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Arabidopsis Histidine Kinase CKI1 Acts Upstream of HISTIDINE PHOSPHOTRANSFER PROTEINS to Regulate Female Gametophyte Development and Vegetative Growth [C] [W]

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Cytokinin signaling is mediated by a multiple-step phosphorelay. Key components of the phosphorelay consist of the histidine kinase (HK)-type receptors, histidine phosphotransfer proteins (HP), and response regulators (RRs). Whereas overexpression of a nonreceptor-type HK gene *CYTOKININ-INDEPENDENT1* (*CKI1*) activates cytokinin signaling by an unknown mechanism, mutations in *CKI1* cause female gametophytic lethality. However, the function of *CKI1* in cytokinin signaling remains unclear. Here, we characterize a mutant allele, *cki1-8*, that can be transmitted through female gametophytes with low frequency (~0.17%). We have recovered viable homozygous *cki1-8* mutant plants that grow larger than wild-type plants, show defective megagametogenesis and rarely set enlarged seeds. We found that *CKI1* acts upstream of *AHP* (*Arabidopsis HP*) genes, independently of cytokinin receptor genes. Consistently, an *ahp1,2-2,3,4,5* quintuple mutant, which contains an *ahp2-2* null mutant allele, exhibits severe defects in megagametogenesis, with a transmission efficiency of <3.45% through female gametophytes. Rarely recovered *ahp1,2-2,3,4,5* quintuple mutants are seedling lethal. Finally, the female gametophytic lethal phenotype of *cki1-5* (a null mutant) can be partially rescued by *IPT8* or *ARR1* (a type-B *Arabidopsis* RR) driven by a *CKI1* promoter. These results define a genetic pathway consisting of *CKI1*, *AHPs*, and type-B *ARRs* in the regulation of female gametophyte development and vegetative growth.

INTRODUCTION

The plant phytohormone cytokinin is a key growth regulator involved in the regulation of a wide range of developmental processes, including root and shoot growth, photomorphogenesis, flowering timing, senescence, and seed development (Davies, 1995; Mok and Mok, 2001). It is generally believed that cytokinin executes these physiological activities by regulating cell division and cell differentiation through a two-component system (TCS) signaling pathway (Hwang et al., 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005; Müller and Sheen, 2007). Key components of the TCS have been well defined by genetic and biochemical studies. In *Arabidopsis thaliana*, upon binding of cytokinin, three homologous *Arabidopsis* histidine kinases (AHK2, 3, and 4; AHK4 is also known as CYTOKININ RESPONSE1 [CRE1] or WOODEN LEG [WOL]) are activated by autophosphorylation at a conserved His residue. Subsequently, the phosphoryl group is transferred to *Arabidopsis* histidine phosphotransfer proteins (AHP1 through AHP5). AHP proteins were suggested to localize in the cytoplasm in the absence of cytokinin and, upon treatment with cytokinin, to translocate into the nucleus (Hwang and Sheen, 2001). However, a recent study demonstrated that AHP proteins maintained a constant nuclear/cytosolic distribution by balancing active transport and that this subcellular pattern was independent of cytokinin signaling (Punwani et al., 2010). Nevertheless, the nuclear-localized and phosphorylated AHPs presumably activate a class of MYB transcription factors, designated as type-B *Arabidopsis* response regulators (ARR). These type-B ARRs directly activate expression of type-A *ARR* genes that, in turn, negatively regulate type-B ARRs (Hwang et al., 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005; Müller and Sheen, 2007; Punwani et al., 2010). CRE1 is a bifunctional protein exerting kinase or phosphatase activities on AHPs in the presence or absence of cytokinin, respectively (Mähönen et al., 2006a). In addition, a pseudophosphotransfer protein, AHP6, was characterized as a negative regulator of cytokinin signaling (Mähönen et al., 2006b). This TCS-based phosphorelay establishes the framework of cytokinin signaling.

Mutations in the cytokinin receptor genes (*ahk2,3,4* triple mutant) (Inoue et al., 2001; Yamada et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006) and *AHP* genes (*ahp1,2,3,4,5* quintuple mutant) (Suzuki et al., 2002; Hutchison

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et al., 2006) cause a pleiotropic phenotype, including abolished sensitivity to cytokinins, a reduced meristematic activity, severely inhibited growth of roots and shoots, as well as impaired reproductive development with reduced fertility. However, given the critical role of cytokinin in plant growth and development, it is somewhat unexpected that *ahk2,3,4* triple mutants have little, if any, effects on gametogenesis and embryogenesis (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). In addition, an *ahp1,2,3,4,5* quintuple mutant can set seeds with reduced fertility (Hutchison et al., 2006), further challenging a possibly essential role of cytokinin signaling in plant growth and development.

In addition to the three cytokinin receptors, an additional *AHK* gene, *CYTOKININ INDEPENDENT1* (*CKI1*), has been implicated in cytokinin signaling (Kakimoto, 1996). *CKI1* was identified as a positive regulator that was capable of promoting shoot regeneration from hypocotyl explants in the absence of cytokinin and therefore was proposed to act in the early steps of cytokinin signaling (Kakimoto, 1996). The observation that *CKI1* induces a typical cytokinin response (Kakimoto, 1996; Hwang and Sheen, 2001; Zheng et al., 2006) and that CKI1 is able to dephosphorylate AHP1 and AHP2 (Nakamura et al., 1999) or phosphorylate AHP1, 2, 3, and 5 (Mähönen et al., 2006a) implies that *CKI1* plays an important role in cytokinin signaling. In addition, CKI1 has been found to interact with AHP2 and AHP3 in a yeast two-hybrid assay (Urao et al., 2000). However, CKI1 is incapable of binding to cytokinins (Yamada et al., 2001), suggesting that CKI1 is unlikely to act as a receptor. Loss-of-function mutations in *CKI1* impair megagametogenesis, indicating an essential role in female gametophyte development (Pischke et al., 2002; Hejátko et al., 2003). Analysis of *CKI1* RNA interference transgenic plants suggests that a reduced *CKI1* expression level causes defects in procambial cell maintenance in shoots (Hejátko et al., 2009). In spite of these progresses, the role of CKI1 in cytokinin signaling has long been questioned, largely owing to the lack of genetic evidence to link *CKI1* to the TCS components. Given the essential role of *CKI1* in female gametogenesis, it remains unclear if cytokinin signaling is directly involved in or is required for female gametophyte development. In addition, because the *cki1* mutant exhibits female gametophytic lethality, the functions of *CKI1* during postgametophytic growth and development remain unknown.

In this study, we present evidence that *CKI1*-activated signaling is dependent on *AHP* genes and that this regulatory scheme is essential for female gametophyte development. Moreover, *CKI1* also plays an important role during vegetative growth by regulating cell expansion. We demonstrate that the *CKI1-AHP*–dependent activity in female gametophyte development can be functionally substituted by the activation of cytokinin receptor-mediated signaling events. These results reveal an essential role of TCS-mediated signaling in plant growth and development.

RESULTS

Characterization of *cki1-7* and *cki1-8* Mutants

We previously reported the partial characterization of two mutants, *cki1-7* and *cki1-8* (Zheng et al., 2006). In these two mutants, an estradiol-inducible activation tagging vector was inserted 994 bp (*cki1-7*) and 498 bp (*cki1-8*) upstream of the putative translation start codon of *CKI1*, respectively (Figure 1A). In these two mutants, expression of *CKI1* was highly inducible by estradiol in a dose-dependent manner (see Supplemental Figure 1A online), which caused a typical cytokinin response, including the inhibition of shoot growth and primary root elongation, delayed leaf senescence induced by dark, and the induction of the cytokinin primary response genes *ARR6* and *ARR7* (see Supplemental Figures 1B to 1E online). The phenotypic strength is correlated with the *CKI1* expression levels induced by different concentrations of estradiol (see Supplemental Figures 1B and 1C online), mimicking wild-type plants treated with different concentrations of cytokinin. These gain-of-function mutant phenotypes could be recapitulated by overexpression of a *CKI1* or a *CKI1-green fluorescent protein* (*GFP*) transgene in wild-type plants, and the transgenic phenotype was correlated with the accumulation of CKI1-GFP fusion protein induced by estradiol (see Supplemental Figure 2 online).

In addition to the gain-of-function mutant phenotype induced by estradiol as highlighted above, the T-DNA insertion in *cki1-8* also caused severe abnormalities owing to the loss-of-function mutation (see below). Hereafter, we refer to mutant plants treated with estradiol as *CKI1*-OXi (for *CKI1* overexpression inducible). Most overexpression experiments described below were performed using the *cki1-7* mutant allele unless otherwise specified.

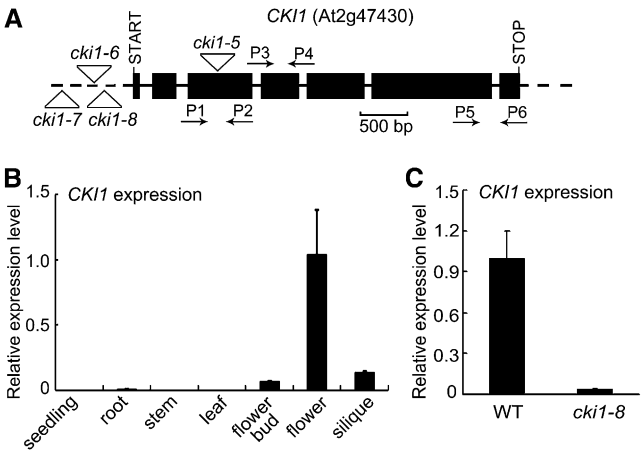

Figure 1. Expression of *CKI1* in Wild-Type and *cki1-8* Mutant Plants.

(A) A schematic map of the *CKI1* gene and *cki1* mutant alleles. Filled boxes and solid lines denote exons and introns, respectively. Untranscribed and untranslated regions are indicated as dashed lines. Putative translation start and stop sites, and positions of the T-DNA insertions in *cki1* mutant alleles are shown. Relative positions and orientation of primers (P1 through P6; see Supplemental Table 2 online for sequences) used for qRT-PCR are shown.

(B) Analysis of expression of *CKI1* in different organs and tissues of wild-type plants by qRT-PCR using primers P3 and P4 shown in **(A)**. Data presented are mean values of three biological repeats with standard deviations. Relative expression level of *CKI1* was normalized using *ACTIN7* (At5g09810) as an internal control.

(C) *CKI1* expression in wild-type and *cki1-8* flowers analyzed by qRT-PCR as described in **(B)**.

On the other hand, plants grown under noninductive conditions are referred to as *cki1-7* and *cki1-8*, which represent loss-of-function mutants by T-DNA insertional mutations.

Under normal growth conditions, *cki1-7* plants had no detectable abnormalities. The position of the T-DNA insertion in *cki1-8* was similar to that in *cki1-6*, which carried a T-DNA 587 bp upstream from the translation start codon and showed a female gametophytic lethal phenotype (Pischke et al., 2002). Consistent with this result, our initial attempts failed to recover *cki1-8* plants homozygous for the T-DNA insertion. To analyze the *cki1-8* phenotype more precisely, we performed reciprocal crosses between *CKI1*/*cki1-8* and wild-type plants. In *CKI1*/*cki1-8* plants, the transmission efficiency of the T-DNA insertion was 48.76% through male gametophytes (n = 252), suggesting that male gametogenesis was not affected by the *cki1-8* mutation. However, the T-DNA insertion was transmitted through female gametophytes at an extremely low frequency in *CKI1*/*cki1-8* plants (0.17%; n = 596). Note that *cki1-6*, which carried a T-DNA at a more distal location in the *CKI1* promoter than *cki1-8* (Figure 1A), did not appear to be capable of being transmitted through female gametophytes. This difference is most likely due to different sizes of F1 populations assayed (596 in *cki1-8* and 173 in *cki1-6*) (Pischke et al., 2002). These results suggest that *cki1-8* is not a null allele, and *cki1-8* homozygous plants indeed showed residual expression of *CKI1* (see below). Similar to the observation made in other *cki1* mutant alleles (Pischke et al., 2002; Hejátko et al., 2003), confocal laser scanning microscopy (CLSM) revealed that ~50% of ovules in *CKI1*/*cki1-8* siliques showed various defects, including collapsed, degenerate, or multinucleated female gametophytes (see Supplemental Figure 3 online). Collectively, these results indicate that the *cki1-8* mutation impairs female gametophyte development.

In correlation with the *cki1* mutant phenotype, a quantitative RT-PCR (qRT-PCR) analysis revealed that *CKI1* is predominantly expressed in flowers, followed by flower buds and siliques (Figure 1B). Expression of *CKI1* was barely detectable in vegetative tissues by qRT-PCR (Figure 1B), although a recent study revealed *CKI1* expression in specific tissues of stem vascular bundles (Hejátko et al., 2009). Whereas our qRT-PCR results are consistent with previous studies by in situ hybridization and reporter gene analyses (Pischke et al., 2002; Hejátko et al., 2003), we could not exclude the possibility that *CKI1* is expressed in certain vegetative tissues at a lower level. To test this possibility, we employed a highly sensitive method to analyze *CKI1* expression in wild-type seedlings. Poly(A) RNA was isolated and then used as a template for RT-PCR. Whereas expression of *WUSCHEL* (*WUS*), a meristem preferentially expressed gene (Mayer et al., 1998), was easily detected under the assay conditions, expression of *CKI1* was undetectable (see Supplemental Figure 4 online). The RT-PCR products were then subjected to DNA gel blot analysis using a *CKI1* cDNA fragment as a probe. Using a *CKI1* cDNA clone as a positive control in the PCR–DNA gel blot analysis, we found that this method could detect 1 fmol *CKI1* cDNA fragment added to the PCR reaction. However, no *CKI1* expression could be detected in wild-type seedlings under the assay conditions (see Supplemental Figure 4 online), suggesting that the *CKI1* expression level in vegetative tissues is under the detection limit of the current method.

Identification and Characterization of the *cki1-8* Homozygous Mutant

Because the *cki1-8* mutation can be transmitted through female gametophytes at low frequency, it should be possible to recover *cki1-8* plants. Because of its defective female gametophyte development, a *cki1-8* plant is expected to set few or no seeds. Therefore, we screened >1000 individual plants derived from self-pollinated *CKI1*/*cki1-8* plants by visual inspection, from which we recovered a plant with significantly reduced fertility. Genotyping revealed that this plant was indeed homozygous for the T-DNA insertion at the *CKI1* locus. We have been able to harvest ~15 seeds from this plant, from which we subsequently amplified the *cki1-8* mutant over several generations and have collected several thousand seeds for the experiments described below. Typically, a *cki1-8* plant produced ~10 to 40 seeds.

To evaluate the strength of the *cki1-8* mutation, we analyzed *CKI1* expression in wild-type and *cki1-8* plants by qRT-PCR and in situ RNA hybridization. Compared with expression in the wild type, expression of *CKI1* was 20-fold lower in *cki1-8* flowers, as revealed by qRT-PCR (Figure 1C). In mature female gametophytes, *CKI1* expression is confined mainly to the central cell nucleus and, to a lower level, to the egg cell nucleus (Pischke et al., 2002; Hejátko et al., 2003). However, we were unable to detect a similar signal by in situ RNA hybridization in *cki1-8* ovules (see Supplemental Figures 5A to 5C online), indicating a lower *CKI1* mRNA level in the mutant female gametophytes than in the wild-type gametophytes. These results indicate that *cki1-8* is a weak mutant allele with residual *CKI1* expression.

The *cki1-8* Mutant Phenotype during Vegetative Growth

When germinated and grown under both continuous white light and in the dark, *cki1-8* plants were larger than wild-type plants (Figures 2A and 2B). Quantitative analysis showed that *cki1-8* had longer primary roots than wild-type seedlings under both the light and dark conditions (Figures 2A and 2B). True leaves were initiated in *cki1-8* at a similar rate and timing as in wild-type seedlings. However, true leaves of *cki1-8* plants were larger than those of wild-type plants, both in length and width (Figures 3A and 3B).

To explore the cellular basis of the enlarged *cki1-8* leaves, we analyzed the cell size and the number of cells in the mutant in comparison with wild-type leaves. Cells in *cki1-8* leaves were larger than those in wild-type leaves (Figures 3C and 3D). However, similar cell numbers were observed in *cki1-8* and wild-type leaves (Figure 3E). These results suggest that *CKI1* may negatively regulate cell expansion in leaves. Note that our RT-PCR–DNA gel blot analysis was unable to detect *CKI1* expression in wild-type seedlings (see Supplemental Figure 4 online). Whereas *CKI1* may be expressed at an extremely low level in vegetative tissues, an alternative explanation could be that CKI1 protein synthesized in embryos and seeds, rather than *CKI1* mRNA, remained active for a specific window during postgerminative growth.

A recent study revealed that *CKI1* RNA interference transgenic plants showed defects in shoot procambial cell maintenance (Hejátko et al., 2009). In serial sections prepared from stems of

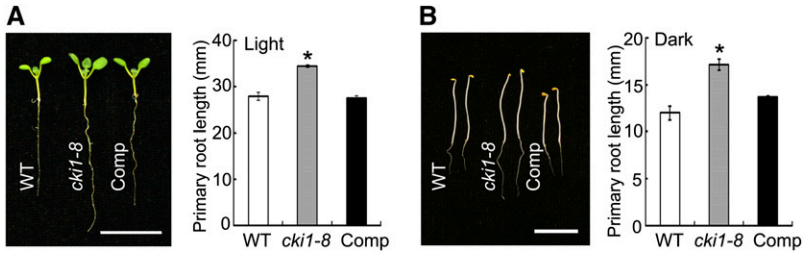

Figure 2. The *cki1-8* Mutant Phenotype.

(A) Left: Seven-day-old seedlings of the wild type, *cki1-8*, and *cki1-8* carrying a *CKI1*:*CKI1* transgene (Comp) germinated and grown under continuous white light. Right: Quantitative analysis of the primary root length of 7-d-old seedlings.
(B) Left: Five-day-old seedlings of wild-type, *cki1-8*, and *cki1-8* plants carrying a *CKI1*:*CKI1* transgene (Comp) germinated and grown in the dark for 5 d. Right: Quantitative analysis of the primary root length of 5-d-old seedlings.
Bar = 10 mm. Data presented are mean values of three independent experiments ($n > 20$ in each experiment) and standard deviations are given in the graphs. Asterisk indicates statistically significant differences compared with wild-type plants (Student's *t* test, $P < 0.01$).
[See online article for color version of this figure.]

cki1-8 plants ($n = 5$), vascular development appeared to be relatively normal compared with that of wild-type shoots (see Supplemental Figure 6 online; see also below). However, we could not exclude the possibility that analysis of additional samples or under certain assay conditions would be able to reveal specific phenotypes in *cki1-8* during shoot vascular development. On the other hand, transgenic plants expressing an antisense *CKI1* transgene were reported to show retarded vegetative growth under nutrition-restricted conditions (Glover et al., 2008). Under our assay conditions, no phenotype similar to that of the *CKI1* antisense transgenic lines was observed in *cki1-8* plants under both normal and nutrition-restricted conditions (Figures 2A and 2B; see Supplemental Figure 7 online).

The *cki1-8* Mutant Phenotype during Reproductive Development

The *cki1-8* plants could produce normal-looking flowers, of which both the number and the identity of the floral organs appeared to be normal (see Supplemental Figure 8A online). Scanning electron microscopy revealed that the initiation and development of the inflorescence meristem are normal (see Supplemental Figure 8B online). However, when the floral inflorescence meristem became inactive and flower production was terminated in wild-type plants, *cki1-8* plants continued to grow and produced new flowers or flower-like structures for an additional 3 to 5 weeks, leading to a substantially increased size of the mutant plant (Figure 4A). Flowers at the inflorescence tips were often transformed into carpel-like structures (Figure 4B). It has been proposed that a signal derived from developing fruits triggers the arrest of proliferative activity at the inflorescence meristems, thereby ceasing flower production on all inflorescence branches. This global proliferative arrest of the inflorescence meristem does not occur in the *male-sterile1* mutant or in wild-type plants when siliques are manually removed. The lack of the global proliferative arrest consequently results in an enlarged plant size and the formation of carpel-like structures in these plants (Hensel et al., 1994). Likewise, the *cki1-8* mutant phenotype during the flowering stage is attributed to a similar mechanism, owing to the formation of abortive fruits (see below).

To explore if the *cki1-8* reproductive phenotype is a secondary effect caused by defects in early development, we performed the following experiments. Wild-type and *cki1-8* plants were germinated and grown in the presence of various concentrations of estradiol. Upon treatment with estradiol, the *cki1-8* mutant phenotype was partially suppressed, and the seedling size was smaller than untreated *cki1-8* and was comparable to wild-type seedlings (see Supplemental Figure 9A online). We found that treatment with 10 nM estradiol resulted in *cki1-8* plants with a growth phenotype similar to that of wild-type seedlings under the assay conditions (see Supplemental Figures 9A and 9B online). A higher concentration of estradiol caused growth arrest in *cki1-8* plants (see Supplemental Figure 9A online). We then transferred the partially rescued *cki1-8* seedlings treated with 10 nM estradiol into soil and continued to grow these plants until maturation. Defective floral development was observed in these estradiol-treated *cki1-8* plants, similar to that in untreated *cki1-8* plants (see Supplemental Figure 9C online; Figure 4B). These results suggest that abnormal reproductive development in *cki1-8* is not directly related to defects in early seedling development.

In *cki1-8* flowers, pollen development was normal, as revealed by Alexander staining (see Supplemental Figure 8C online). However, >98% of ovules showed defective female gametophyte development, as determined by CLSM. Consequently, remarkably smaller siliques were formed in *cki1-8* plants than in the wild type (Figure 4C), and these mutant siliques produced no seeds in most cases, or occasionally set seeds (Figure 4D). *cki1-8* seeds were larger than wild-type seeds (Figure 4E), a phenotype similar to that observed in the *ahk2*,*3*,*4* triple mutant (Riefler et al., 2006) and the *ahp1*,*2*,*3*,*4*,*5* quintuple mutant (Hutchison et al., 2006) as well as of transgenic plants overexpressing cytokinin oxidase genes (Werner et al., 2003). This result suggests that *CKI1* may play a similar role as cytokinin and the cytokinin signaling components in seed development. Taken together, these results suggest that, whereas flower development and male gametogenesis remain relatively normal, female

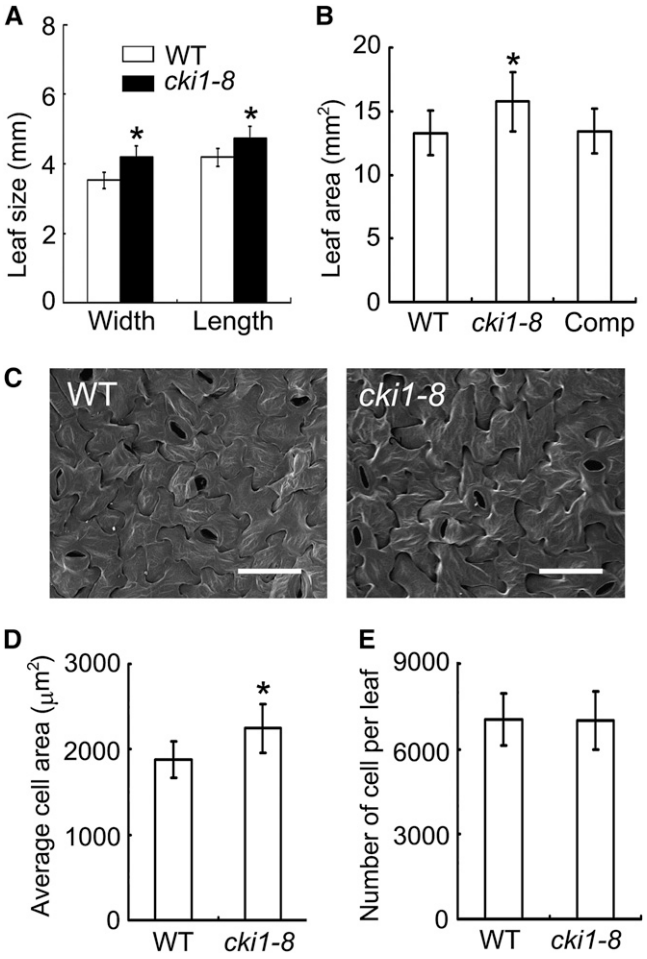

Figure 3. The Cellular Phenotype of the *cki1-8* Leaves.

(A) Size comparison of wild-type and *cki1-8* leaves. Data presented are mean values of two independent experiments ($n > 20$ in each experiment) and standard deviations are indicated by bars.

(B) The leaf blade area (in mm^2) of wild-type and *cki1-8* leaves. Data presented are mean values of two independent experiments ($n > 15$; collected from different seedlings), and standard deviations are indicated by bars.

(C) Scanning electron microscopy analysis of wild-type and *cki1-8* leaves. Images show abaxial leaf surfaces. Bar = 50 μm.

(D) Average size of abaxial epidermal cells. Data presented are mean values of two independent experiments (nine and five leaves collected from different seedlings were measured in two experiments, respectively), and standard deviations are indicated by bars.

(E) The number of abaxial epidermal cells. Data presented are calculated by dividing leaf area (shown in **[B]**) with the average cell size (shown in **[D]**). Standard deviations are indicated by bars.

All data presented were obtained using the second true leaves derived from wild-type, *cki1-8*, and *cki1-8* seedlings carrying a *CKI1*:*CKI1* transgene (Comp; **[B]** only) germinated and grown under continuous white light for 14 d. Asterisks in **(A)**, **(B)**, and **(D)** indicate statistically significant differences compared with wild-type plants (Student's *t* test, $P < 0.01$).

gametogenesis and seed development are impaired in the *cki1-8* mutant.

All of these developmental abnormalities of *cki1-8* could be fully rescued by a *CKI1*:*CKI1* transgene. In the 18 independent transgenic lines analyzed, we were able to recover fully fertile *cki1-8* plants carrying a *CKI1* transgene in all 18 transgenic lines, which showed normal growth and development throughout the life cycle (Figures 2A, 2B, 3B, 4A, and 4C to 4E). Similarly, 17 out of 21 analyzed transgenic lines carrying a *CKI1*:*CKI1-GFP* transgene were also able to fully rescue the *cki1-8* mutant phenotype, indicating that the CKI1-GFP fusion protein is functional in planta.

cki1-8 Responds Normally to Cytokinin

Previous studies showed that overexpression of *CKI1* mimicked cytokinin effects (Kakimoto, 1996; Hwang and Sheen, 2001; Zheng et al., 2006) (see Supplemental Figures 1 and 2 online). These observations prompted us to investigate the cytokinin response in *cki1-8*. Cytokinin inhibited primary root growth in the wild type, and this inhibitory effect was nearly unaltered in *cki1-8* seedlings (see Supplemental Figure 10A online). Cytokinin specifically eliminates the protoxylem cell linage in root vascular tissues (Mähönen et al., 2006b). In the absence or presence of cytokinin, root protoxylem development in *cki1-8* showed a pattern similar to that of the wild type (see Supplemental Figure 10B online). Moreover, cytokinin delayed dark-induced leaf senescence in wild-type and *cki1-8* leaves by a similar degree (see Supplemental Figure 10C online). In a shoot formation assay, *cki1-8* hypocotyl explants had a similar response to cytokinin as the wild type (see Supplemental Figure 10D online). Lastly, cytokinin-induced expression of *ARR6* and *ARR7* was comparable in the wild type and *cki1-8* (see Supplemental Figure 10E online). Collectively, these results indicate that *cki1-8* has a similar response to cytokinin as wild-type plants.

The *CKI1*-Induced Cytokinin Phenotype Is Independent of *CRE1/WOL*

Because CKI1, a histidine kinase, is structurally and functionally similar to cytokinin receptors, we then asked if *CKI1* could genetically interact with *CRE1/WOL* in the regulation of cytokinin signaling. The *wol* mutant protein is incapable of binding cytokinin, thus exerting a dominant-negative effect and resembling the *ahk2*,*3*,*4* triple mutant phenotype (Mähönen et al., 2006a, 2006b). We generated a *CKI1*-OXi *wol* double mutant by crossing *cki1-7* and *wol* and then analyzed the *CKI1*-induced cytokinin phenotype of the double mutant. The *wol* mutation inhibited seedling growth and primary root elongation, and this inhibitory effect was reduced in the *CKI1*-OXi *wol* double mutant in the presence of estradiol (Figures 5A and 5B). When grown under higher concentrations of estradiol, *CKI1*-OXi *wol* exhibited a phenotype similar to that of *CKI1*-OXi (Figures 5A and 5B). In particular, *CKI1*-OXi *wol* had longer primary roots than *wol* at all tested concentrations of estradiol (Figure 5B), indicating that overexpression of *CKI1* is able to partially complement the *wol* phenotype in primary root growth.

The *wol* mutation converts all cell files of the root vascular cylinder into protoxylem, whereas cytokinin exerts an opposite

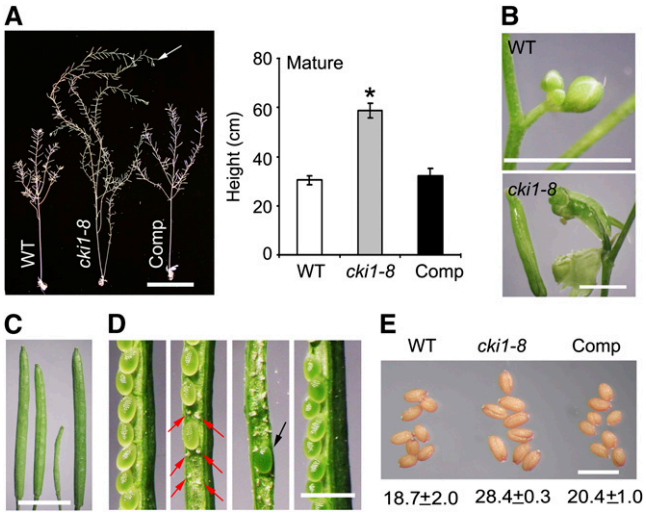

Figure 4. The Reproductive Development Phenotype of the *cki1-8* Mutant.

(A) Left: Ten-week-old plants of the wild type, *cki1-8*, and *cki1-8* carrying a *CKI1*:*CKI1* transgene (Comp) germinated and grown under continuous white light. Note that *cki1-8* continues to produce flowers (indicated by an arrow). Bar = 10 cm. Right: Quantitative analysis of the height of 10-week-old plants (n = 8). Standard deviations are indicated by bars. Asterisk indicates statistically significant differences compared with wild-type plants (Student's t test, $P < 0.01$).
(B) Inflorescences derived from 8-week-old (wild type) or 10-week-old (*cki1-8*) plants. Bar = 2 mm.
(C) Siliques (from left to right) of the wild type, *CKI1*/*cki1-8*, *cki1-8*, and *cki1-8* carrying a *CKI1*:*CKI1* transgene. Bar = 5 mm.
(D) Seed development in siliques. From left to right: wild type, *CKI1*/*cki1-8* (red arrows show abortive ovules), *cki1-8* (a rarely set seed indicated by a black arrow), and *cki1-8* carrying a *CKI1*:*CKI1* transgene. Bar = 1 mm.
(E) Increased seed weight in *cki1-8*. Average weight (μg/seed; at least 500 seeds were analyzed in each genotype) and standard deviations are given below the image. Bar = 1 mm.

effect by inhibiting protoxylem differentiation (Scheres et al., 1995; Mähönen et al., 2000, 2006b). *CKI1*-OXi roots treated with estradiol showed a phenotype similar to that of wild-type plants treated with cytokinin (Figure 5C; see Supplemental Figure 11A online). When treated with cytokinin in the absence of estradiol, the *cki1-7 wol* double mutant showed a phenotype similar to that of *wol*; both mutants contained protoxylem but lacked metaxylem (see Supplemental Figure 11A online). However, when treated with estradiol, 76% of *CKI1*-OXi *wol* roots (n = 21) did not have protoxylem cell files but contained metaxylem (Figure 5C). Therefore, overexpression of *CKI1* partially rescued the *wol* phenotype in xylem development.

In a shoot regeneration assay, while *wol* showed a reduced response to cytokinin (see Supplemental Figure 11B online), *CKI1*-OXi *wol* explants showed a similar phenotype as *CKI1*-OXi explants (Figure 5D). Moreover, expression of *ARR6* and *ARR7* was highly inducible in *CKI1*-OXi *wol* plants treated with estradiol (Figure 5E). Taken together, these results suggest that *CKI1* is able to induce cytokinin responses independently of *CRE1/WOL*.

The *CKI1*-Induced Cytokinin Phenotype Is Dependent on *AHP1-5*

The data presented above indicated that *CKI1* is capable of activating cytokinin signaling independently of *CRE1/WOL*. We next investigated if *AHP* genes are functionally required for *CKI1* activity. Previous studies indicated that single *ahp* mutants did not have a detectable phenotype, and a quintuple mutant *ahp1,2,3,4,5* showed various developmental defects and was insensitive to cytokinin (Hutchison et al., 2006). Therefore, we generated and analyzed a *CKI1*-OXi *ahp1,2,3,4,5* hextuple mutant. In the presence of estradiol, the hextuple mutant showed a phenotype indistinguishable from that of the *ahp1,2,3,4,5* mutant (Figures 6A and 6B), indicating that the *CKI1*-OXi gain-of-function phenotype requires *AHP* genes. In terms of xylem development, the estradiol-treated *CKI1*-OXi *ahp1,2,3,4,5* hextuple mutant showed a phenotype similar to the *ahp1,2,3,4,5* quintuple mutant, with overproliferated protoxylem and no differentiation of metaxylem (Figure 6C; see Supplemental Figure 11A online). This phenotype differs from the phenotype of *CKI1*-OXi roots, which lack the protoxylem cell lineage (Figure 5C). In contrast with *CKI1*-OXi, *CKI1*-OXi *ahp1,2,3,4,5* explants were incapable of regenerating shoots when treated with estradiol (Figure 6D). Moreover, *CKI1*-induced expression of *ARR6* and *ARR7* was substantially reduced in the *CKI1*-OXi *ahp1,2,3,4,5* hextuple mutant (Figure 6E). These results demonstrate that *AHP* genes are essential for *CKI1*-activated cytokinin signaling.

The Cytokinin Receptor Genes Are Not Required for Female Gametophyte Development

The data presented above indicate that *CKI1* acts upstream of *AHP* to regulate cytokinin signaling in a cytokinin receptor-independent manner. Because gametophyte development appears to be unaffected in various cytokinin receptor mutants (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), we reasoned that the *CKI1-AHP* pathway, rather than the cytokinin receptor-*AHP* pathway, may be required for female gametogenesis. To test this hypothesis, we first analyzed female gametogenesis in *wol* and *ahk2,3,4* mutants by CLSM analysis. No apparent abnormalities were observed in *wol* ovules and in ovules derived from *ahk2,3/+,4* (the T-DNA insertions were heterozygous at the *AHK3* locus) flowers that should produce 50% of *ahk2,3,4* haploid female gametophytes (see Supplemental Figure 12 online). These results are consistent with the observation made in the genetic analysis of the *ahk2/+,3,4* and *ahk2,3/+,4* mutants, in which the T-DNA insertion at the *AHK2* and *AHK3* loci is segregated as a typical Mendelian trait in a 3:1 ratio, respectively (Nishimura et al., 2004). Taken together, these results indicate that gametophyte development is not affected by the receptor triple mutations.

AHP Genes Play a Critical Role in Female Gametophyte Development

Given that *CKI1* and *AHP* act in a linear pathway and that *AHP* genes are essential for *CKI1* activity, one would expect that the *ahp1,2,3,4,5* quintuple mutation may also cause abnormal

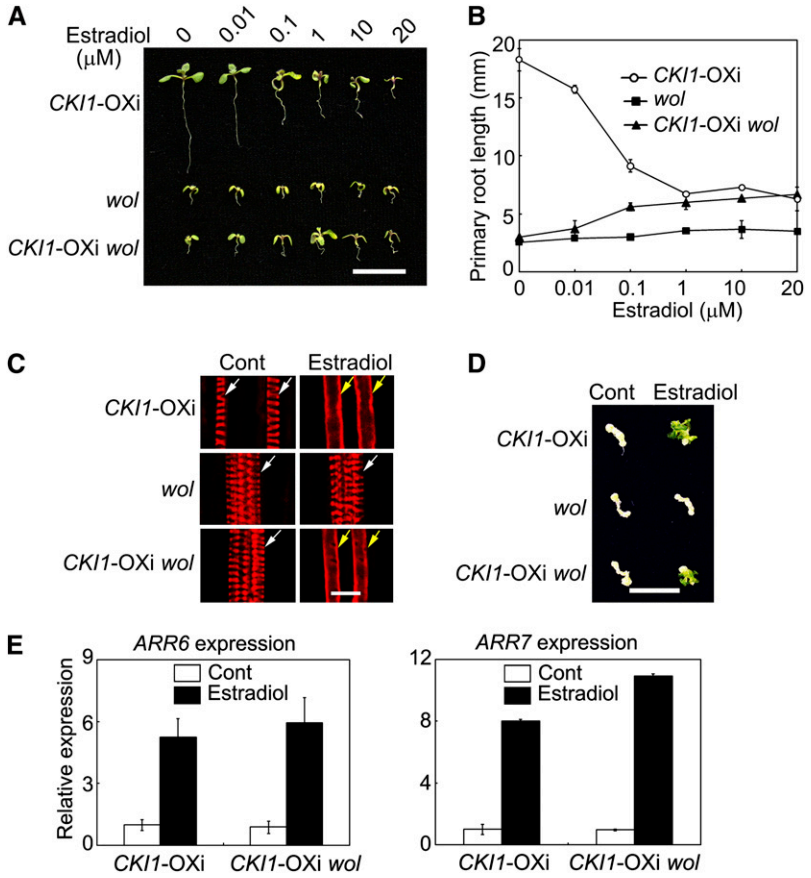

Figure 5. Overexpression of *CKI1* Partially Rescues the *wol* Mutant Phenotype.

(A) Two-week-old seedlings germinated and grown in the presence of various concentrations of estradiol as indicated on the top of the panel. Bar = 10 mm.

(B) Primary root length of *CKI1*-OXi, *wol*, and *CKI1*-OXi *wol* seedlings germinated and grown as described in **(A)**. Data presented are mean values of three independent experiments ($n > 15$ in each experiment) with standard deviations.

(C) Xylem development in *CKI1*-OXi, *wol*, and *CKI1*-OXi *wol* roots. Five-day-old seedlings were germinated and grown on germination medium (GM) containing DMSO (Control, Cont; dimethylsulfoxide, a solvent for estradiol) or 20 μM estradiol. The seedlings were stained with basic Fuchsin Red and then analyzed by confocal microscopy. White and yellow arrows denote protoxylem and metaxylem, respectively. Bar = 10 μm.

(D) Shoot regeneration assay of *CKI1*-OXi, *wol*, and *CKI1*-OXi *wol* hypocotyl explants treated with or without (Control, Cont) 20 μM estradiol. Bar = 10 mm.

(E) Expression of *ARR6* and *ARR7* as analyzed by qRT-PCR as described in Figure 1B. Total RNA prepared from 2-week-old seedlings treated with DMSO or estradiol (20 μM) for 12 h was used for qRT-PCR. Data presented are mean values of three biological repeats with standard deviations. Relative expression level of *ARR6* and *ARR7* was normalized using *ACTIN7* (At5g09810) as an internal control.

All *CKI1*-OXi *wol* plants are in the *cki1-7*/*cki1-7* background.

development of female gametophytes. Among the analyzed *ahp1*,*2*,*3*,*4*,*5* ovules, 38.2% ($n = 178$) showed severe defects in female gametophyte development at various stages, similar to those observed in *cki1* mutants (see Supplemental Figure 13). In wild-type flowers 48 h after emasculation, most female gametophytes (98%) were at FG7 (female stage 7; the developmental stages were defined according to Christensen et al. [1997]). However, at the same stage, 37.3% ($n = 110$) of normal-looking female gametophytes in *ahp1*,*2*,*3*,*4*,*5* siliques stayed at around FG5 to FG6, suggesting that the mutation also affects the progression of female gametogenesis. Moreover, in flowers derived from *ahp1*,*2*,*3*/+,*4*,*5* plants (the T-DNA insertions were heterozygous at the *AHP3* locus), 14.29% of ovules ($n = 70$) showed various defects, similar to those observed in *ahp1*,*2*,*3*,*4*,*5* flowers (Figure 7).

Similar to *cki1-8*, no abnormality was observed in pollen development of the *ahp1*,*2*,*3*,*4*,*5* mutant plants (see Supplemental Figure 14A online). Moreover, the number and identity of floral organs in the *ahp1*,*2*,*3*,*4*,*5* quintuple mutant appeared to be normal (see Supplemental Figure 14B online). Again, similar to *cki1-8*, the *ahp1*,*2*,*3*,*4*,*5* mutant plant produced enlarged seeds (Hutchison et al., 2006). Taken together, these results suggest that *CKI1* and *AHP* genes function similarly in the regulation of reproductive development.

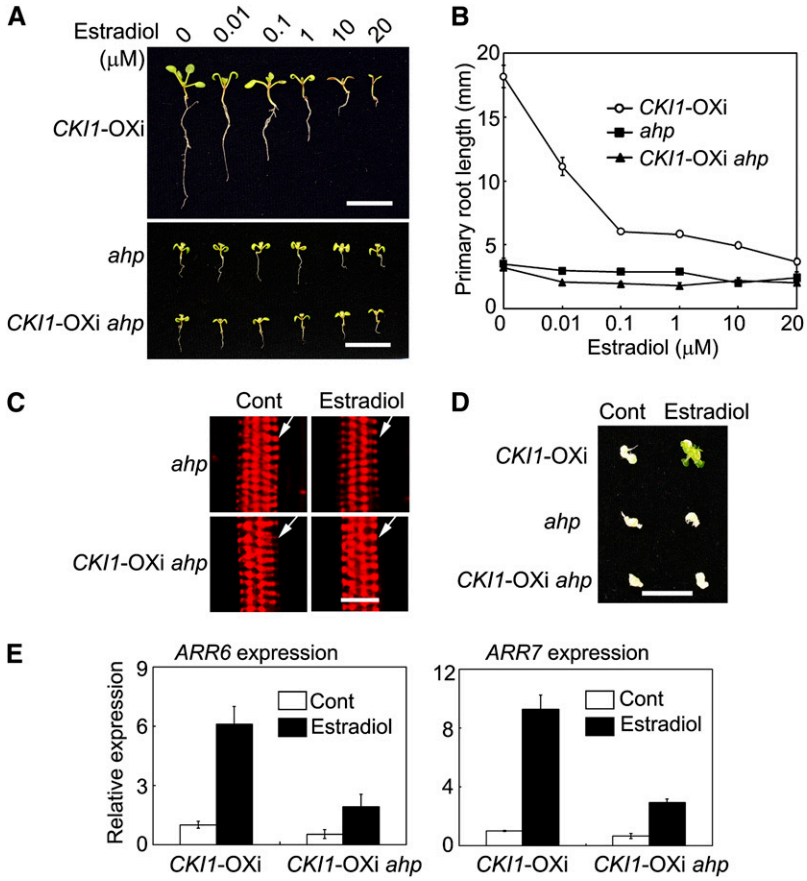

Figure 6. *CKI1*-Mediated Signaling Is Dependent on *AHP* Genes.

(A) Two-week-old seedlings germinated and grown in the presence of various concentrations of estradiol. Note that *ahp* in **(A)** through **(E)** denotes the *ahp1,2-1,3,4,5* quintuple mutation. Bar = 10 mm.

(B) Primary root length of *CKI1*-OXi, *ahp1,2-1,3,4,5*, and *CKI1*-OXi *ahp1,2-1,3,4,5* seedlings germinated and grown as described in **(A)**. Data presented are mean values of three independent experiments ($n > 15$ in each experiment) with standard deviations.

(C) Xylem development in *ahp1,2-1,3,4,5* and *CKI1*-OXi *ahp1,2-1,3,4,5* roots. See Figure 5C for technical details. All analyzed (31) *CKI1*-OXi *ahp1,2-1,3,4,5* roots treated with estradiol show a phenotype similar to that of *ahp1,2-1,3,4,5*. White arrow denotes protoxylem. Bar = 10 μm.

(D) Shoot regeneration assay of *CKI1*-OXi, *ahp1,2-1,3,4,5*, and *CKI1*-OXi *ahp1,2-1,3,4,5* hypocotyl explants treated with or without (Control, Cont) 20 μM estradiol. Bar = 10 mm.

(E) Expression of *ARR6* and *ARR7* as analyzed by qRT-PCR, as described in Figure 1B. Total RNA prepared from 2-week-old seedlings treated with DMSO or estradiol (20 μM) for 12 h was used for qRT-PCR. Data presented are mean values of three biological repeats with standard deviations. Relative expression level of *ARR6* and *ARR7* was normalized using *ACTIN7* (At5g09810) as an internal control.

To analyze the transmission efficiency of T-DNA insertions through gametophytes in the *ahp1,2,3,4,5* quintuple mutant, we constructed and characterized two different multiple mutants, *ahp1,2,3/+,4,5* and *ahp1,2,3,4,5/+*. These two multiple mutants were reciprocally crossed to wild-type plants, and transmission efficiency of the T-DNA insertion at either *AHP3* or *AHP5* was analyzed in F1 plants. Unexpectedly, the transmission efficiency for both female and male gametophytes in *ahp1,2,3,4,5* was only marginally altered compared with that of wild-type plants (Table 1). This result was not fully consistent with the CLSM analysis, in which 14.29% *ahp1,2,3,4,5* ovules were found to be defective. This result raises the possibility that abnormal female gametophyte development in the *ahp1,2,3,4,5* mutant might be caused by sporophytic defects. To clarify this question, we performed the following experiments.

We noticed that the *ahp2* allele used for the construction of the *ahp1,2,3,4,5* quintuple mutant contained a T-DNA insertion in intron 3, which was not a null mutation, as it showed residual expression of *AHP2* (Hutchison et al., 2006). Hereafter, we refer to this allele characterized by Hutchison et al. (2006) as *ahp2-1*. To more precisely define *AHP* function, we identified and characterized an additional mutant allele, *ahp2-2* (SALK_019024), in which a T-DNA is inserted in exon 4 (Figure 8A). Whereas *AHP2* expression was easily detectable in *ahp2-1* by qRT-PCR analysis, as previously observed (Hutchison et al., 2006), essentially no *AHP2* expression was detected in

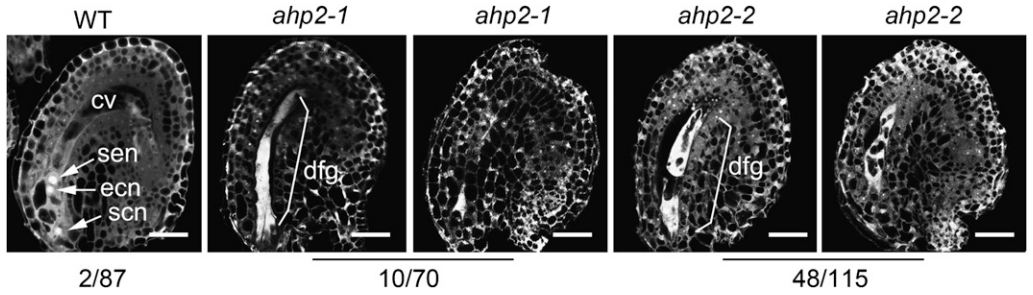

Figure 7. Female Gametophyte Development in the *ahp1,2,3,4,5* Mutants.

CLSM of ovules derived from wild-type, *ahp1,2-1,3,4,5*/+ (*ahp2-1*), and *ahp1,2-2,3*/+*,4,5* (*ahp2-2*) flowers, as indicated on the top of the images. Numbers below the images denote abnormal female gametophytes out of the examined ovules. cv, central cell vacuole; ecn, egg cell nucleus; dfg, degenerated female gametophyte; scn, synergid cell nucleus; sen, secondary nucleus. Bar = 25 μm.

ahp2-2 (Figure 8B), indicating that *ahp2-2* is a null mutant allele.

We used *ahp2-2* to construct *ahp* multiple mutants and analyzed *ahp1,2-2,3*/+*,4,5* and *ahp1,2-2,3,4,5*/+ in detail. No apparent developmental defects were observed in *ahp1,2-2,3*/+*,4,5* and *ahp1,2-2,3,4,5*/+ plants except for defects in female gametophyte development (see below). These two multiple *ahp* mutants were reciprocally crossed to wild-type plants, respectively, and the transmission efficiency through female or male gametophytes was examined. We found that the T-DNA insertion at either the *AHP3* or *AHP5* locus was transmitted through male gametophytes with a transmission efficiency of ~50% (Table 1), indicating that male gametophyte development is not affected by the *ahp* multiple mutations. However, the transmission efficiency of the T-DNA insertion through female gametophytes was dramatically reduced in both combinations (3.45 and 1.06% in *ahp1,2-2,3*/+*,4,5* and *ahp1,2-2,3,4,5*/+, respectively; Table 1). Consistent with this result, CLSM revealed the presence of 41.74% (n = 115) abortive ovules in *ahp1,2-2,3,4,5*/+ flowers, a significantly higher phenotypic penetrance than that of the weaker allele *ahp1,2-1,3*/+*,4,5* (14.29%; Figure 7). Taken together, these results indicate that the *ahp1,2-2,3,4,5* quintuple mutation causes severe defects in female gametophyte development.

We noticed that T-DNA insertions were present in the introns of *ahp1* and *ahp4* in both *ahp1,2-1,3,4,5* and *ahp1,2-2,3,4,5* mutants. Although no full-length transcripts of *AHP1* and *AHP4* were detected by RT-PCR (Hutchison et al., 2006), one cannot exclude the possibility that either or both of these genes has residual expression. Indeed, we have been able to detect residual *AHP4* expression in *ahp1,2-1,3,4,5* seedlings using two different primer sets (see Supplemental Figure 15 online). This result indicates that residual AHP4 activities are present in both *ahp1,2-1,3,4,5* and *ahp1,2-2,3,4,5* mutants. Therefore, an *ahp1,2,3,4,5* quintuple mutant carrying all five null mutations most likely has a stronger phenotype than *ahp1,2-2,3,4,5*.

The *ahp1,2-2,3,4,5* Quintuple Mutant Is Seedling Lethal

To gain more insight into the function of *AHPs* during vegetative growth, we attempted to identify *ahp1,2-2,3,4,5* quintuple mutant plants. In progeny obtained from self-pollinated *ahp1,2-2,3*/+*,4,5* plants, 2.34% of seedlings (n = 598) showed a mutant phenotype, significantly lower than that obtained from *ahp1,2-1,3*/+*,4,5* (22.00%; n = 559) and *ahp1,2-1,3,4,5*/+ (22.66%; n = 384) plants, consistent with the observation that *ahp2-2* is a stronger mutant allele than *ahp2-1*. In progeny obtained from self-pollinated *ahp1,2-2,3,4,5*/+ plants, no mutants were recovered from 596 analyzed plants. This result is consistent with the observation that *ahp1,2-2,3,4,5*/+ plants show a lower transmission efficiency of T-DNA through female gametophytes than *ahp1,2-2,3*/+*,4,5* plants (Table 1).

In wild-type-like progeny derived from self-pollinated *ahp1,2-2,3*/+*,4,5* and *ahp1,2-2,3,4,5*/+ plants, genotyping revealed that ~50% of plants were heterozygous for the T-DNA insertion at the *AHP3* (50.00%; n = 58) or *AHP5* (51.72%; n = 116) locus, and the remaining half were wild type at the *AHP3* or *AHP5* locus,

Table 1. Genetic Analysis of Transmission Efficiency of *ahp1,2-1,3,4,5* and *ahp1,2-2,3,4,5*

Parent		F1 Genotype		
Female	Male	*AHP/AHP*	*AHP/ahp*	T-DNA TE (%)
Col-0	*ahp1,2-1,3*/+*,4,5*	51	42	45.16
Col-0	*ahp1,2-1,3,4,5*/+	31	36	53.73
Col-0	*ahp1,2-2,3*/+*,4,5*	47	38	44.71
Col-0	*ahp1,2-2,3,4,5*/+	47	47	50.00
ahp1,2-1,3/+*,4,5*	Col-0	83	69	45.39
ahp1,2-1,3,4,5/+	Col-0	83	72	46.45
ahp1,2-2,3/+*,4,5*	Col-0	196	7	3.45
ahp1,2-2,3,4,5/+	Col-0	93	1	1.06

The *ahp* mutants used in crosses were heterozygous for the T-DNA insertion at *AHP3* (*ahp1,2,3*/+*,4,5*) or *AHP5* (*ahp1,2,3,4,5*/+) and were homozygous at other loci. Genotypes of both parents and the resulting progenies were determined by PCR analysis as described in Methods. Success of crossing was confirmed by genotyping F1 progenies using appropriate simple sequence length polymorphism markers (approximately three to five markers were used in each assay). Transmission efficiency (TE) is calculated as 100 × heterozygous/(heterozygous + wild type).

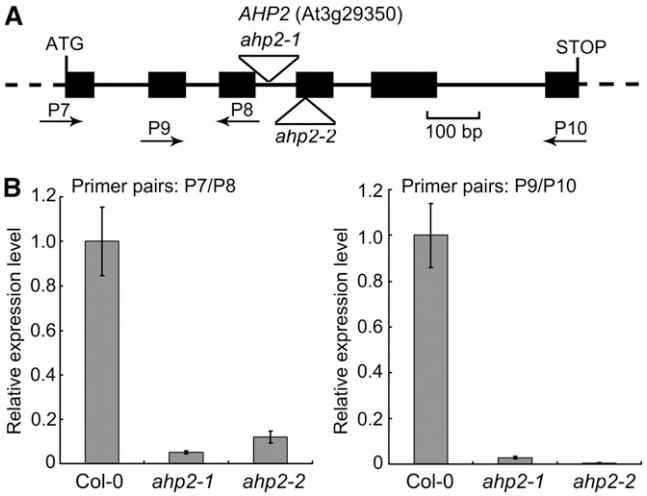

Figure 8. Characterization of the *ahp2-2* Mutant.

(A) A schematic map of the *AHP2* gene and *ahp2* mutant alleles. Filled boxes and solid lines denote exons and introns, respectively. Untranscribed and untranslated regions are indicated as dashed lines. Putative translation start and stop sites and positions of the T-DNA insertions in *ahp2* mutant alleles are shown. P7 through P10 denote relative positions and orientation of primers used in qRT-PCR (see Supplemental Table 2 online for sequences).

(B) *AHP2* expression in wild-type (Col-0), *ahp1,2-1,3,4,5* (labeled as *ahp2-1*), and *ahp2-2* seedlings (2 weeks old) analyzed by qRT-PCR using primer pairs as indicated in each graph. Data presented are mean values of three biological repeats with standard deviations. Relative expression level of *AHP2* was normalized using *ACTIN7* (At5g09810) as an internal control.

respectively. No homozygous plants for the T-DNA insertion at the respective loci were recovered among the analyzed population, consistent with the gametophytic defective nature of the *ahp1,2-2,3,4,5* quintuple mutation.

Compared with poorly developed *ahp1,2-1,3,4,5* plants as previously described (Hutchison et al., 2006), the rarely recovered *ahp1,2-2,3,4,5* mutant plants (derived from self-pollinated *ahp1,2-2,3/+,4,5* plants) showed a stronger phenotype. When germinated and grown on Murashige and Skoog medium, both *ahp1,2-1,3,4,5* and *ahp1,2-2,3,4,5* mutants were significantly smaller than wild-type seedlings and were delayed in the initiation of true leaves (Figure 9A). Upon longer culture, the *ahp1,2-1,3,4,5* mutant grew slowly and occasionally grew as wild-type seedlings. However, the growth and development of *ahp1,2-2,3,4,5* seedlings were completely arrested, and these plants eventually died. Whereas *ahp1,2-1,3,4,5* seedlings were able to grow and develop to maturity, albeit with reduced fertility, upon transfer to soil, *ahp1,2-2,3,4,5* seedlings did not grow further and eventually died (Figures 9B and 9C). Therefore, the *ahp1,2-2,3,4,5* mutation causes seedling lethality.

AHP Genes Are Required for Cell Division and Cell Expansion

The *ahp1,2-1,3,4,5* plants were smaller than wild-type plants (Figures 9A and 9B). Leaves of the quintuple mutant plants were substantially smaller than wild-type leaves (Figure 9D). At the cellular level, we found that both the cell size and the cell number are reduced in *ahp1,2-1,3,4,5* leaves (Figures 9E and 9F). Notably, the cell number in *ahp1,2-1,3,4,5* leaves was only 27.04% that of wild-type leaves (Figure 9F). The dramatically reduced cell number in *ahp1,2-1,3,4,5* leaves is similar to that observed in *ahk2,3,4* leaves (Nishimura et al., 2004). However, the cell size of *ahk2,3,4* leaves was slightly increased compared with that of wild-type leaves (Nishimura et al., 2004), a phenotype similar to that of *cki1-8* leaves (Figure 3D). Therefore, the *AHP* genes appear to play a more dominant role than the cytokinin receptor genes and *CKI1*, being involved in the regulation of both cell division and cell expansion during plant growth and development.

ARR1 Is Able to Partially Rescue the *cki1-5* Mutant Phenotype

Because *CKI1* acts upstream of *AHP* genes to regulate cytokinin signaling as well as plant growth and development, we then asked if the activation of a downstream component of the phosphorelay was able to rescue the *cki1* mutant phenotype. To test this possibility, a *CKI1:ARR1* construct was made and initially transformed into *CKI1/cki1-8* heterozygous plants. In later experiments, we found that floral dipping with *Agrobacterium tumefaciens* cultures, that were either transformed with a binary vector or not transformed, was able to cause partial recovery of homozygous *cki1-8* mutant plants. We found that *Agrobacterium* cultures could significantly induce *CKI1* expression in *cki1-8* plants for unknown reasons in an allele-specific manner that did not occur in wild-type and *cki1-5* flowers (see Supplemental Figure 16 online). Presumably, the partial rescue effect on the *cki1-8* mutant was likely caused by the *Agrobacterium*-induced expression of *CKI1*. Therefore, we used the null *cki1-5* mutant (Pischke et al., 2002) in all of the related experiments described below.

In *CKI1/cki1-5* plants transformed with an empty vector (pER8), no homozygous *cki1-5* plants were recovered from the tested population (159 independent transgenic lines; Table 2). We analyzed 100 independent transgenic lines (T1) of *CKI1/cki1-5* plants transformed with a *CKI1:ARR1* transgene that were resistant to hygromycin (conferred by the binary vector pER8). Seven of these transgenic lines exhibited enlarged plant size and remarkably reduced fertility, a phenotype similar to that of *cki1-8* (Table 2). Genotyping confirmed that these seven lines were indeed homozygous for the T-DNA insertion at the *cki1-5* locus, indicating that the *CKI1:ARR1* transgene is able to partially rescue the *cki1-5* mutant phenotype. When an *ARR1*ΔDDK transgene, which was shown to be a constitutively activated form (Sakai et al., 2001), was used in the experiment, a similar rescue frequency (8.96%) was observed (Table 2).

We also transformed *CKI1/cki1-5* plants with two additional mutant *ARR1* transgenes, in which the conserved Asp-94 (D94) residue in the receiver domain was substituted with a Glu (D94E) or an Asn (D94N). The Asp-94 residue is presumed to receive a phosphoryl group from AHP proteins upon the activation of the cytokinin pathway. Therefore, ARR1D94E, similar to ARR1ΔDDK, may act as a constitutively active mutant by

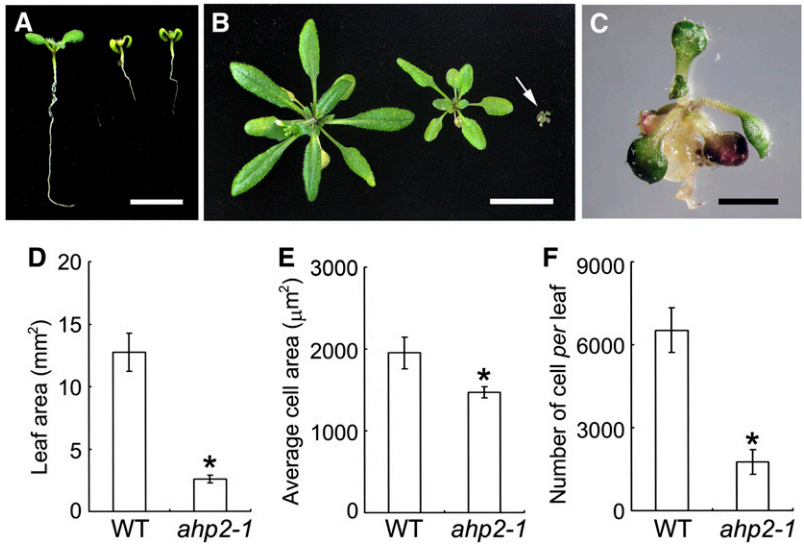

Figure 9. Characterization of the *ahp1,2,3,4,5* Quintuple Mutant.

(A) Seven-day-old seedlings of the wild type (Col-0; left), *ahp1,2-1,3,4,5* (middle), and *ahp1,2-2,3,4,5* (right). Bar = 5 mm.
(B) Four-week-old seedlings of the wild type (Col-0; left), *ahp1,2-1,3,4,5* (middle), and *ahp1,2-2,3,4,5* (right; denoted by an arrow). Bar = 20 mm.
(C) An enlarged view of an *ahp1,2-2,3,4,5* seedling grown in soil for 4 weeks. Bar = 2 mm.
(D) to **(F)** The cellular phenotype of the *ahp1,2-1,3,4,5* leaf. Leaves of the wild type and *ahp1,2-1,3,4,5* (*ahp2-1*) were analyzed as described in Figure 3. Asterisks indicate statistically significant differences compared with wild-type plants (Student's *t* test, $P < 0.01$).
(D) The leaf blade area.
(E) Average size of abaxial epidermal cells.
(F) The number of abaxial epidermal cells.

mimicking phosphorylation, whereas ARR1D94N is presumably an inactive form due to its inability to be phosphorylated at Asp-94. We have recovered homozygous *cki1-5* T1 transgenic plants transformed with *CKI1:ARR1D94E* or *CKI1:ARR1D94N* at a similar efficiency as obtained in the *CKI1:ARR1*-transformed *CKI1/cki1-5* plants (Table 2), suggesting that both transgenes were able to rescue the *cki1-5* mutant phenotype. In a previous study, a mutant transgene *ARR2D80N*, analogous to *ARR1D94N* in this study, also showed a phenotype similar to wild-type *ARR2*, and the *ARR2D80N* mutation did not affect its ability to enhance *ARR6-LUC* expression in the absence or the presence of cytokinin (Hwang and Sheen, 2001). It has been proposed that ectopic overexpression of *ARR2D80N* may bypass the negative regulation of the cytokinin pathway and thus activate the cytokinin pathway without an input signal (Hwang and Sheen, 2001). An alternative explanation could be that nonphosphorylated ARR1 and ARR2, when ectopically overexpressed, may have the basal transcriptional competence to allow them to bind to and subsequently activate their target genes. Nevertheless, these results suggest that the *ARR1* transgene, but not an empty vector, can partially rescue the *cki1-5* mutant phenotype.

Because of the low frequency of seed setting (~10 to 40 seeds per plant; see below), we amplified these transgenic lines (at least two independent lines for each construct) for an additional two to three generations (T2 through T4) and obtained thousands of seeds for the analyses described below. All of these rescued *cki1-5* plants displayed a phenotype similar to *cki1-8* plants, with characteristics of an enlarged size of seedlings/plants, longer primary roots, delayed termination of flower production, and abortive female gametogenesis, which resulted in setting seeds at low frequency (Figures 10A to 10D). In *cki1-5* plants rescued with the *ARR1* transgene, development of shoot procambial cells appeared to be normal (see Supplemental Figure 6 online).

No *CKI1* expression was detected in the flowers of the rescued *cki1-5* plants (transformed with *ARR1* or *ARR1*ΔDDK) (see Supplemental Figure 17A online), thus ruling out the possibility that the rescued phenotype was caused by residual expression of *CKI1*. Instead, expression of the *ARR1* or *ARR1*ΔDDK transgenes was detected in flowers of the rescued *cki1-5* plants, using primer pairs specific for these transgenes by qRT-PCR analysis (see Supplemental Figures 17B and 17C online). Taken together, these results indicate that *ARR1* is able to partially complement the *cki1-5* mutant phenotype.

Ectopic Expression of *IPT8* in the *CKI1*-Expressing Domain Partially Rescues the *cki1-5* Mutant Phenotype

Although the cytokinin receptors are dispensable in female gametogenesis, we asked if CKI1 activity could be functionally substituted by receptor-activated signaling. If this is the case, an increased cytokinin level in the *CKI1*-expressing domain, which presumably activates cytokinin receptor-mediated signaling, should be able to rescue the *cki1* phenotype. To test this possibility, we constructed a *CKI1:IPT8* transgene and transformed it into *CKI1/cki1-5* heterozygous plants. *IPT8* encodes an isopentenyl transferase that catalyzes the first rate-limiting

Table 2. Rescue of the *cki1-5* Mutant Phenotype by *ARR1* and *IPT8* Transgenes

Genotype[a]	Construct[b]	Tested[c]	*cki1-5*/−[d]	Percentage
cki1-5/+	–	>500	0	0.00
cki1-5/+	Empty vector	159	0	0.00
cki1-5/+	*ARR1*	100	7	7.00
cki1-5/+	*ARR1*ΔDDK	201	18	8.96
cki1-5/+	*ARR1*D94E	47	4	8.51
cki1-5/+	*ARR1*D94N	233	15	6.44
cki1-5/+	*IPT8*	58	2	3.45

[a]Heterozygous *cki1-5*/+ plants were transformed with different constructs.

[b]All transgenes were placed under the control of the *CKI1* promoter and were cloned into the binary vector pER8 (Zuo et al., 2000) that carried a hygromycin selectable marker gene. "Empty vector" refers to the use of pER8 in the transformation. "–" Denotes self-pollinated nontransgenic plants.

[c]"Tested" refers to the tested T1 transgenic lines that were resistant to hygromycin and carried a *cki1-5* mutation. For nontransgenic plants, the number refers to the analyzed progeny obtained from self-pollinated *cki1-5*/+ plants that carried a *cki1-5* mutation.

[d]"*cki1-5*/−" refers to homozygous lines (transgenics) or families (self-pollinated) that were obtained from the tested populations by PCR genotyping and phenotypic analysis.

reaction of the cytokinin biosynthesis pathway, and overexpression of *IPT8* causes an elevated cytokinin level in planta (Sun et al., 2003). Among the 58 tested independent transgenic lines, we recovered two lines that are homozygous for the T-DNA insertion at the *cki1-5* locus (Table 2).

Because of the relatively low rescue frequency of the *cki1-5* phenotype by *IPT8*, we then analyzed progeny obtained from self-pollinated transgenic T1 lines that were heterozygous for the T-DNA insertion at the *CKI1* locus (*cki1-5*/+). If the *IPT8* transgene is able to partially rescue the *cki1-5* phenotype, homozygous *cki1-5*/− plants should be recovered from self-pollinated T2 *cki1-5*/+ plants. Among 10 T1 lines transformed by an empty vector, no *cki1-5*/− homozygous plants were recovered by analyzing 580 T2 plants. In 11 T1 lines transformed with the *CKI1*:*IPT8* transgene, *cki1-5*/− homozygous plants were recovered from seven lines, and the rescue frequency ranged from 1.25 to 11.48% (see Supplemental Table 1 online). Taken together, these observations indicate that the *CKI1*:*IPT8* transgene can partially rescue the *cki1-5* female gametophytic defects.

T2 and T3 progenies derived from these *IPT8*-rescued plants showed a phenotype similar to that of *cki1-8* and c*ki1-5* plants rescued by an *ARR1* or an *ARR1*ΔDDK transgene (Figures 10A to 10C). CLSM revealed the presence of normal or relatively normal ovules in flowers of the *IPT8*-rescued *cki1-5* plants (Figure 10E). Moreover, rarely-set seeds were observed in the siliques of the *IPT8*-rescued *cki1-5* plants (Figure 10D).

In flowers of the *IPT8*-rescued transgenic plants, expression of the *IPT8* transgene, but not of *CKI1*, was detected as analyzed by qRT-PCR (see Supplemental Figures 17A and 17D online). These results indicate that expression of *IPT8* in the *CKI1*-expressing domain partially rescues the *cki1-5* mutant phenotype, presumably by locally activating receptor-mediated cytokinin signaling.

DISCUSSION

The identification of CKI1 implied the possible involvement of a TCS-based mechanism in cytokinin signaling (Kakimoto, 1996). Subsequent studies revealed the pivotal role of the TCS-mediated phosphorelay in cytokinin signaling (Hwang et al., 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005; Müller and Sheen, 2007). Despite these progresses, several key questions on the function of *CKI1* remain unanswered. CKI1 shares considerable homology with the cytokinin receptors, and overexpression of *CKI1* is able to evoke typical cytokinin responses that are indistinguishable from those in cytokinin-treated wild-type plants (Kakimoto, 1996; Hwang and Sheen, 2001; Yamada et al., 2001; Zheng et al., 2006). However, CKI1 does not appear to be a cytokinin receptor, owing to its incapability to bind to cytokinins and its cytokinin-independent manner of action. Therefore, it has long been questioned whether CKI1 is directly involved in cytokinin signaling during plant growth and development (Haberer and Kieber, 2002; Hwang et al., 2002; Kakimoto, 2003; Hwang and Sakakibara, 2006; Klumpler et al., 2009). Whereas *CKI1* plays an essential role in female gametogenesis, it is an open question if this function of CKI1 in female gametophyte development is dependent on the TCS-mediated signaling or on an independent pathway (Pischke et al., 2002; Hejátko et al., 2003). Lastly, again because of female gametophytic lethality, the function of *CKI1* beyond the gametophytic stage remains completely unknown.

In this study, we show that overexpression of *CKI1* induces the cytokinin response in a *CRE1*/*WOL*-independent manner and is able to partially rescue the *wol* mutant phenotype. These results suggest that *CKI1* may function downstream of *CRE1*/*WOL* or independently of the cytokinin receptors. However, biochemical analyses indicate that both CRE1/WOL and CKI1 are able to phosphorylate AHP1, 2, 3, and 5 (Mähönen et al., 2006a) and that CKI1 can dephosphorylate AHP1 and AHP2 (Nakamura et al., 1999), suggesting that these two histidine kinases act directly on downstream targets and do not need an intermediate component. In addition, the *wol* mutant and *ahk2*,*3*,*4* triple mutants do not show phenotypic similarity with the *cki1-8* mutant in vegetative growth and female gametogenesis, thus disfavoring a hypothesis that cytokinin receptor genes and *CKI1* act in a linear pathway. More importantly, whereas *ahk2*,*3*,*4* triple mutants have no response to cytokinin (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), the *cki1-8* mutant shows a normal response to cytokinin. Lastly, genetic analysis reveals that *AHP* genes are essential for *CKI1* activity. Together, these results suggest that the cytokinin receptors and CKI1 may act in parallel branches and that AHPs act as a converging point of TCS-mediated signaling that is independently activated by the cytokinin receptors and CKI1.

The TCS has also been implicated in certain physiological activities other than cytokinin signaling. For example, AHK1, another homolog of the cytokinin receptors, has been shown to be involved in both the stress response and seed maturation (Urao et al., 1999; Tran et al., 2007; Wohlbach et al., 2008). Interestingly, several type-A *ARR* genes are coexpressed with *AHK1*, and different combinations of multiple *arr* mutants show altered responses to osmotic stress (Wohlbach et al., 2008).

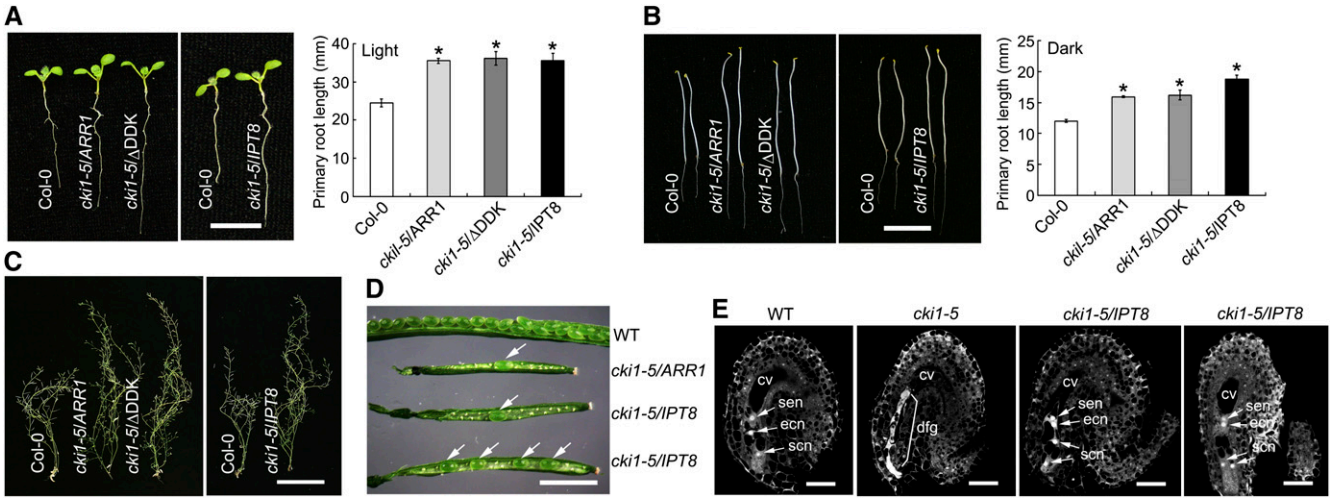

Figure 10. *ARR1* and *IPT8* Transgenes Partially Rescue the *cki1-5* Mutant Phenotype.

(A) Left: Seven-day-old seedlings of the wild type (Col-0) and *cki1-5* carrying *CKI1:ARR1* (*cki1-5/ARR1*), *CKI1:ARR1*/ΔDDK (*cki1-5*/ΔDDK), or *CKI1:IPT8* (*cki1-5/IPT8*) transgenes, respectively. T2 (*CKI1:IPT8*) or T3 (other transgenics) seedlings grown on GM under continuous white light are shown. Right: Primary root length of wild-type (Col-0) and *cki1-5* transgenic plants. Data presented are mean values of three independent experiments ($n > 15$ in each experiment) with standard deviations. Asterisk indicates statistically significant differences compared with wild-type plants (Student's *t* test, $P < 0.01$).
(B) Left: Five-day-old seedlings germinated and grown on GM in the dark. See **(A)** for other details. Right: Primary root length of wild-type (Col-0) and *cki1-5* transgenic plants. Data presented are mean values of three independent experiments ($n > 15$ in each experiment) with standard deviations. Asterisk indicates statistically significant differences compared with wild-type plants (Student's *t* test, $P < 0.01$).
(C) Eight-week-old soil-grown plants of the wild type (Col-0) and *cki1-5* carrying different transgenes, as shown in the panel.
(D) Seed development in siliques of wild-type and *cki1-5*/− plants carrying a *CKI1:ARR1* (*cki1-5/ARR1*) or *CKI1:IPT8* (*cki1-5/IPT8*) transgene. Arrows denote seeds in the *cki1-5/ARR1* and *cki1-5/IPT8* siliques.
(E) CLSM of ovules derived from wild-type, *cki1-5*/+ (*cki1-5*), and *cki1-5*/− plants transformed with the *CKI1:IPT8* transgene (*cki1-5/IPT8*) flowers as indicated on the top of the images. Approximate 50% of ovules in *cki1-5*/+ flowers show various defects as previously observed (Pischke et al., 2002). Among 222 examined ovules in *cki1-5/IPT8* flowers, 13 show relatively normal development as shown in the figure. cv, central cell vacuole; ecn, egg cell nucleus; dfg, degenerated female gametophyte; scn, synergid cell nucleus; sen, secondary nucleus.
Bars = 10 mm in **(A)** and **(B)**, 10 cm in **(C)**, 2 mm in **(D)**, and 25 μm in **(E)**.

These observations suggest that AHK1 may also adopt TCS to activate downstream responses, although it remains unknown if this response is dependent on AHPs and ARRs (Tran et al., 2010). In contrast with *AHK1*, however, *CKI1* induces all examined cytokinin responses, and these responses are strictly dependent on the *AHP* loci. Moreover, the *cki1-5* and *ahp1,2,3,4,5* mutants show similar defects in female gametophyte development, and these defects in *cki1-5* can be partially rescued by the activation of cytokinin signaling. Therefore, although both CKI1 and AHK1 use TCS to transmit specific signals, these two structurally related histidine kinases execute distinctive physiological activities, involving the cytokinin response and the stress response, respectively. Taken together, these observations indicate that CKI1 is capable of specifically activating the cytokinin signaling pathway.

Several lines of evidence presented in this study demonstrate that *CKI1*-regulated development of female gametophytes is dependent on the activation of the TCS-based signaling pathway. First, whereas *CKI1*-activated signaling is dependent on *AHP* genes, the *ahp1,2,3,4,5* mutants show defective female gametophyte development, similar to that observed in *cki1* mutants. Moreover, *cki1* mutants and the *ahp1,2,3,4,5* mutants also show a similar phenotype in flower development and male gametogenesis. These results suggest that *CKI1* acts upstream of *AHP* and that *CKI1-AHP*–dependent signaling is essential for female gametophyte development. Second, ectopic expression of *ARR1* in the *CKI1*-expressing domain partially rescues the *cki1-5* mutant phenotype, indicating that cell- or tissue-specific activation of TCS-mediated signaling is able to bypass the essential requirement of the activity of *CKI1* and *AHP* genes during female gametophyte development. Third, given that TCS-based signaling is required for female gametogenesis and that this requirement is functionally fulfilled by *CKI1* in ovules, the activation of receptor-mediated cytokinin signaling should complement, at least partly, the *cki1* mutant phenotype. Indeed, ectopic expression of *IPT8* in the *CKI1*-expressing domain partially rescues the *cki1-5* mutant phenotype, providing an additional line of evidence to support the essential role of the TCS-activated signaling in female gametophyte development. Notably, whereas the *cki1* mutant shows impaired female gametogenesis, the receptor mutations do not appear to affect gametophyte development. The use of CKI1, instead of the conventional cytokinin-activated receptors, during female gametophyte development may represent a default signaling shortcut, which facilitates specific and rapid activation of the pathway in a small group of cells in a relatively short developmental

window. Collectively, these results suggest that *CKI1* positively regulates the TCS-based signaling via *AHP* and type-B *ARR* genes, and this genetic framework is essential for female gametophyte development.

Compared with the severe developmental abnormalities observed in the receptor mutants (*ahk2*,*3*,*4* and *wol*) and the *ahp1*,*2*,*3*,*4*,*5* mutant, *cki1-8* shows a moderate phenotype during vegetative growth. However, in contrast with the small-sized *ahk2*,*3*,*4* and *ahp1*,*2*,*3*,*4*,*5* mutants, *cki1-8* plants grow larger than wild-type plants. It is worth noting that, whereas the *ahk2*,*3* double mutants have reduced growth of shoots and leaves (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), *cre1/ahk4* single and *ahk2*,*3* double mutants have longer primary roots and more lateral roots than wild-type plants (Riefler et al., 2006). Compared with these receptor mutants, *cki1-8* shows a similar phenotype in root growth but an opposite phenotype in shoot and leaf growth, suggesting that the cytokinin receptors and CKI1 play overlapping yet distinctive roles during vegetative growth and development. Consistent with these developmental differences, the cytokinin receptors positively regulate both cell division and cell expansion (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), whereas *CKI1* appears to negatively regulate cell growth in leaves, with little, if any, effects on cell division during vegetative growth. Notably, the *ahp1*,*2-1*,*3*,*4*,*5* mutants show a similar phenotype as *ahk2*,*3*,*4* and *wol* during vegetative growth but similar to that of *cki1* mutants during female gametophyte development. By contrast, *cki1-8* and *ahp1*,*2-1*,*3*,*4*,*5* show similar reproductive phenotypes, but exhibit different phenotypes during vegetative growth. Based on these observations, we propose that, whereas *CKI1-AHP*-dependent signaling is essential for megagametogenesis, cytokinin receptor-*AHP*–mediated signaling mainly regulates vegetative growth and possibly other developmental programs as well.

Finally, because both cytokinin receptors and CKI1 are able to activate cytokinin signaling via AHPs, it is likely that these two types of HKs function redundantly, at least partially, in certain developmental stages. Notably, the *ahk2*,*3*,*4* triple mutants are neither gametophyte nor embryo lethal, as one would expect for essential roles of cytokinin signaling in plant growth and development (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). In this regard, the presence of a cytokinin receptor-independent signaling mechanism mediated by the CKI1-AHP scheme may explain concerns on the viability of *ahk2*,*3*,*4* triple mutants. Presumably, the cytokinin receptor activity is, at least partly, substituted by CKI1 during reproductive development. In agreement with this notion, the *ahp1*,*2-2*,*3*,*4*,*5* mutant, which lacks *AHP2* expression, but has residual *AHP4* expression, has severe defects in female gametophyte development similar to those observed in *cki1-8*, and the rarely recovered *ahp1*,*2-2*,*3*,*4*,*5* seedlings die during early developmental stages. In summary, the results presented here indicate that the cytokinin receptors and CKI1 function independently and complementarily to regulate the TCS-based signaling pathway upon perceiving distinctive signals during different developmental stages. In particular, the CKI1-AHP–dependent signaling pathway is crucial for female gametophyte development and vegetative growth.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana wild types Wassilewskija (Ws) and Columbia-0 (Col-0) were used in this study. The *cki1-7* and *cki1-8* mutants (Ws) have been previously described (Zheng et al., 2006). The *cki1-5* (Col-0; CS6360) and *ahp2-2* (Col-0; SALK_019024) mutants were obtained from the ABRC. The *wol*, *ahk2,3,4*, and *ahp1*,*2*,*3*,*4*,*5* (all in Col-0) mutant seeds were kindly provided by Ykä Helariutta, Thomas Schmülling, and Joseph Kieber, respectively. Seeds were imbibed at 4°C for 3 d prior to germination. Unless indicated otherwise, plants were grown under continuous white light (100 μmoles m^{-2} s^{-1}) at 22°C in soil or on GM (1× Murashige and Skoog salts, 1% sucrose, 1× B5 vitamin, 0.05% MES-KOH, and 0.3% Phytagel).

Agrobacterium tumefaciens–mediated transformation of *Arabidopsis* plants was performed by the floral dip method (Bechtold and Pelletier, 1998). The agrobacterial strain GV3101 was used in all transformation experiments.

Genetic Analyses and Genotyping

Reciprocal crosses and genetic analysis were performed as described (Pischke et al., 2002). Genotyping of *cki1-5*, *cki1-7*, *cki1-8*, and *ahp1*,*2*,*3*,*4*,*5* mutants was performed by PCR with three primers as previously described (Hutchison et al., 2006; Zheng et al., 2006). Genotyping of *wol* was performed by digesting a PCR fragment spanning the mutated region with *Spe*I. This *Spe*I site was introduced by the PCR primer in the wild-type genome but not in the *wol* sequence (a derived cleaved amplified polymorphism sequence marker; see Supplemental Table 2 online for sequences of all primers used in this study).

Assays of Cytokinin Responses

Root elongation and shoot formation assays were performed as previously described (Sun et al., 2003; Zheng et al., 2006). Analysis of root vasculature was performed by basic Fuchsin Red staining according to Mähönen et al. (2000). After the staining, the sample was analyzed under a confocal microscope. Dark-induced leaf senescence was analyzed as described previously (Dong et al., 2007; Feng et al., 2007).

Microscopy Analyses

CLSM of ovule development was performed according to Christensen et al. (1997). Scanning electron microcopy was performed as previously described (Feng et al., 2007). Analysis of leaf development, including the analysis of cell size and the calculation of cell number per leaf, was performed essentially according to Nishimura et al. (2004).

Analysis of vascular development of inflorescence stems was performed essentially according to Hejátko et al. (2009). Semithin sections were made from the base of the inflorescence stem when the first silique appeared, following the same methods as described (Hejátko et al., 2009).

In Situ RNA Hybridization

In situ RNA hybridization was performed as previously described (Chen et al., 2007). To prepare probes, a 299-bp cDNA fragment amplified by PCR with primers P3 and P4 (see Supplemental Table 2 online for primer sequences) was subcloned in a pBluescript II SK− vector in two different orientations. The resulting constructs were used as templates for PCR amplification of linearized DNA fragments, using primer pairs M13F/P3 (for sense probe; with M13F anchored on the vector) and M13F/P4 (for antisense probe), respectively. The PCR products were gel purified and

then used for in vitro transcription of sense or antisense probes with T7 RNA polymerases in the presence of digoxigenin-11-dUTP.

RT-PCR–DNA Gel Blot Analysis of *CKI1* Expression

Poly(A) RNA was prepared from fresh or frozen plant materials using the Oligotex mRNA mini kit (Qiagen) according to the manufacturer's instructions. One microgram of poly(A) RNA was used as a template for the RT reaction using oligo(dT) as a primer. The resulting reactions were then subjected to PCR for ~20 to 25 cycles (cycling condition: 94°C, 30 s; 56°C, 30 s; 72°C, 25 s), using appropriate primer pairs. Aliquots of the PCR products were separated on a 2% agarose gel, stained with ethidium bromide, photographed using a UV light box, and then subjected to DNA gel blot analysis. The probe was double labeled by radioactive ^{32}P-dATP and ^{32}P-dCTP using the Megaprime DNA labeling system kit (Amersham) or labeled by the DIG DNA labeling and detection kit (Roche) according to the manufacturer's instructions. Both methods yielded similar results.

Molecular Manipulations

All molecular manipulations were performed according to standard methods (Sambrook and Russell, 2001). Primer sequences are presented in Supplemental Table 2 online. To make *CKI1*:*CKI1*, a *CKI1* cDNA clone was placed under the control of the putative *CKI1* promoter (containing sequences ~1.5 kb upstream from the translation start codon), and the resulting fragment was cloned into the *Sse8337*I and *Xba*I sites of a binary vector pX7 (Zuo et al., 2001). An ~6.0-kb *CKI1* genomic fragment, which contains the putative promoter sequence (~2.0 kb upstream of the translation start codon) and the coding sequences, was PCR amplified, in-framed fused to a *GFP* cDNA fragment using a *Sma*I site, and then cloned between the *Xho*I and *Spe*I sites of pER8 (Zuo et al., 2000) to yield *CKI1*:*CKI1-GFP*. To generate constructs in which the *CKI1* promoter controlled *ARR1*, *ARR1*ΔDDK (Sakai et al., 2001), *ARR1*D94E, *ARR1*D94N, and *IPT8* transgenes, the *CKI1* promoter and 5′-untranslated region sequences (the same sequences as for the *CKI1*:*CKI1-GFP* construct) were PCR amplified and then ligated to PCR-amplified cDNA (*ARR1* and its mutated versions) or genomic sequences (*IPT8*; Sun et al., 2003) of these tested genes using a *Bam*HI restriction site embedded in the PCR primers. For genotyping the *ARR1*D94E and *ARR1*D94N mutant genes, a *Hin*dIII site 15 bp downstream of the Asp-94 codon was eliminated during PCR, and this change (AAGCTT to AAaCTT) did not alter the encoded amino acid residue (both AAG and AAA encode Lys).

Analysis of gene expression by qRT-PCR was performed as previously described (Dong et al., 2007; Feng et al., 2007). Briefly, the first-strand cDNA was synthesized using ~1 to 2 μg of total RNA primed by oligo(dT). SuperScript II (Invitrogen) was used for the RT reaction according to the manufacturer's instructions. After incubated with RNase H for 30 min at 37°C, 1 μL of the diluted RT reaction (usually ~5- to 10-fold) was then used as a template of qPCR. Real-time qPCR was performed using ExTaq DNA polymerase (Takara Biotechnology) and the fluorescent intercalating dye SYBR-Green (iQ SYBR Green Super mix; Bio-Rad) according to the manufacturer's instructions. The reaction was run in a PTC200 DNA Engine gradient thermal cycler (MJ Research). *ACTIN7* was used as an internal control in the qRT-PCR analysis. Relative expression of the target genes was analyzed with the delta-delta Ct method.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *CKI1* (At2g47430), *AHK2* (At5g35750), *AHK3* (At1g27320), *CRE1/WOL/AHK4* (At2g01830), *AHP1* (At3g21510), *AHP2* (At3g29350), *AHP3* (At5g39340), *AHP4* (At3g16360), *AHP5* (At1g03430), *ARR1* (At3g16857), *ARR6* (At5g62920), *ARR7* (At1g19050), *IPT8* (At3g19160), *WUS* (At2g17950), and *ACT7* (At5g09810).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The *CKI1*-OXi Gain-of-Function Mutant Phenotype.

Supplemental Figure 2. Phenotype of Transgenic Plants upon Inducible Overexpression of a *CKI1-GFP* Transgene.

Supplemental Figure 3. Female Gametophyte Development in *cki1-8* Ovules.

Supplemental Figure 4. *CKI1* Expression in Wild-Type Seedlings Analyzed by RT-PCR–DNA Gel Blot Analysis.

Supplemental Figure 5. Expression of *CKI1* in Wild-Type and *cki1-8* Ovules Analyzed by in Situ RNA Hybridization.

Supplemental Figure 6. Vascular Development of Inflorescence Stems in *cki1-8* and *cki1-5*/*ARR1.*

Supplemental Figure 7. The *cki1-8* Phenotype under the Nutrition-Restricted Condition.

Supplemental Figure 8. Reproductive Development Phenotype of the *cki1-8* Mutant.

Supplemental Figure 9. Floral Development in *cki1-8* Plants Induced with Estradiol.

Supplemental Figure 10. The Cytokinin Response in *cki1-8.*

Supplemental Figure 11. Root Xylem Development and Shoot Regeneration Phenotype in *wol* and *ahp1*,*2-1*,*3*,*4*,*5* Mutants.

Supplemental Figure 12. Female Gametophyte Development in *wol* and *ahk2*,*3*,*4* Mutants.

Supplemental Figure 13. Female Gametophyte Development in *ahp1*,*2-1*,*3*,*4*,*5.*

Supplemental Figure 14. Reproductive Development in the *ahp1*,*2*,*3*,*4*,*5* Mutants.

Supplemental Figure 15. *AHP4* Expression in *ahp1*,*2-1*,*3*,*4*,*5* Seedlings.

Supplemental Figure 16. *Agrobacterium*-Induced *CKI1* Expression in *cki1-8* Flowers.

Supplemental Figure 17. Expression of *CKI1* and the *ARR1* and *IPT8* Transgenes in Transgenic Plants.

Supplemental Table 1. Rescue of the *cki1-5* Mutant Phenotype by *CKI1*:*IPT8* Transgene in T2 Transgenic Plants.

Supplemental Table 2. Primers Used in This Study.

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