 to 13 DAG), the cells in the triple mutant were slightly larger than those in other plants (Figure 6C), suggesting an earlier onset of cell expansion. The results thus demonstrated that the reduced leaf size of the multiple *ahk* mutants is primarily because of the reduced number of cells, and not of the cell size, and that the cell division activity in leaves is decreased.

Reproductive Growth of the *ahk2-1 ahk3-1 ahk4-1* Triple Mutant Is Severely Impaired

The *ahk2-1 ahk4-1* and *ahk3-1 ahk4-1* mutants showed no apparent defective phenotypes as to growth and development during the reproductive phase (i.e., onset of flowering, growth rate and final length of influorescences, number and morphology of flowers, and fertility). In the *ahk2-1 ahk3-1* double mutant, the bolting time was slightly (∼2 to 3 d) delayed and the influorescence stem length was also reduced (Figure 7A) compared with in the wild-type controls (Col and Ws). However, the morphology and fertility of the flowers were quite normal. In contrast with the moderate phenotypes in the double mutant, the growth of the *ahk2-1 ahk3-1 ahk4-1* triple mutant was severely impaired in the reproductive growth phase. The onset of flowering of the triple mutant varied with each individual plant. The earliest bolted plant was observed >1 week later than in the case of the wild-type controls. Even after an 8-week culture, ∼90% of the plants (39 per 45 plants) had generated influorescences, the rest remained in the vegetative phase. The influorescences of the bolted plants were thin and short (Figure 7B). The triple mutant formed only a few flowers of smaller size but with the correct numbers of floral organs (Figure 7E). Despite the regular structure of the flowers, they were sterile. The anthers contained only a small amount of viable pollen grains and showed no anther dehiscence (data not shown). We tried to cross the triple mutant and the wild type as female and male parents, respectively, but the seed setting was unsuccessful (data not shown), indicating that certain processes during male and female gametogenesis are somehow inhibited in the triple mutant. The results thus suggested that the establishment and activity of the influorescence meristem is deeply dependent on the function of AHKs. Of course, the possibility that the growth defect in the vegetative phase secondly, not directly, affects the subsequent growth and development cannot be ruled out at present.

Expression of *AHK* Genes

To gain a further insight into the biological roles of the three cytokinin receptors, we examined expression patterns using a set of *AHK* promoter:β-glucuronidase (*GUS*) fusion constructs as probes. As shown in Figure 8, the promoter activity profiles were similar to each other. In various organs, the *AHK* genes are expressed nearly ubiquitously but at low levels. In seedlings, strong staining was mainly localized in meristematic tissues, such as the SAM, root tips, and growing leaf and lateral root
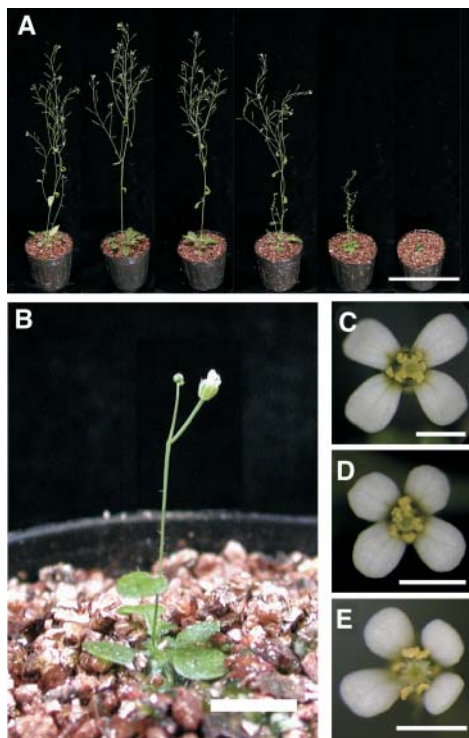
Figure 7. Mutational Phenotypes of the *ahk* Multiple Mutants in the Reproductive Growth Phase.

(A) Morphology of 5-week-old plants. From left to right are Col, Ws, *ahk2-1 ahk4-1*, *ahk3-1 ahk4-1*, *ahk2-1 ahk3-1*, and *ahk2-1 ahk3-1 ahk4-1* plants. Bar = 10 cm.
(B) Close-up view of the 7-week-old *ahk2-1 ahk3-1 ahk4-1* triple mutant. Bar = 1 cm.
(C) to **(E)** Close-up views of Col **(C)**, Ws **(D)**, and *ahk2-1 ahk3-1 ahk4-1* **(E)** flowers. Bars = 1 mm.

primordia (Figures 8A to 8F). In the root tips, strong staining was associated with various cells in the entire meristem and the basal part of the meristem for *AHK2:GUS* and *AHK3:GUS*, respectively, whereas strong expression of *AHK4:GUS* was relatively restricted to the vascular tissues in the apical part of the meristem (Figures 8G to 8I). Vascular systems were also strongly stained throughout the whole plant, such as those in roots (Figures 8A to 8C), leaves (Figures 8J to 8L), influorescence stems (Figures 8M to 8O), and several floral organs (Figures 8P to 8R). In the floral organs, carpels and developing ovules were also stained in the *AHK2:GUS* and *AHK4:GUS* transgenic plants. From these data, it may be concluded that all the *AHK* genes are predominantly expressed in cells comprising meristematic and vascular tissues in a nearly completely overlapping manner.

DISCUSSION

In this study, we obtained the first genetic evidence that AHK2 and AHK3 function as positive regulators for the cytokinin-signaling pathway. The results of a series of phenotypic analyses

on *ahk* multiple mutants allowed us to determine the relevant biological functions not only of the *AHK* cytokinin receptor gene family but also of plant hormone cytokinins.

In Planta Roles of Each AHK Cytokinin Receptor

Our results confirmed the idea that there is functional redundancy among the three *AHK* genes, which has been expected from their highly conserved primary structures (Ueguchi et al., 2001a). The lack of a phenotype in the mutant carrying the intact *AHK2* or *AHK3* gene alone as well as the expression profiles strongly suggests the complete overlapping of their biological roles. AHK2 and AHK3 may thus function as the main cytokinin receptors that support normal growth and development throughout whole tissues and all growth phases. In this regard, AHK4 seems to have some different features. First, although *AHK4* expression is observed in all the main tissues, as demonstrated by the *AHK4:GUS* fusion gene, the abundance of the *AHK4* transcript is controlled in tissue-specific and regulatory manners. Indeed, *AHK4* expression is observed predominantly in roots and is induced by exogenous cytokinins (Mähönen et al., 2000; Ueguchi et al., 2001a; Che et al., 2002; Rashotte et al., 2003). Second, cellular responses to exogenously supplied cytokinins are largely dependent on the AHK4 function because shoot formation by root explants in vitro and inhibition of root growth in vivo were strongly affected even in the *ahk4-1* single mutant, which showed no apparent phenotype as to growth and development. Third, a recent report demonstrated that AHK4 is involved in the phosphate starvation response, suggesting its additive role in an adaptive response to external stimuli (Franco-Zorrilla et al., 2002). Therefore, AHK4 may play a certain role by modulating the sensitivity of particular cells to cytokinins in response to various environmental stimuli, resulting in adaptation of the plants to altered growth conditions.

Role of the AHK Cytokinin Receptors in Cell Division

What is the function of AHKs at the cellular level? The impaired leaf development in the *ahk2 ahk3* double mutant and *ahk2 ahk3 ahk4* triple mutants revealed to us their primary function in cells. During leaf morphogenesis, cell division occurs in an early phase and then ceases with the initiation of cell expansion and differentiation. In the *ahk* multiple mutants, cells tend to lose cell division activity and differentiate earlier than those in the wild-type controls. The primary functions of AHKs may thus be activation of cell division and maintenance of the meristematic competence of cells to prevent subsequent differentiation until a sufficient number of cells has accumulated during leaf morphogenesis. It is likely that the decreased density of veins is also because of the loss of cell division activity in vascular tissues in an early period of leaf development. The root phenotype of the *ahk2 ahk3 ahk4* triple mutant may be explained in a similar manner as the leaf phenotype. In the mutant roots, cells generated from the root apical meristem tend to expand significantly earlier than wild-type root cells, suggesting that the mutant cells fail to maintain cell division activity and quickly undergo differentiation. Although further investigation is required to determine
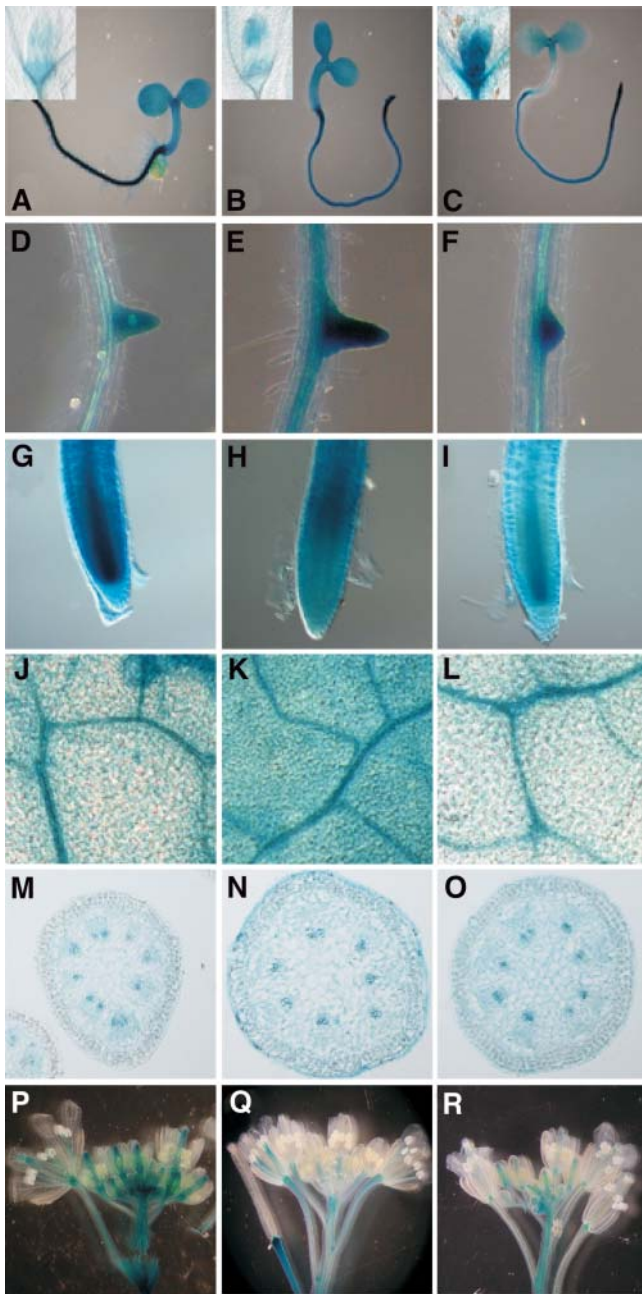
Figure 8. Expression of *AHK:GUS* Fusion Genes.

GUS staining of *AHK2:GUS* (left column), *AHK3:GUS* (middle column), and *AHK4:GUS* (right column) is shown. Plants were grown on MS gellan gum plates or on soil with 16-h-light/8-h-dark fluorescent illumination at 22°C.

(A), **(B)**, and **(C)** Five-day-old seedlings and close-up views of SAM and young leaf primordia (insets).

(D), **(E)**, and **(F)** Growing lateral root primordia of 5-d-old seedlings.

(G), **(H)**, and **(I)** Root tips of 5-d-old seedlings.

(J), **(K)**, and **(L)** Close-up views of the surface of first mature leaves. Ten-day-old rosette plants were stained.

(M), **(N)**, and **(O)** Cross-sections of influorescences of 4-week-old plants.

(P), **(Q)**, and **(R)** Floral tissues of 4-week-old plants.

whether or not the decreased cell division activity in the mutant roots is a direct consequence of the mutations, AHKs are likely to maintain cell division activity in various tissues to guarantee organs of sufficient size.

Reduced leaf size and inhibition of root growth have also been reported for transgenic plants, in which cell cycle progression is inhibited through modulation of the expression or gene function of various components in cell cycle regulation (Umeda et al., 2000; De Veylder et al., 2001b; Zhou et al., 2002). In addition, exogenous cytokinins have been shown to trigger the G2/M transition in tissue and cell cultures (Zhang et al., 1996; Laureys et al., 1998). It has also been reported that exogenous cytokinins induce cyclin D3 expression, which is required for the G1/S transition in cell cycle regulation (Riou-Khamlichi et al., 1999). Although we do not know at present whether or not such cytokinin actions involve AHK cytokinin receptors, AHKs may regulate the expression and/or activity of certain components in cell cycle regulation. Further investigation regarding the target gene(s) of the AHKs is needed to clarify the mechanism underlying the cell cycle regulation by endogenous cytokinins.

It should be mentioned that the final size of leaf cells was not affected by the *ahk* mutations. Enlargement of leaf cells to compensate for the reduced cell number is occasionally observed in the transgenic plants discussed above. The over-expression of a dominant negative derivative of Arabidopsis cdc2a kinase in tobacco (*Nicotiana tabacum*) results in leaves of normal size and shape even though the leaf cell number is greatly reduced (Hemerly et al., 1995). These observations led to the view that an increased cell volume, which is mediated by a communication signal between leaf cells, compensates for the reduction in the cell number caused by inhibited cell division (Tsukaya, 2002; Beemster et al., 2003). The lack of such compensation in the *ahk* multiple mutants suggests that the recognition of cytokinins by AHKs might be involved in the presumed compensatory mechanism.

Role of Endogenous Cytokinins in Root Growth

Hormone-insensitive plants, in general, are expected to show particular defective phenotypes similar to and overlapping those of hormone-deficient plants. In this regard, it would be quite intriguing to compare the growth phenotypes of the *ahk* multiple mutants with those of recently reported cytokinin-deficient transgenic plants, in which the cytokinin content is decreased to ∼30 to 50% of that in wild-type controls because of overexpression of cytokinin oxidase genes (Werner et al., 2001, 2003; Yang et al., 2003). The aerial parts of the transgenic plants show similar defective phenotypes to those in the *ahk2 ahk3 ahk4* triple mutant, such as small leaves with a reduced cell number as well as incomplete formation of higher ordered veins; a relatively small SAM structure with a slightly impaired function; delayed timing and a reduced rate of flowering; and a reduced number of flowers with the correct morphology but reduced fertility. The phenotypic similarities strongly suggest that endogenous cytokinins act through the functions of AHKs, at least in shoots. However, there is a great difference between cytokinin-deficient transgenic plants and the *ahk* triple mutant in root growth. The growth of main roots

as well as the formation of lateral roots is significantly inhibited in the *ahk* triple mutant, which is associated with decreased cell division activity, whereas the main root growth in cytokinin-deficient transgenic plants is rather enhanced because of enlargement of the meristematic region in the root tips, and propagation and growth of lateral roots are also stimulated (Werner et al., 2001, 2003; Yang et al., 2003). The enhanced growth in the transgenic plants may suggest that, different from shoot growth, the root growth in the wild-type plants is repressed by cytokinins, presumably because of the higher content of and/or sensitivity to cytokinins in roots, so that the partially reduced content of cytokinins in the transgenic plants is more suitable for stimulating root growth. Because *AHK4* expression is predominantly detected in roots (Mähönen et al., 2000; Ueguchi et al., 2001a), the presumed higher sensitivity to cytokinins might be accounted for by a higher content of the cytokinin receptors in roots than in shoots. This view therefore does not necessarily lead us to the idea, previously proposed on the basis of the transgenic studies, that the function of cytokinins in roots is opposite to that in shoots (Werner et al., 2001, 2003). It is probable that complete, not partial, loss of endogenous cytokinins in root tissues leads to inhibition of root growth, as observed in the *ahk2 ahk3 ahk4* triple mutant, whose cells are unable to sense extracellular cytokinins. We thus concluded that endogenous cytokinins function in roots in a similar manner to that in shoots.

Role of AHKs in SAM Formation and Its Maintenance

The effect of cytokinins on shoot formation from undifferentiated cells in vitro has led us to regard cytokinins as essential hormones that also play a role in the initiation of the SAM during in vivo embryogenesis (Mok and Mok, 2001; Howell et al., 2003). Our results with the *ahk2 ahk3 ahk4* triple mutant almost confirm the important roles of cytokinins in the growth, development, and functions of several organs, such as leaves, roots, and inflorescence meristems. However, mutational lesions do not prevent SAM establishment and only slightly affect the subsequent functioning of the SAM. Even in the triple mutant, embryo patterning and morphogenesis proceeded almost normally except for a slight reduction of cells in the hypocotyl epidermis and vascular tissues. As observed in the leaves and root tips, embryogenesis in a cytokinin-insensitive mutant should also be somehow affected by reduced meristematic activity. The moderate effect on embryogenesis in the triple mutant thus raised the question of whether endogenous cytokinins or the AHK cytokinin receptors are essential for particular biological processes, such as SAM formation. One might imagine that cytokinins are important but dispensable for these processes. However, we feel this is not the case. In the cytokinin-deficient transgenic Arabidopsis discussed above, different from in the triple mutant, the growth of plants exhibiting the severe phenotype is arrested in an early period during the vegetative growth phase even in the presence of a residual amount of cytokinins, suggesting the functional essentiality of cytokinins at least in the maintenance of SAM activity (Werner et al., 2003).

An alternative idea explaining the lack of a severe phenotype in embryogenesis is that the T-DNA mutants used in this study still allow some of their molecular functions in cytokinin signaling. Because of the undetectable level of the *AHK2* transcript in the *ahk2-1* mutant and the T-DNA insertion site in the *ahk3-1* mutant, both alleles may be regarded as null mutations. By contrast, it is possible that the C-terminally truncated AHK4 protein encoded by the *ahk4-1* allele has a residual function carried by its intact transmitter domain. If this is the case, signal transduction might operate through the direct phosphotransfer reactions between the transmitter domain of AHK4 and the receiver domains of several ARRs or of other hybrid histidine kinases. Or, alternatively, signal transduction might be mediated by a factor(s) other than a two-component system, as in the case of ethylene signaling (Clark et al., 1998). In this regard, it should be mentioned that the *ahk4-1* single mutant does not exhibit the short root phenotype observed for the different alleles of the *AHK4* gene, such as *wol*. Because the root phenotype of the *wol* mutant (i.e., inhibited root elongation with a reduced number of vascular cell files) is quite similar to that of the *ahk2-1 ahk3-1 ahk4-1* triple mutant, it is likely that the mutated AHK4 caused by the missense *wol* mutation inactivates other AHKs (i.e., AHK2 and AHK3) through heterodimerization, resulting in the severe phenotype. Therefore, the C-terminally truncated AHK4 in the *ahk4-1* mutant might be completely inactive as to heterodimerization with other intact AHKs or partially active, as discussed above, even in the heterodimers. In any event, these possibilities suggesting a more complex nature of cytokinin signaling are worth investigating further.

Finally, it should be mentioned that there is another explanation; for example, cytokinin receptor(s) other than AHKs compensate for the lack of the *AHK* gene products during embryogenesis. In this regard, it is worth mentioning that putative receptor proteins and signaling systems other than the two-component signal transduction system have been proposed recently to mediate cytokinin signal perception and transduction. Transgenic Arabidopsis expressing the antisense *GCR1* gene, encoding a seven-transmembrane receptor, shows reduced cytokinin sensitivity (Plakidou-Dymock et al., 1998). The cytokinin-inducible *ARR5* expression is suppressed by treatment with primary alcohols, suggesting the involvement of phospholipase D activity in cytokinin signaling (Romanov et al., 2002). Riou-Khamlichi et al. (1999) demonstrated that cyclin D3 expression is directly induced by cytokinins in vivo and in vitro and that regulatory phosphorylation, not phosphorelay in the two-component system, is involved in the induction. Of course, we do not know whether or not these candidates are actually involved in the cytokinin signaling at present. However, cytokinin signal perception and transduction might be more complex than expected and involve a signaling pathway other than that including the *AHK* gene products.

In this study, we demonstrated that the *AHK* gene family plays an important role in the postembryonic development of various shoot and root organs through maintenance of the meristematic activity of cells. This view also highlights the functional importance of endogenous cytokinins. However, clarification and elucidation of the entire picture of the cytokinin signaling system as well as the biological significance of cytokinins must await further investigation.

METHODS

Plant Materials

The Col and Ws ecotypes as well as derivatives of *Arabidopsis thaliana* were used in this study. Plants were grown with 16 h light/8 h dark or continuous fluorescent illumination at 22°C. Plants were germinated and grown on soil, rockwool (Nittobo, Tokyo, Japan), or MS medium comprising MS salts (Wako, Osaka, Japan), 2% sucrose, 1/100 volume of 5% 2-(*N*-morpholino)-ethanesulfonic acid (adjusted to pH 5.7 with KOH), 0.5 μg/mL of thiamine-HCl, 5 μg/mL of nicotinic acid, 0.5 μg/mL of pyridoxine-HCl, 100 μg/mL of myo-inositol, and 0.8% agar or 0.3% gellan gum (Wako). Hygromycin (20 μg/mL) and kanamycin (25 μg/mL) were included in the medium when necessary.

Screening of T-DNA tagged mutations of the *AHK2* and *AHK3* genes was performed according to protocol for the PCR-based screening system at the Kazusa DNA Research Institute and Wisconsin University, respectively. Note that *ahk2-1* and *ahk2-2* were Kazusa DNA Research Institute (KG26095) and SALK Institute (SALK_052531) lines, respectively, and that the *ahk3-1* and *ahk3-2* mutants were Wisconsin University lines. These mutants were used for further analyses after being backcrossed two times.

Transformation of Arabidopsis was performed essentially according to the *Agrobacterium tumefaciens*–mediated vacuum infiltration method (Bechtold and Pelletier, 1998).

Genotyping

The wild-type *AHK2*, *ahk2-1*, and *ahk2-2* alleles were detected by means of PCR reactions using the following pairs of primers: AHK2-1F (5′-GGGGATATAAGTGAGCAGCATATAA-3′) and AHK2-1R (5′-AAAA-CTCCATTCATCGGAGTCCGTA-3′) or AHK2-2F (5′-ACCTTCCCCACC-TAGATGATGAGAT-3′) and AHK2-2R (5′-TCACTGCTCGACGACTT-CTTAGCTT-3′) for the wild-type *AHK2* allele; AHK2-1F and P06RB (5′-TTCCCTTAATTCTCCGCTCATGATC-3′) for the *ahk2-1* allele; and LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and AHK2-2R for the *ahk2-2* allele.

The wild-type *AHK3*, *ahk3-1*, and *ahk3-2* alleles were detected by means of PCR reactions using the following pairs of primers: AHK3-1F (5′-GAGACAGCATCATCTCCGGAAAGTA-3′) and AHK3-1R (5′-TTCAA-CACGTGGAACTACTTCGACT-3′) or AHK3-2F (5′-GTATCTCGGGG-GAGTGTTTGACATT-3′) and AHK3-2R (5′-TGTGACTTTGCAACATCTG CTGCTT-3′) for the wild-type *AHK3* allele; AHK3-1F and JL202 (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′) for the *ahk3-1* allele; and AHK3-2 and JL202 for the *ahk3-2* allele.

The wild-type *AHK4* and *ahk4-1* alleles were detected by means of PCR reactions as described previously (Ueguchi et al., 2001b).

Plasmid Construction

To construct a set of *AHK* promoter-protein:*GUS* fusion genes, a plasmid carrying the GUS open reading frame was first constructed. The *Eco*RI site of pUC118 was substituted with a *Not*I site by linker insertion, which yielded pCU97. A 0.3-kb *Not*I-*Eco*RI fragment encompassing the nopaline synthetase terminator was prepared from pSK1 (Kojima et al., 1999), blunt-ended with T4 DNA polymerase, and then inserted into the previously blunt-ended *Sac*I site of pCU97, yielding pCU98. GUS open reading frame was amplified from pBI101 using primers 5′-TTAACCCGG-GATGTTACGTCCTGTAGAAACC-3′ and 5′-TTAAGGTACCTCATTGTT TGCCTCCCTGCTG-3′. Note that the underlined nucleotides hereafter were added to create appropriate restriction sites. The resultant fragment was digested with *Sma*I and *Kpn*I and then inserted between the *Sma*I and *Kpn*I sites of pCU98. After confirmation of the sequence, the resultant plasmid was named pCU101.

To construct a *AHK2*:*GUS* fusion, a 1056-bp genomic DNA fragment encompassing the 3′ portion of the *AHK2* gene was amplified by means of a PCR reaction from the P1 clone MXH1 (GenBank accession number AB011485) using the following pair of primers: 5′-ACTAAGCTTGGATCC-GCATGCCTAGCTATTCAGGAGTTTACAGGAT-3′ and 5′-TTTCCCGGG-ACAAGGTTCAAAGAATCTTGCTACCGCT-3′. The resultant fragment was digested with *Hind*III and *Sma*I and then inserted between the *Hind*III and *Sma*I sites of pCU101 to create an in-frame protein fusion of the 3′ portion of AHK2 and GUS. The sequence of the amplified fragment was confirmed by sequencing. The resultant plasmid was digested with *Sph*I (note that one *Sph*I site was created by the forward PCR primer and another *Sph*I site is within the AHK2-coding region), and then a 7.8-kb genomic *Sph*I fragment encompassing the putative promoter as well as the 5′ portion of the AHK2-coding region was cloned between the *Sph*I sites, yielding an *AHK2* promoter-protein:*GUS* fusion gene. The resultant construct was then cloned into pBIBHm (Becker, 1990) to yield pCUA246.

To construct a *AHK3*:*GUS* fusion, a 1979-bp genomic DNA fragment encompassing the 5′ portion of the AHK3-coding region was amplified by means of a PCR reaction from the BAC clone F17L21 (GenBank accession number AC004557) using the following pair of primers: 5′-GTGGATGAGTCTGTTCCATGTGCTA-3′ and 5′-AATTCCCGGGTT-TATCACAATCTTCTTCAACTTTG-3′. The resultant fragment was digested with *Sma*I, treated with T4 kinase, and then inserted into the *Sma*I sites of pCU101 to create an in-frame protein fusion of the 5′ portion of AHK3 and GUS. After confirmation of the cloned sequence, the resultant plasmid was digested with *Bam*HI and *Nde*I, and then a 5.0-kb genomic *Bam*HI/*Nde*I fragment encompassing the putative promoter as well as the 5′ portion of AHK3-coding region was cloned between the *Bam*HI and *Nde*I sites, yielding a *AHK3*:*GUS* fusion gene, in which the N-terminal membrane-anchored domain of AHK3 is fused with GUS. The resultant construct was then cloned into pBIBHm to yield pCUA168.

To construct an *AHK4*:*GUS* fusion, a 776-bp genomic DNA fragment encompassing the 3′ portion of the AHK4-coding region was amplified by means of a PCR reaction from the BAC clone T23K3 (GenBank accession number AC007069) using the following pair of primers: 5′-AATTCCCGGGGGAAACGTTCATCACAAGTCTC-3′ and 5′-AATTCC-CGGGCGACGAAGGTGAGATAGGATT-3′. The resultant fragment was digested with *Sma*I and then inserted into the *Sma*I site of pCU101 to create an in-frame protein fusion of 3′ portion of AHK4 and GUS. After confirmation of the cloned sequence, the resultant plasmid was digested with *Bam*HI and *Xho*I. A 6.6-kb genomic *Bam*HI/*Xho*I fragment encompassing the putative promoter as well as the 5′ portion of the AHK4-coding region was prepared from pCUA88 and then cloned between the *Bam*HI and *Nde*I sites in the above *AHK4*:*GUS* fusion plasmid, yielding an *AHK4*:*GUS* fusion gene. The resultant construct was then cloned into pBIBHm to yield pCUA243.

RT-PCR

Total RNA was isolated by the SDS-phenol method (Palmiter, 1974). Five micrograms of the total RNA was treated with RNase-free DNase I at 25°C for 30 min, and then 100 ng of the resultant RNA was reverse transcribed with oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After the RT reaction, the cDNA was subjected to PCR reactions. The following pairs of primers were used: 5′-GTTTTGCGTCCCGAGATGTTAGATA-3′ and 5′-AGCTGCGAGTAGATATCATTAGCTT-3′ for *ARR5*, 5′-CTCAA-GAGGTTCTCAGCAGTA-3′ and 5′-TCACCTTCTTCATCCGCAGTT-3′ for *TUB2* (At5g62690), and 5′-CCTTGTCAATGGCAAGAAGAGGCAA-3′ and 5′-CTTGCTACCGCTGTGTAGAGCACTT-3′ for *AHK2*. The PCR reaction comprised 33 cycles (or 40 cycles for *AHK2*) of 0.5 min at 94°C, 0.5 min at 55°C, and 1 min at 72°C.

Cytokinin Assays

The effects of cytokinins on greening and shoot induction from calli and elongation of main roots were examined as described previously (Ueguchi et al., 2001b).

The effects of cytokinins on *ARR5* expression were examined as follows. Plants were grown in vitro on MS agar plates with 16-h-light/8-h-dark fluorescent illumination at 22°C for 2 weeks. The plants were sprayed with a 100-μM *t*-zeatin solution or 0.36% methanol, the solvent for *t*-zeatin, and then incubated under the same conditions. Rosette leaves were then harvested, and total RNA was isolated and subjected to RT-PCR analysis.

Histological Analysis of Seedlings

Plants were germinated on MS gellan gum plates with 16-h-light/8-h-dark fluorescent illumination at 22°C. Five-day-old seedlings were fixed overnight in a solution comprising 90% (v/v) ethanol and 10% (v/v) acetic acid at room temperature. After fixation, samples were hydrated in an ethanol series and then cleared overnight with a chloral hydrate:glycerol:water solution (8:1:2, w:v:v) at 4°C. Microscopic analysis was performed with a Nomarski microscope (LEITZ DM RBE; Leica, Wetzlar, Germany). Images were taken using a digital camera (DC300; Leica) and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA). For SAM observation, tissues containing SAM of 12-d-old seedlings were fixed and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), and then 2-μm sections were stained with 0.1% toluidine blue.

Histological Analysis of Roots

Plants were grown on MS gellan gum plates with continuous fluorescent illumination at 22°C. Five-day-old seedlings were stained with a propidium iodide solution (10 μg/mL) for 10 s, followed by washing with sterilized water. Samples were observed under a confocal microscanning laser microscope (FV500; Olympus, Tokyo, Japan) equipped with a digital camera (DP50; Olympus). For DAPI staining, roots were fixed with an ethanol:acetic acid (6:1, v:v) solution overnight at room temperature, stained with a DAPI solution (1 μg/mL) for 15 s, and then washed three times with water. Samples were observed as described above.

Kinematic Analysis of Leaf Development

The kinematic analysis of leaf development was performed essentially according to the method of De Veylder et al. (2001a). Plants were grown on MS gellan gum plates with continuous fluorescent illumination at 22°C. The second mature leaves were harvested from six plants every day, fixed, and then cleared as described above. Microscopic analysis was performed with a Nomarski microscope (LEITZ DM RBE). Images were photographed using a digital camera (DC300) and processed with Adobe Photoshop and Image J (version 1.30; http://rsb.info.nih.gov/ij/). The cell size was determined as the average for at least 10 abaxial epidermal cells located at 25 and 75% of the distance between the tip and the base of the leaf blade and halfway between the midvein and the margin, respectively. The total cell number per leaf was estimated by dividing the leaf size by the average cell size.

GUS Staining

T3 transgenic plants each carrying the *AHK*:*GUS* fusion homozygously were used. Sample tissues were fixed in 90% ice-cold acetone for 1 h, washed with 50 mM phosphate buffer (pH 7.0) three times, and then immersed in a solution comprising 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100, and 0.5 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 37°C. After staining, samples were destained with ethanol overnight at room temperature. Samples were observed by a stereomicroscope (MS5; Leica) or the Nomarski microscope (LEITZ DM RBE) with either a Nikon COOLPIX 990 (Tokyo, Japan) or Leica DC300 digital camera, respectively.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AB011485, AC004557, and AC007069.

REFERENCES

Bechtold, N., and Pelletier, G. (1998). In planta *Agrobacterium*-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol. Biol. **82,** 259–266.

Becker, D. (1990). Binary vectors which allow the exchange of plant selectable markers and reporter genes. Nucleic Acids Res. **18,** 203.

Beemster, G.T.S., Fiorani, F., and Inzé, D. (2003). Cell cycle: The key to plant growth control? Trends Plant Sci. **8,** 154–158.

Brandstatter, I., and Kieber, J.J. (1998). Two genes with similarity to bacterial response regulators are rapidly and specifically induced by cytokinin in Arabidopsis. Plant Cell **10,** 1009–1019.

Che, P., Gingerich, D.J., Lall, S., and Howell, S.H. (2002). Global and hormone-induced gene expression changes during shoot development in Arabidopsis. Plant Cell **14,** 2771–2785.

Clark, K.L., Larsen, P.B., Wang, X., and Chang, C. (1998). Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc. Natl. Acad. Sci. USA **95,** 5401–5406.

De Veylder, L., Beeckman, T., Beemster, G.T.S., Krols, L., Terras, F., Landrieu, I., Van Der Schueren, E., Maes, S., Naudts, M., and Inzé, D. (2001a). Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell **13,** 1653–1667.

De Veylder, L., Beemster, G.T.S., Beeckman, T., and Inzé, D. (2001b). *CKS1At* overexpression in *Arabidopsis thaliana* inhibits growth by reducing meristem size and inhibiting cell-cycle progression. Plant J. **25,** 617–626.

Franco-Zorrilla, J.M., Martin, A.C., Solano, R., Rubio, V., Leyva, A., and Paz-Ares, J. (2002). Mutations at *CRE1* impair cytokinin-induced repression of phosphate starvation responses in *Arabidopsis*. Plant J. **32,** 353–360.

Gendreau, E., Traas, J., Desnos, T., Grandjean, O., Caboche, M., and Höfte, H. (1997). Cellular basis of hypocotyls growth in *Arabidopsis thaliana*. Plant Physiol. **114,** 295–305.

Hemerly, A., de Almeida Engler, J., Bergounioux, C., Van Montagu, M., Engler, G., Inzé, D., and Ferreira, P. (1995). Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J. **14,** 3925–3936.

Heyl, A., and Schmülling, T. (2003). Cytokinin signal perception and transduction. Curr. Opin. Plant Biol. **6,** 480–488.

Howell, S., Lall, S., and Che, P. (2003). Cytokinins and shoot development. Trends Plant Sci. **8,** 453–459.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in *Arabidopsis* cytokinin signal transduction. Nature **413,** 383–389.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409,** 1060–1063.

Kojima, S., Banno, H., Yoshioka, Y., Oka, A., Machida, C., and Machida, Y. (1999). A binary vector plasmid for gene expression in plant cells that is stably maintained in *Agrobacterium* cells. DNA Res. **6,** 407–410.

Laureys, F., Dewitte, W., Witters, E., Van Montagu, M., Inzé, D., and Van Onckelen, H. (1998). Zeatin is indispensable for the G2-M transition in tobacco BY-2 cells. FEBS Lett. **426,** 29–32.

Mähönen, A.P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P.N., and Helariutta, Y. (2000). A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root. Genes Dev. **14,** 2938–2943.

Mok, D.W.S., and Mok, M.C. (2001). Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. **52,** 89–118.

Palmiter, R.D. (1974). Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. Biochemistry **13,** 3606–3615.

Plakidou-Dymock, S., Dymock, D., and Hooley, R. (1998). A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. Curr. Biol. **8,** 315–324.

Rashotte, A.M., Carson, S.D.B., To, J.P.C., and Kieber, J. (2003). Expression profiling of cytokinin action in Arabidopsis. Plant Physiol. **132,** 1998–2011.

Riou-Khamlichi, C., Huntley, R., Jacqmard, A., and Murray, J.A.H. (1999). Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science **283,** 1541–1544.

Romanov, G.A., Kieber, J.J., and Schmülling, T. (2002). A rapid cytokinin response assay in *Arabidopsis* indicates a role for phospholipase D in cytokinin signaling. FEBS Lett. **515,** 39–43.

Sakai, H., Honma, T., Aoyama, T., Sato, S., Kato, T., Tabata, S., and Oka, A. (2001). ARR1, a transcription factor for genes immediately responsive to cytokinins. Science **294,** 1519–1521.

Sheen, J. (2002). Phosphorelay and transcription control in cytokinin signal transduction. Science **296,** 1650–1652.

Suzuki, T., Miwa, K., Ishikawa, K., Yamada, H., Aiba, H., and Mizuno, T. (2001). The Arabidopsis sensor His-kinase, AHK4, can respond to cytokinins. Plant Cell Physiol. **42,** 107–113.

Taniguchi, M., Kiba, T., Sakakibara, H., Ueguchi, C., Mizuno, T., and Sugiyama, T. (1998). Expression of *Arabidopsis* response regulator homologs is induced by cytokinins and nitrate. FEBS Lett. **429,** 259–262.

Tsukaya, H. (2002). Interpretation of mutants in leaf morphology: Genetic evidence for a compensatory system in leaf morphogenesis that provides a new link between cell and organismal theories. Int. Rev. Cytol. **217,** 1–39.

Ueguchi, C., Koizumi, H., Suzuki, T., and Mizuno, T. (2001a). Novel family of sensor histidine kinase genes in *Arabidopsis thaliana*. Plant Cell Physiol. **42,** 231–235.

Ueguchi, C., Sato, S., Kato, T., and Tabata, T. (2001b). The *AHK4* gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. Plant Cell Physiol. **42,** 751–755.

Umeda, M., Umeda-Hara, C., and Uchimiya, H. (2000). A cyclin-dependent kinase-activating kinase regulates differentiation of root initial cells in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **97,** 13396–13400.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H., and Schmülling, T. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell **15,** 2532–2550.

Werner, T., Motyka, V., Strnad, M., and Schmülling, T. (2001). Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA **98,** 10487–10492.

Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., and Mizuno, T. (2001). The AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. **42,** 1017–1023.

Yang, S., Yu, H., Xu, Y., and Goh, C.J. (2003). Investigation of cytokinin-deficient phenotypes in *Arabidopsis* by ectopic expression of orchid *DSCKX1*. FEBS Lett. **555,** 291–296.

Zhang, K., Letham, D.S., and John, P.C. (1996). Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. Planta **200,** 2–12.

Zhou, Y., Wang, H., Gilmer, S., Whitwill, S., Keller, W., and Fowke, L.C. (2002). Control of petal and pollen development by the plant cyclin-dependent kinase inhibitor ICK1 in transgenic *Brassica* plants. Planta **215,** 248–257.