In planta functions of the *Arabidopsis* cytokinin receptor family

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Since their discovery as cell-division factors in plant tissue culture about five decades ago, cytokinins have been hypothesized to play a central role in the regulation of cell division and differentiation in plants. To test this hypothesis *in planta*, we isolated *Arabidopsis* plants lacking one, two, or three of the genes encoding a subfamily of histidine kinases (*CRE1*, *AHK2*, and *AHK3*) that function as cytokinin receptors. Seeds were obtained for homozygous plants containing mutations in all seven genotypes, namely single, double, and triple mutants, and the responses of germinated seedlings in various cytokinin assays were compared. Both redundant and specific functions for the three different cytokinin receptors were observed. Plants carrying mutations in all three genes did not show cytokinin responses, including inhibition of root elongation, inhibition of root formation, cell proliferation in and greening of calli, and induction of cytokinin primary-response genes. The triple mutants were small and infertile, with a reduction in meristem size and activity, yet they possessed basic organs: roots, stems, and leaves. These results confirm that cytokinins are a pivotal class of plant growth regulators but provide no evidence that cytokinins are required for the processes of gametogenesis and embryogenesis.

Since the discovery of kinetin in 1956 as a degradation product of DNA that promotes cell division in plants (1), a considerable amount of biochemical, physiological, and, most recently, genetic research has focused on elucidating the diverse roles that cytokinins play in plant growth and development. Perturbations of cytokinin levels in plants via over-expression of bacterial cytokinin synthesis genes (2–4), recovery of mutant plants with a higher-than-normal cytokinin content (5), and characterization of loss-of-function mutants of the cytokinin receptor CYTOKININ RESPONSE 1 (CRE1) (6–9) have implicated cytokinins in a wide variety of processes, including cell division, organ formation and regeneration, senescence, apical dominance, vascular development, response to pathogens, and nutrient mobility. These numerous roles for cytokinins, coupled with the failure of mutant screens to yield plants with nondetectable cytokinin levels, led to the longstanding belief that cytokinins are essential for plant growth and development.

Plants respond to cytokinin through a multistep phosphorelay system, consisting of sensor histidine kinase (HK) proteins, histidine phosphotransfer (HPt) proteins, and effector response regulator (RR) proteins. Over-expression and loss-of-function analyses of particular HK, HPt, and RR proteins in *Arabidopsis* (8–13), combined with transient expression assays in protoplasts (14), have led to a model for cytokinin signaling (for a review, see refs. 15 and 16), beginning with perception of cytokinins by HK proteins.

The *Arabidopsis* genome encodes six nonethylene receptor HKs: CRE1/WOL/AHK4, AHK2, AHK3, AtHK1, CKI1, and CKI2/AHK5. Among them, CRE1, *Arabidopsis* HK2 (AHK2), and *Arabidopsis* HK3 (AHK3) (hereafter called the CRE family) are highly homologous at the amino acid level, especially within the putative cytokinin-binding extracellular domain (≈60% identity). CRE1 was the first cytokinin-signaling component identified. A substantial body of evidence supports a role for CRE1 as a cytokinin receptor: plants carrying loss-of-function mutations in the *CRE1* gene have a reduced sensitivity to cytokinin (8, 9), CRE1 initiates a phosphorelay in response to cytokinins when expressed in heterologous systems (8, 9, 17), and fission yeast expressing CRE1 bind active cytokinins in a specific and saturable manner (18). Similar to CRE1, AHK2 (M.H. and T.K., unpublished data) and AHK3 (18) are also activated by cytokinins when expressed in yeast and bacteria, respectively.

CKI1 was first identified as a gene that induces constitutive cytokinin responses when overexpressed in callus tissue (19). However, all subsequent efforts to detect cytokinin receptor activity of CKI1 have yielded negative results (refs. 14 and 18 and T.K., unpublished work). CKI2 is the only HK lacking a putative extracellular domain, and loss-of-function mutants of *CKI2* have no noticeable phenotype (Y.H. and T.K., unpublished work). AtHK1 has been implicated in osmosensing (20), and the HK activity of AtHK1 in yeast is unchanged by cytokinins. These results indicate that CRE-family members are cytokinin receptors, but that the other nonethylene receptor HKs are less likely to perform a cytokinin-sensing role.

To elucidate the *in planta* roles of the three CRE-family members, as well as the role of cytokinin signaling in plant development, we identified loss-of-function alleles for *CRE1*, *AHK2*, and *AHK3*; created plants containing all possible mutant allele combinations; and characterized their responses in a series of cytokinin assays. These observations are discussed in relation to the relative contributions of each family member to cytokinin action, as well as the overall role of cytokinins in plant growth and development.

Materials and Methods

Plant Materials and Growth Conditions. Unless otherwise indicated, sterilized seeds of *Arabidopsis thaliana*, ecotype Columbia (Col), were incubated at 4°C for 2 days on germination medium (GM) containing full-strength Murashige and Skoog salts (MS) (21); 0.05% (wt/vol) Mes-KOH (pH 5.7); 1% (wt/vol) sucrose; 100 mg/ml inositol; 10 mg/ml thiamine HCl; 1 mg/ml nicotinic acid; 1 mg/ml pyridoxine HCl; and 0.3% (wt/vol) Phytagel (Sigma–

Abbreviations: HK, histidine kinase; AHK2, *Arabidopsis* HK2; AHK3, *Arabidopsis* HK3; BA, benzyl adenine; Col, Columbia; CRE1, cytokinin response 1; SAM, shoot apical meristem; GM, germination medium; MS, Murashige and Skoog salts; GUS, β-glucuronidase; *t*-zeatin, *trans*-zeatin.

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Aldrich); and grown at 22°C under constant illumination (100 μmol/m^2·s). For plastochron measurements, the number of leaves >0.5 mm in width from 11-day-old seedlings was counted under a dissecting microscope. For adventitious root formation assays as well as callus and shoot induction assays, 11-day-old plants grown aseptically under dim light (2.5 μmol/m^2·s) were used. For visualization of root nuclei, roots from 21-day-old plants were fixed with 3.7% (vol/vol) formaldehyde, stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), and photographed on an Olympus BX50 (Melville, NY) microscope with a Roper Scientific Coolsnap HQ/OL digital camera. For analysis of shoot apical meristem (SAM) morphology, seeds were germinated on full-strength MS, 4.5% (wt/vol) sucrose, 0.05% (wt/vol) Mes, and 0.8% (wt/vol) agar (MS + 4.5 suc), and grown for 7 days. Seedlings were fixed, embedded, and sectioned according to Mähönen *et al.* (6). Three-micrometer sections were stained with 0.05% (wt/vol) toluidine blue in water and photographed on an Olympus Provis microscope with an Olympus DP70 digital camera.

Cytokinin Response Assays. For the root elongation assay, seedlings grown under constant light for 8 days on GM supplemented with 1/5,000 volume of appropriate concentrations of benzyl adenine (BA) dissolved in DMSO were removed from plates, and root lengths were measured. Plants that had not germinated within 2 days of culture were excluded from the analysis. For the adventitious root formation assay, plants were separated into upper and lower portions by bisecting the hypocotyl with fine scissors. The upper portions were inserted into GM supplemented with 1/5,000 volume of appropriate concentrations of *trans* (*t*)-zeatin dissolved in DMSO. After 11 days, the presence or absence of adventitious roots near the cut site was observed under a dissecting microscope. For the callus induction assay, hypocotyls were excised with fine scissors and cultured for 24 days on GM supplemented with 30 ng/ml 2,4-dichloriphenoxyaceticacid (2,4-D) and varying concentrations of kinetin. All plates contained 0.02% (vol/vol) DMSO. For the shoot formation assay, excised hypocotyls were cultured for 7 days on GM supplemented with 500 ng/ml 2,4-D and 50 ng/ml kinetin to induce callus formation. Calli were then moved onto GM supplemented with 0.3 μg/ml indolebutyric acid and varying concentrations of *t*-zeatin and cultured for 14 days.

Expression Patterns of Cytokinin Receptor Genes. For RNA gel blot analysis, 5 μg of total RNA was used per lane. DNA fragments corresponding to a region of *CRE1*, *AHK2*, or *AHK3* were amplified by PCR. Primer sequences are available in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. After the T7 promoter was ligated to the 3′ end of each amplified fragment, radiolabeled riboprobes were synthesized by using the MAXIscript T7 Kit (Ambion, Austin, TX). Hybridizations were conducted in PerfectHyb (Toyobo, Dojima, Osaka) according to the manufacturer's instructions. For expression patterns of reporter genes, the promoter sequence for each of the CRE-family genes was cloned in-frame upstream of the *β-glucuronidase* (*GUS*) gene. Details of the reporter gene cloning are available in the *Supporting Materials and Methods*. *CRE1*::*GUS* and *AHK3*::*GUS* were introduced into *Arabidopsis,* ecotype Wassilewskija (Ws), by the floral dip method (22). *AHK2*::*GUS* was similarly introduced into *Arabidopsis* (Col). Expression patterns for each gene were determined according to Miyawaki *et al.* (23).

Screening for T-DNA Insertion Mutants. Multiple alleles of T-DNA insertion mutants were identified. One set of mutants (*cre1–10*, *ahk2–1*, *ahk3–1*) is in the ecotype Ws, whereas another set of mutants (*cre1–12*, *ahk2–2*, and *ahk3–3*) is in the ecotype Col. Double and triple mutants were generated in the same backgrounds. Additional mutant alleles identified in the ecotype Ws include *ahk3–2* and *cre1–11*. Details of the screening process are provided in *Supporting Materials and Methods*.

Expression of Cytokinin Receptor Genes in Mutant Backgrounds. Details of the RT-PCR analysis of CRE-family genes in *cre1–12*, *ahk2–2*, and *ahk3*-3 mutant plants are available in *Supporting Materials and Methods*.

Expression of Cytokinin Primary-Response Genes. For real-time quantitative PCR (qRT-PCR) analysis of cytokinin-inducible gene expression, seeds were germinated on MS + 4.5suc and grown for 6 days. Cytokinin treatment was carried out by incubating seedlings in an MS + 4.5suc solution without agar and supplemented with 10 μM BA for 30 min. Before RNA preparation, three WT Col and five *cre1–12 ahk2–2 ahk3–3* (Col) seedlings were pooled and stored in RNAlater solution (Qiagen, Valencia, CA). Total RNA was extracted by using the RNeasy Plant Mini Kit (Qiagen). TaqMan RT-PCR reagents (Applied Biosystems) were used to synthesize double-stranded cDNA. Unlabeled gene-specific primers and 6-carboxy-fluorescein-labeled gene-specific TaqMan Minor Groove Binder probes were used for qRT-PCR with ABI prism 7700 (Applied Biosystems). The number of *ARR5* and *ARR15* (*Arabidopsis Response Regulator*) transcripts present in two biological replicates each of WT, and *cre1–12 ahk2–2 ahk3–3* seedlings, with or without BA, was determined three separate times. Fold induction of the *ARR5* and *ARR15* cytokinin primary-response gene transcripts was calculated relative to the *SHORT ROOT* (*SHR*) transcript (24), according to the manufacturer's instructions (ABI Prism 7700 Sequence Detection System, User Bulletin #2). Primer and probe sequences are available in *Supporting Materials and Methods*.

Flow Cytometry. Experiments were performed according to Shpak *et al.* (25), with some modifications. Plants were grown vertically on GM plates, with 1.8% (wt/vol) purified agar. Whole roots of 33-day-old plants were finely chopped in 0.5 ml of ice-cold extraction buffer [15 mM Hepes/1 mM EDTA/80 mM KCl/20 mM NaCl/300 mM sucrose/0.5% (vol/vol) Triton X-100/0.5 mM spermine/0.1% (vol/vol) 2-mercaptoethanol), passed through 33-μm nylon mesh, and centrifuged at 3,000 × *g* for 1 min. The pellet was resuspended in 100 μl of staining buffer (1/10,000 dilution of SYBR green I (Molecular Probes)/50 μg/ml RNase A/3.7% (vol/vol) formaldehyde in the extraction buffer) and subjected to FACScan (Becton Dickinson) by using the FL2 channel with a photomultiplier voltage of 300 V.

Results

Expression Patterns of Cytokinin Receptor Genes. RNA gel blot hybridization experiments confirmed that the three CRE-family genes have distinct expression patterns (Fig. 1*A*). *CRE1* expression was highest in the root and low in rosette leaves. *AHK2* was expressed to about the same degree in rosette leaves and the root. *AHK3* expression was highest in rosette leaves, moderate in the root, and low in the silique. Expression of all three cytokinin receptor genes was detected in flowers. Differential expression of the cytokinin receptor genes in root and shoot tissue was confirmed by expression of the *GUS* reporter gene directed by regulatory sequences from *CRE1*, *AHK2*, or *AHK3*. CRE1::GUS activity was high in the root, moderate in the inflorescence stems and pedicels, and low in the leaves (Fig. 1 *B* and *E*). AHK2::GUS activity was high in leaf veins, petioles, inflorescence stems, flowers, and siliques, and moderate in the roots (Fig. 1 *C* and *F*). AHK3::GUS was expressed ubiquitously in root and shoot tissues including leaves, inflorescence stems, and flowers (Fig. 1 *D* and *G*).

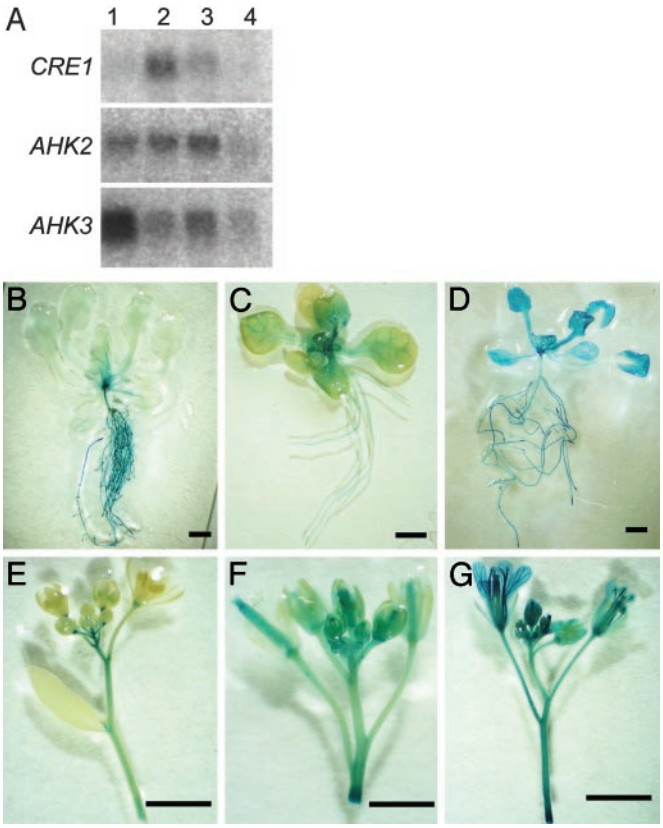

Fig. 1. Expression patterns of the *CRE*-family genes. (*A*) RNA gel blot hybridizations (5 μg of RNA) from different tissues of WT plants, probed with gene-specific probes for *CRE1*, *AHK2*, or *AHK3*. 1, rosette leaves; 2, roots; 3, floral bunches; 4, siliques. (*B*) Expression of the GUS reporter gene under control of regulatory sequences from cytokinin-receptor genes. (*B* and *E*) CRE1::GUS activity. (*C* and *F*) AHK2::GUS activity. (*D* and *G*) AHK3::GUS activity. (Bars = 2 mm.)

Expression of Cytokinin Receptor Genes in the Mutant Background. Multiple independent T-DNA insertion alleles for all members of the *Arabidopsis* CRE cytokinin receptor family (*AHK2*, *AHK3*, and *CRE1*) were identified (Fig. 2). To determine whether full-length transcripts of *CRE1*, *AHK2*, and *AHK3* were present in the T-DNA insertion mutants, RT-PCR analysis was performed on RNA prepared from plants carrying a single mutation in the CRE-family genes by using gene-specific primers flanking the T-DNA insertion site (see Fig. 10, which is published as supporting information on the PNAS web site). Even

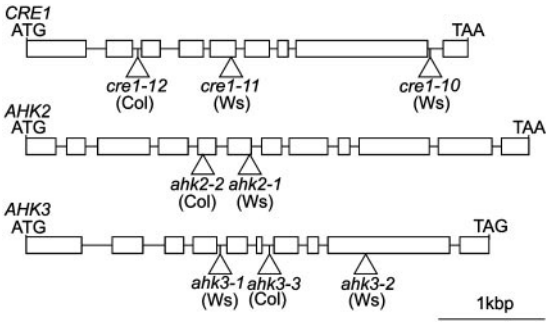

Fig. 2. Description of the *CRE1*, *AHK2*, and *AHK3* T-DNA insertion alleles. Boxes represent exons; horizontal bars, introns; and triangles, T-DNA integration sites.

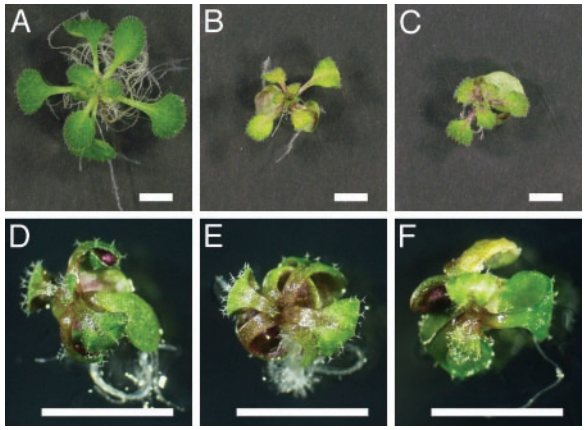

Fig. 3. Phenotype of the triple mutant grown with or without *t*-zeatin. (*A–C*) Thirteen-day-old WT seedlings from the ecotype Col. (*D–F*) Thirteen-day-old *cre1–12 ahk3–3 ahk2–2* (Col) triple mutants. Plants were grown on plates with 0 ng/ml (*A* and *D*), 100 ng/ml (*B* and *E*), 1,000 ng/ml *t*-zeatin (*C* and *F*). (Bars = 5 mm.)

with saturating numbers of PCR cycles, the full-length transcripts of *CRE1* and *AHK2* were not detected in *cre1–12* and *ahk2–2* mutants, respectively (Fig. 10*A*). The full-length transcript of *AHK3* was not detected in the *ahk3–3* mutant with the same number of PCR cycles that yielded near-saturating amplification of *AHK3* in WT plants (Fig. 10*A*). However, the transcript was detected with a greater number of PCR cycles (data not shown). The full-length transcripts of *CRE1*, *AHK2*, or *AHK3* were not detected in *cre1–10* or *cre1–11, ahk2–1*, *ahk3–1*, or *ahk3–2* mutants, respectively (data not shown). Recovery of *Arabidopsis* plants containing multiple independent T-DNA insertion alleles for each of the CRE-family genes, lacking expression of a full-length transcript, indicates that these T-DNA insertion mutants are null alleles. The greatly reduced level of *AHK3* expression detected in the *ahk3–3* mutant is unlikely to contribute to cytokinin signaling, because the cytokinin-response phenotypes of the *ahk3–3* mutant are the same as those observed for the *ahk3–1* and *ahk3–2* null mutants. Likewise, the cytokinin-response phenotypes of the *cre1–12 ahk3–3* and *ahk2–2 ahk3–3* double mutants are the same as those observed for the *cre1–10 ahk3–1* and *ahk2–1 ahk3–1* double mutants, respectively.

Overall Appearance of Cytokinin Receptor Mutants. When grown on soil, plants with a single mutation in *AHK2*, *AHK3*, or *CRE1* grew normally (see Fig. 11, which is published as supporting information on the PNAS web site). When grown on soil, plants carrying the *cre1–12 ahk2–2*, *cre1–12 ahk3–3* (Fig. 11), *cre1–10 ahk2–1*, and *cre1–10 ahk3–1* (not shown) mutations exhibited no noticeable phenotype. The *ahk2–2 ahk3–3* (Fig. 11) and *ahk2–1 ahk3–1* (not shown) double mutants had smaller leaves and shorter stems than did the WT plants. This result indicates that *AHK2* and *AHK3* functions dominate in the shoot. Roots of double mutants for any mutant combination were normal. Surprisingly, triple mutants were recovered in both the Ws background (*cre1–10 ahk2–1 ahk3–1*, and *cre1–11 ahk2–1 ahk3–1*) (not shown) and the Col background (*cre1–12 ahk2–2 ahk3–3*) (Figs. 3*D* and 11), indicating that seeds can germinate and seedlings can grow for a limited period without any of the *CRE-family* genes being expressed. The shoot and root growth of triple mutants was very slow, and leaf numbers were decreased (see below). The triple mutants occasionally produced an inflorescence stem with abnormal and nonfunctional flowers, but did not produce seeds. Supplementing media with 1 μg/ml *t*-zeatin

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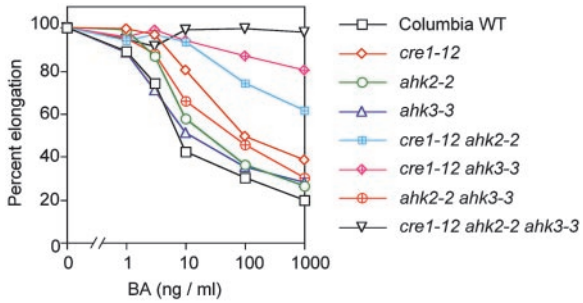

Fig. 4. Elongation of roots of cytokinin-receptor mutants in the presence of increasing concentrations of BA. Root length of each genotype without cytokinin was set at 100%. Lengths of roots in the absence of BA were: WT, 2.58 ± 0.29 (mean ± SD); *cre1–12*, 2.65 ± 0.32; *ahk2–2*, 2.18 ± 0.28; *ahk3–3*, 2.81 ± 0.38; *cre1–12 ahk2–2*, 2.57 ± 0.38; *cre1–12 ahk3–3*, 3.01 ± 0.35; *ahk2–2 ahk3–3*, 2.69 ± 0.35; and cre1–12 *ahk2–2 ahk3–3*, 0.65 ± 0.09.

severely inhibited the growth of WT seedlings but did not affect the growth of triple mutants (Fig. 3), suggesting that the triple mutants lack a mechanism for cytokinin perception. The insensitivity of the triple mutants to cytokinins was verified with several cytokinin-response assays, as shown below.

Cytokinin Sensitivity in the Root Growth Assay. Exogenous cytokinins normally inhibit root elongation. As previously reported (8), mutations in *CRE1* caused a reduced sensitivity to cytokinin (*cre1–12*, Fig. 4; *cre1–10* and *cre1–11*, not shown). The *ahk2–2* or *ahk3–3* mutants exhibited normal or slightly reduced sensitivity (Fig. 4). Additive effects were seen in the double mutants; that is, *cre1–12 ahk2–2*, *cre1–12 ahk3–3*, and *ahk2–2 ahk3–3* double mutants were less sensitive to cytokinin than was either single mutant (Fig. 4). Similarly, *cre1–10 ahk2–1* and *cre1–10 ahk3–1* double mutants were less sensitive than was either single mutant (not shown). The triple mutant had a shorter root with respect to WT plants, and the root length was not affected by BA up to 1 μg/ml. These data indicate that CRE1, AHK2, and AHK3 have redundant functions in cytokinin signaling in roots.

Cytokinin Sensitivity in the Adventitious Root Formation Assay. Cytokinins normally inhibit adventitious root formation near the cut end of hypocotyls (26). The *cre1–12* and *ahk3–3* single mutants were less sensitive to cytokinins in the adventitious root formation assay, whereas the *ahk2–2* mutant exhibited a normal sensitivity (Fig. 5). The effect of mutations in both *CRE1* and *AHK3* was synergistic; that is, *cre1–12 ahk3–3* roots were completely resistant to all cytokinin concentrations tested (up to a nonphysiological concentration of 3 μg/ml *t*-zeatin) (Fig. 5). These data indicate that CRE1 and AHK3 are key regulators of

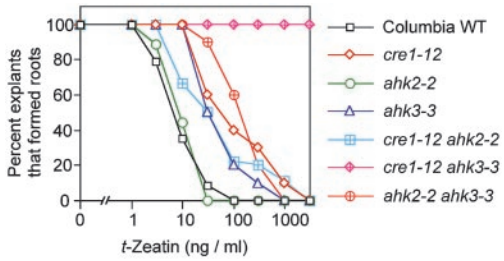

Fig. 5. Cytokinin inhibition of adventitious root formation in cytokinin-receptor mutants. The percent of explants per genotype producing adventitious roots on increasing concentrations of *t*-zeatin is presented. At least eight plants were used for each data point.

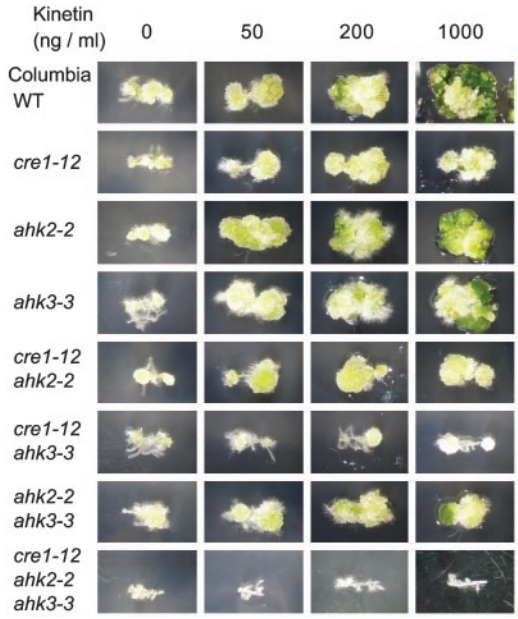

Fig. 6. Induction of callus formation on hypocotyl segments, from cytokinin-receptor mutants, on different concentrations of kinetin.

cytokinin-induced inhibition of adventitious root formation in *Arabidopsis*.

Cytokinin Sensitivity in the Callus Induction Assay. Cytokinins normally stimulate cell division and greening of calli (1). Similar to previously published work (8), cytokinin-induced cell division and greening of hypocotyl-derived calli were partially inhibited in *cre1–12* mutants (Fig. 6), as well as in *cre1–10* and *cre1–11* mutants (data not shown). The *ahk2–2* and *ahk3–3* mutants responded normally to cytokinin in this assay (Fig. 6). Similar results were seen with *ahk2–1*, *ahk3–1*, and *ahk3–2* mutants (data not shown). Mutations in *AHK2* and *AHK3*, in combination with a mutation in *CRE1,* enhanced the effect of the *cre1–12* mutation (Fig. 6). Finally, the triple cytokinin receptor mutant showed no significant response in this assay (Fig. 6). These results indicate that CRE1, AHK2, and AHK3 have redundant function in callus induction.

Cytokinin Sensitivity in the Shoot Formation Assay. Cytokinins induce shoot formation and inhibit root formation on calli (27). The *cre1–12*, *ahk2–2*, and *ahk3–3* single mutants exhibited normal or slightly reduced sensitivity to cytokinins in a shoot induction assay (see Fig. 12, which is published as supporting information on the PNAS web site). Similar results were seen for the *cre1–10*, *cre1–11*, *ahk2–1*, *ahk3–1*, and *ahk3–2* single mutants (data not shown). Additive effects were observed for all CRE-family mutant combinations (Fig. 12). This result indicates that CRE1, AHK2, and AHK3 have redundant functions in cytokinin-induced shoot formation.

Cytokinin Induction of Primary-Response Genes Is Absent in the Triple Mutant. Cytokinins normally induce the transcription of type A response regulator genes in *Arabidopsis* (28, 29). To determine whether induction of these cytokinin primary-response genes was compromised in the triple mutant, reverse transcription, and real-time quantitative PCR analysis was performed on RNA prepared from WT and *cre1–12 ahk2–2 ahk3–3* triple mutants, before and after a 30-min cytokinin treatment. Cytokinin treatment of WT seedlings induced transcription of the *ARR5* and *ARR15* transcripts by ≈14- and 13-fold, respectively (Fig. 7).

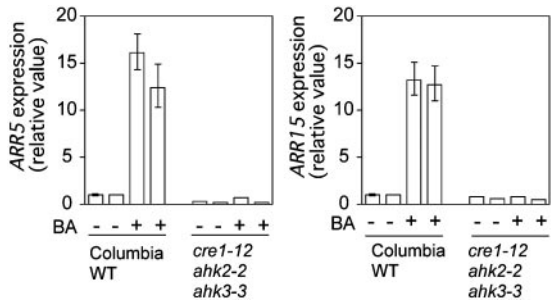

Fig. 7. Induction of cytokinin primary-response genes in the triple mutant. Fold induction of the *ARR5* and *ARR15* (*Arabidopsis Response Regulator*) transcripts in WT (Col) and the *cre1–12 ahk3–3 ahk2–2* (Col) triple mutant, in response to BA, is presented. Results are based on real-time quantitative PCR analysis, and normalized to the *SHR (SHORT ROOT*) transcript level.

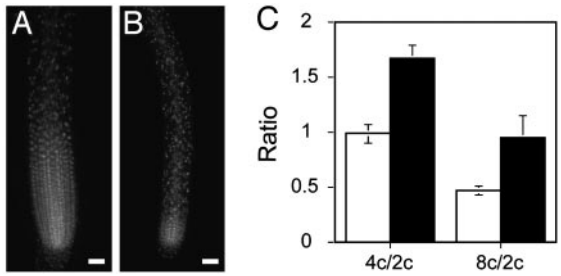

Fig. 9. Root meristem activity of the triple mutant. (*A* and *B*) One microgram per milliliter 4′,6-diamidino-2-phenylindole staining of root cells of WT (Col) (*A*) and *cre1–12 ahk2–2 ahk3–3* (Col) triple mutant (*B*). (Bars = 50 μm.) (*C*) tetraploid (4C/2C) and octaploid (8C/2C) ratios ± standard deviation (n = 4). Open column, WT; filled column, *cre1–12 ahk2–2 ahk3–3* mutant.

Cytokinin treatment of the triple mutant produced no change in *ARR5* or *ARR15* transcript levels (Fig. 7).

The Size and Activity of SAMs Are Decreased in the Triple Mutant. The diameter of the SAM was almost three times smaller in the triple mutant (29 ± 7 μm; n = 5) when compared to WT (82 ± 7 μm; n = 9) (Fig. 8). Fewer cell layers were seen in the SAM of triple mutants, as well as fewer cells per layer. One function of the SAM is to produce leaf primordia. Typically, plants with an enlarged SAM have a shorter interval of leaf production, or more rapid plastochron, than plants with a smaller SAM (5, 30, 31). As expected from the reduced SAM, the triple mutants had a prolonged plastochron with respect to WT plants. The leaf number of the *cre1–12 ahk2–2 ahk3–3* (Col) triple mutants was 4.0 ± 0.0 (mean ± standard deviation, n = 7) after 11 days of culture, whereas that of WT (Col) was 9.13 ± 0.83 (n = 7). The leaf numbers of single and double mutants were not significantly different from those of WT.

The size and activity of the root apical meristem of the triple mutant were also decreased with respect to WT plants (Fig. 9). The reduction in activity was seen in fluorescence-activated cell sorting experiments of root cells whose DNA had incorporated SYBR green I dye (see Fig. 13, which is published as supporting information on the PNAS web site). As shown in Fig. 9*C*, the triple mutant had a reduced diploid (2C) content of DNA with respect to WT plants, indicating that root cells of the triple mutant are delayed in the transition from $G_2 \rightarrow M$ phase of the cell cycle.

Discussion

The main purpose of this study was to determine whether CRE-family members are the only cytokinin receptors in *Arabidopsis* by identifying a plant lacking expression of all three genes, and noting any developmental consequences, should this plant be viable. Indeed, plants homozygous for a T-DNA insertion in *CRE1*, *AHK2*, and *AHK3* were recovered. The severe developmental abnormalities of these plants confirm that cytokinins are key regulators of plant growth and development. However, the phenotype of the triple mutant calls into question the longstanding belief that cytokinins are essential plant hormones, because an embryo is formed and a seedling produced without expression of the three known cytokinin receptors. It should be noted that our results do not eliminate the possibility that additional yet-unknown cytokinin receptors function in gametogenesis and embryogenesis.

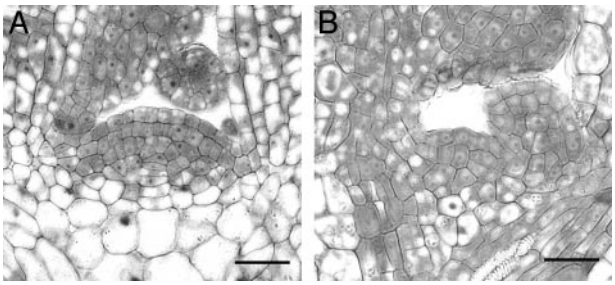

Fig. 8. Microscopic analysis of median longitudinal sections from the SAM of WT (Col) (*A*) and the *cre1–12 ahk2–2 ahk3–3* (Col) mutant (*B*). (Bar = 25 μm.)

Functions of CRE-Family Genes in the Root. The resistance of *cre1* mutants to cytokinin-induced inhibition of root growth and adventitious root formation indicates that CRE1 functions dominate in the root. Mutations in either *AHK2* or *AHK3* had only minor effects on cytokinin-induced inhibition of root growth. However, either of these mutations in combination with the *cre1* mutation enhanced the effect of *cre1* mutation, suggesting that AHK2, AHK3, and CRE1 have redundant functions in the root. The important roles cytokinins play in root development can best be seen in the triple mutant, which has shorter, narrower roots than do WT plants (data not shown).

The size and activity of the root apical meristem were markedly reduced in the triple mutant. Cytokinins were first discovered for their role in promoting cell division (1) and have since been implicated in stimulating both the $G_1 \rightarrow S$ phase transition (32) as well as the $G_2 \rightarrow M$ phase transition of the cell cycle (33). The reduced meristem activity seen in the triple mutant roots seems to be a consequence of a delay in the $G_2 \rightarrow M$ phase, as demonstrated by FACS experiments. Interestingly, a sharp increase in the levels of zeatin, zeatin riboside, and zeatin riboside-5′-monophosphate was reported in tobacco cell cultures at the $G_2 \rightarrow M$ phase transition (34).

Our data are consistent with a role for cytokinins as positive regulators of root apical meristem activity. This is inconsistent with the findings of Werner *et al.* (35, 36), who demonstrated that over-expression of several members of the *Arabidopsis* cytokinin oxidase family led to an increase in root meristem size and activity. A possible explanation for this difference is to assume that cytokinins have two opposing effects on root growth, one inhibitory and one stimulatory. An inhibitory effect on root elongation could be mediated by cytokinin-induced ethylene production, because it is known that cytokinins induce ethylene production, ethylene inhibits root growth, and roots of ethylene-resistant mutants are also resistant to cytokinins (37). Another role for cytokinins in roots could be as a stimulator of cell division. Most likely, cell-division defects in the root occur only when cytokinin signaling is severely inhibited, as seen in the triple mutant.

***CRE1, AHK2,* and *AHK3* Functions in the Shoot.** The observation that *ahk2 ahk3* double mutants have shorter inflorescence stems and smaller leaves than do WT plants, yet normal root growth, indicates that AHK2 and AHK3 functions dominate in the shoot. The severely stunted growth of the aerial portions of triple cytokinin receptor mutants indicates a redundant function for CRE1 in the shoot as well. Histological analyses showed that the SAM of the triple mutant was 3-fold smaller than normal. The organization of the SAM also appeared to be disrupted, because only the outermost layer of the tunica (L1) was recognizable. The interval of leaf formation was prolonged in the triple mutant, providing evidence of reduced SAM activity. Thus, our results support the finding of Werner *et al.* (35, 36) that cytokinins play a positive role in the SAM.

CRE1, AHK2, and AHK3 Function in *de Novo* Organ Formation. Since the late 1950s, scientists have recognized the ability of relative concentrations of the hormones cytokinin and auxin to induce plant cells to form particular tissues: undifferentiated callus, shoot structures, root structures, or a whole plant (21, 27). The ability to respond appropriately to cytokinin in an organ induction assay was retained in the *cre1*, *ahk2*, and *ahk3* single mutants, suggesting redundant functions for the CRE-family members in organ formation. Among the single mutants, callus formation was most compromised in the *cre1* mutant, indicating that CRE1 may play an important role in the process of dedifferentiation. The ability to appropriately respond to cytokinin in callus and shoot induction assays was lost in all three double mutant combinations, indicating that no single cytokinin receptor is sufficient for organ formation. Strikingly, the triple mutant did not respond to cytokinins at all, indicating that there may be no other cytokinin receptors that function in cell division and differentiation under tissue culture conditions.

What Is the Role of Cytokinins in the Formation of a Basic Vegetative Body Plan? The complete lack of cytokinin responses in the triple mutant, including the absence of cytokinin primary-response gene induction, could suggest that no other mechanism for cytokinin sensing exists in these plants. The retarded growth and sterility of the triple mutants indicate that cytokinins are very important growth regulators. In light of the inability of the triple mutant to form organs in tissue culture, that these plants can germinate and produce the basic plant organs induced *in vitro* by cytokinin and auxin is surprising. One possible explanation for this result is that, despite the well documented requirement for cytokinin in organogenesis during tissue culture, cytokinin-mediated regulation may not be required *in planta* for the formation of a basic vegetative body plan. Less radical interpretations include that there is another cytokinin receptor important for the earliest stages of plant development, or that the cytokinin production and responses of maternal tissues are sufficient for gametogenesis, embryogenesis, and germination of diploid offspring.

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