Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in *Arabidopsis*

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In plants, multistep component systems play important roles in signal transduction in response to environmental stimuli and plant growth regulators. *Arabidopsis* contains six nonethylene receptor histidine kinases, and, among them, *AHK1/ATHK1*, *AHK2*, *AHK3*, and *CRE1* were shown to be stress-responsive, suggesting their roles in the regulation of plant response to abiotic stress. Gain- and loss-of-function studies in *Arabidopsis* indicated that AHK1 is a positive regulator of drought and salt stress responses and abscisic acid (ABA) signaling. Microarray analysis of the *ahk1* mutant revealed a down-regulation of many stress- and/or ABA-inducible genes, including AREB1, ANAC, and DREB2A transcription factors and their downstream genes. These data suggest that AHK1 functions upstream of AREB1, ANAC, and DREB2A and positively controls stress responses through both ABA-dependent and ABA-independent signaling pathways. In addition, AHK1 plays important roles in plant growth because the *ahk1 ahk2 ahk3* triple mutant showed further reduced growth. Unlike AHK1, loss-of-function analysis of *ahk2*, *ahk3*, and *cre1* implied that the stress-responsive AHK2, AHK3, and CRE1 act as negative regulators in ABA signaling. AHK2 and AHK3 also negatively control osmotic stress responses in *Arabidopsis* because *ahk2*, *ahk3*, and *ahk2 ahk3* mutants were strongly tolerant to drought and salt stress due to up-regulation of many stress- and/or ABA-inducible genes. Last, cytokinin clearly mediates stress responses because it was required for CRE1 to function as a negative regulator of osmotic stress.

cross-talk | microarray | osmotic stress | plant growth

Plants are constantly exposed to environmental stresses that frequently impose constraints on their growth and productivity. Cells have developed elaborate and sensitive protection systems that enable them to rapidly signal, respond, and properly adapt to various stresses, including drought and high salinity (1–5). Phosphorylation, which is catalyzed by protein kinases, is a key mechanism for intracellular signal transduction in both eukaryotic and prokaryotic cells. Exposure of *Saccharomyces cerevisiae* to a high-osmolarity environment leads to rapid phosphorylation and activation of the MAPK kinase Hog1 through either the SLN1 or SHO1 branch of the HOG pathway (6). Overexpression of *Arabidopsis* histidine kinase *AHK1/ATHK1* in *S. cerevisiae sln1* and *sho1* deletion mutants enables the yeast mutant to grow normally under high-salinity conditions, suggesting that the histidine kinase (HK) AHK1 can sense and transduce a signal of external osmolarity to downstream targets (7). Moreover, the AHK1 transcript accumulated in *Arabidopsis* in response to changes in external osmolarity, suggesting the functional importance of AHK1 for the efficient sensing of environmental signals. However, it is still not known whether AHK1 functions as an osmosensor in plants.

Beside the nonethylene receptor AHK1, the *Arabidopsis* genome encodes 10 other putative HKs: the ethylene receptor (ER) HKs ETR1, ETR2, EIN4, ERS1, and ERS2 and the nonethylene receptor (NER) HKs AHK2, AHK3, CRE1/AHK4, CKI1, and CKI2/AHK5 (8). Regarding the NER kinases, AHK2, AHK3, and CRE1 have all been shown to function in cytokinin (CK) signaling (9, 10) and were consequently named CK receptor HKs. Other reports revealed that these three HKs also function in shoot growth, leaf senescence, leaf longevity, seed size, germination, and root development (11, 12). CKI1 function is required for megagametophyte development (13). Recently, CKI2 has been implicated in root elongation through an ETR1-dependent abscisic acid (ABA) and ethylene signaling pathway (14). The NER HKs play the central role in the multistep His–Asp phosphorelay system consisting of sensor HKs, histidine phosphotransfers (HPt), and effector response regulators. On the other hand, the ER HKs are atypical in that they modulate the function of the downstream CTR1, which does not belong to the His–Asp phosphorelay family (for reviews, see refs. 15–19). Interestingly, CRE1 exhibits a dual function depending on the presence or absence of CK. In the presence of CK, CRE1 phosphorylates the HPt. Conversely, it removes phosphate from HPt in the absence of CK (20).

To understand the *in planta* role of AHK1 in osmotic stress and ABA signaling, as well as in plant development, we used both gain-of-function and loss-of-function genetic approaches. Multiple mutants of *ahk1*, *ahk2*, *ahk3*, and *cre1* were constructed to elucidate the function of AHK1 in plant growth and development. To investigate the possible involvement of additional NER HKs, including CK receptor HKs, in stress and ABA signaling, we examined the functions for all of the remaining NER HKs as osmosensors in the *sln1 sho1* yeast mutant. We also studied the expression of genes encoding the NER HKs under various stress conditions and analyzed the ABA-, drought-, and high-salinity-responsive phenotypes of the *ahk2*, *ahk3*, and *cre1* single mutants as well as the dwarf *ahk2 ahk3* double mutant. Microarray analyses were then carried out to analyze the function of AHK1 and CK HKs in the regulation of stress-related transcriptional networks. On the basis of our results, we discuss the functions of

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Data deposition: The complete set of microarray data has been deposited in the European Bioinformatics Institute ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession nos. E-MEXP-1154 and MEXP-1155).

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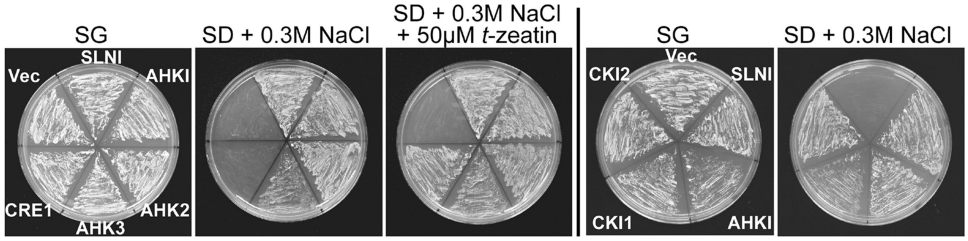

Fig. 1. The NER HKs act as osmosensors in yeast. cDNA fragments encoding the NER HKs were cloned into the YepGap vector, and the plasmids were introduced into the yeast *sln1 sho1* double mutant. Yeast transformants were investigated on various media as indicated. SG medium contains galactose and raffinose instead of glucose (SD).

AHK1, AHK2, AHK3, and CRE1 in ABA and osmotic stress signalings.

Results

The NER Kinases Confer High-Osmolarity Tolerance to the *sln1 sho1* Yeast Double Mutants. The two upstream branches SLN1 and SHO1 in the HOG pathway are redundant and respond independently to the osmotic status of the environment. Under high-osmolarity conditions, mutants lacking both SLN1 and SHO1 are lethal (6). We have previously shown that AHK1 can complement the function of SLNI. Specifically, introduction of AHK1 into the *sln1 sho1* yeast mutant allowed normal growth and activation of the HOG1 MAPK cascade under high osmolarity (7). We were interested to determine whether the remaining NER kinases have catalytic activity similar to those of AHK1 and SLN1 under high-salinity conditions. Therefore, we introduced the AHK2, AHK3, CRE1, CKI1, and CKI2 cDNAs into the *sln1 sho1* mutant. Yeast transformants containing AHK2, AHK3, CKI1, or CKI2 grew as well as those having AHK1 or SLN1 on minimal medium containing 0.3 M NaCl. When CRE1 was introduced into the yeast mutant, transformants could grow under high-salt concentration only in the presence of CK (Fig. 1). The results indicated that the NER kinases can activate the HOG1 pathway, giving rise to high-osmolarity tolerance to the *sln1 sho1* mutant. Similarly, we also tested the ability of the ER HKs in yeast by transforming the *sln1 sho1* mutant with cDNAs encoding all five ER HKs. None of the ER HKs were able to complement the SLN1 function even in the presence of amynocyclopropane carboxylic acid (data not shown).

Expression of the NER HK Genes. To examine the *in planta* function of the NER HKs under abiotic stresses, we initially analyzed the expression patterns of the NER HK genes under various stresses and hormone treatments by RNA gel blot hybridization (Fig. 2). All four *AHK1*, *AHK2*, *AHK3*, and *CRE1* transcripts were rapidly induced by dehydration just after 10 min. Expression of *AHK2* also appeared to be influenced by NaCl and ABA treatments. Furthermore, induction of the *AHK3* mRNA was observed during high salinity and cold stresses. Because expression of *CKI1* and *CKI2* could not be detected by Northern blot analysis, quantitative real-time PCR (qRT-PCR) was used to examine the expression of these two genes. We found that expression of *CKI1* and *CKI2* was not induced by any treatment (data not shown). These results indicated that among six NER HKs, *AHK1*, *AHK2*, *AHK3*, and *CRE1* may function during abiotic stress responses.

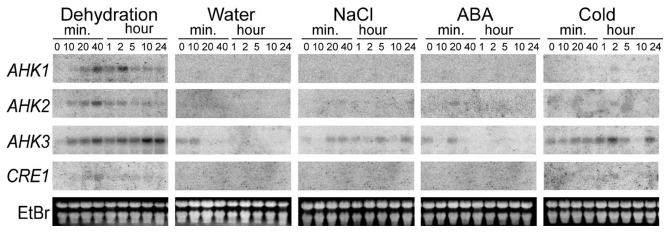

Fig. 2. Expression of NER HK genes under various stress treatments. Twenty micrograms of RNA of 3-week-old plants, which had been treated as indicated, were subjected to RNA blot hybridization with gene-specific probes. EtBr, ethidium bromide. The expression of *CKI1* and *CKI2* was not detectable by RNA gel blot hybridization (data not shown).

Gain of Function of AHK1 Enhances Drought Tolerance in *Arabidopsis*. To investigate whether AHK1 functions in stress signaling, we generated *Arabidopsis* transgenic plants in which *AHK1* was overexpressed by using its own promoter. To check the expression level of the *AHK1* in the transgenic plants under dehydration, 16 independent transgenic lines were subjected to Northern blot analysis. Two stable lines showing higher dehydration-induced *AHK1* transcript were chosen for further studies (Fig. 3 *A* and *B*). Under normal conditions, these Pro_{AHK1}:*AHK1* plants displayed similar morphological phenotypes regarding the size of rosette leaves and inflorescences [supporting information (SI) Fig. 8]. The drought tolerance of the Pro_{AHK1}:*AHK1* plants was compared with that of the control plants as a means to assess the effect of *AHK1* overexpression on stress tolerance. More than 75% of the plants overexpressing *AHK1* survived as compared with ≈18% of the control plants (Fig. 3 *C–E*). These data suggest that AHK1 plays an important role, probably as a positive regulator, in drought stress signaling.

Isolation of *ahk1* Knockdown Mutants for the Loss-of-Function Approach. In our next line of investigation, we searched for *ahk1* mutants to further dissect the function of AHK1 in stress signaling by using a loss-of-function approach. We identified two *ahk1* knockdown mutants—one T-DNA insertion line and one Ds transposon insertion line—from data banks. Although the T-DNA and Ds transposon insertions happened at −215 and −246, respectively, from putative transcription start, RNA gel blot and qRT-PCR confirmed that *AHK1* expression was markedly reduced in both mutants (SI Fig. 9 *A–C*). Phenotypic analyses for both lines suggested that these mutants are not affected in growth or morphology under normal conditions (SI Fig. 9 *D* and *E*).

AHK1 Positively Regulates Plant Growth. Like the *ahk1* mutant, the *ahk2*, *ahk3*, and *cre1* mutants did not alter significantly morphological phenotypes (9, 10). However, in comparison to WT plants, the *ahk2 ahk3* double and the *ahk2 ahk3 cre1* triple mutants displayed dwarf phenotypes (9, 10) (SI Fig. 10). Because expression of *AHK1* is induced by CK as that of CK receptor HKs (data not shown), we were interested in carefully determining whether AHK1 is involved in regulation of plant growth. We constructed multiple mutants of *ahk1*, *ahk2*, *ahk3*, and *cre1* in the same ecotype Col-0 background in every possible combination, and the morphological phenotypes of the multiple mutants were carefully examined. In comparison to both WT and the *ahk2*

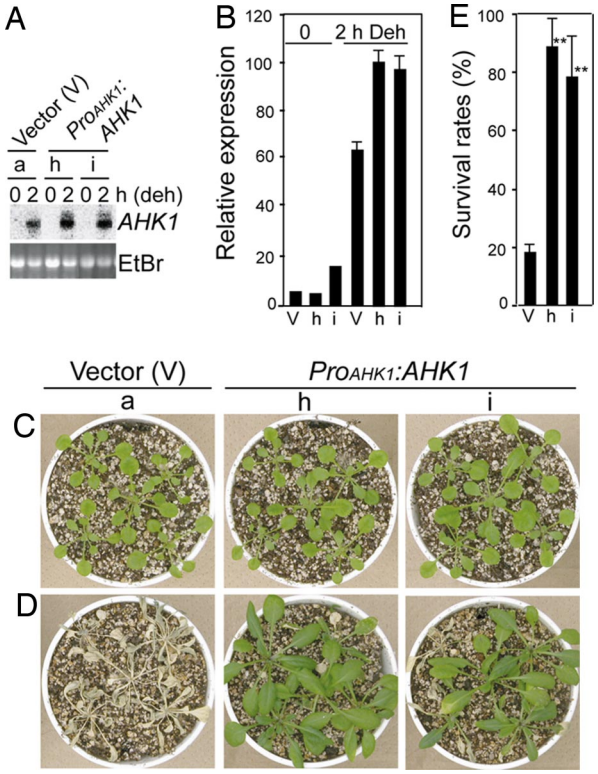

Fig. 3. Drought-tolerant phenotype of P_{AHK1}:*AHK1* transgenic plants. (*A*) Expression of *AHK1* in P_{AHK1}:*AHK1* plants as shown by RNA gel blot hybridization using the AHK1-specific probe. EtBr, ethidium bromide. (*B*) Expression of *AHK1* in P_{AHK1}:*AHK1* plants as determined by qRT-PCR. The highest expression level was set to 100. The data represent the means and standard errors of three replications. (*C*) Vector control and P_{AHK1}:*AHK1* plants (*Center* and *Right*) were grown on a GM-Bar [selective germination medium for *bar* gene (5 μg/ml phosphinotrycin)] plate for 8 days, then transferred to GM plates and grown for an additional 15 days. Thereafter, the plants were transferred to soil and grown for an additional 4 days. (*D*) A drought stress was then imposed by withholding water until the majority of control plants were dead. Photos were taken 3 days subsequent to rewatering the drought-stressed plants. (*E*) Survival rates and standard deviations (error bars) were calculated from results of three independent experiments ($n = 20$ for each experiment). Asterisks indicate significantly higher survival rates than WT plants as determined by χ^2 test ($P < 0.001$).

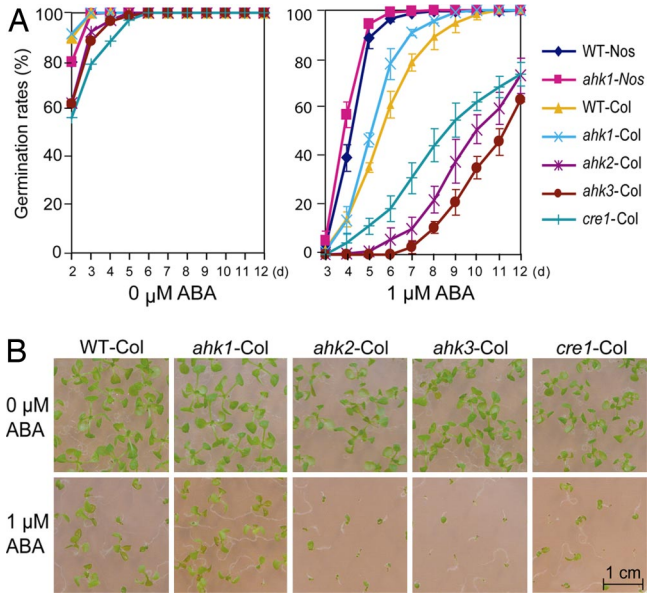

Fig. 4. Response of *ahk1*, *ahk2*, *ahk3*, and *cre1* mutants to ABA treatment. (*A*) Seeds were sown on GM/1% sucrose with or without the addition of 1 μM ABA. Germination rates were quantified at the indicated time points by counting the number of opened cotyledons. Standard deviations (error bars) were calculated from results of three independent experiments ($n > 50$ for each experiment). (*B*) Plates in *A* were incubated at 22°C for 12 days. Photographs were taken to document phenotypes.

ahk3 background, down-regulation of *AHK1* expression in the *ahk2 ahk3* background significantly reduced the size of the plant and retarded growth (SI Fig. 10). These data demonstrated that AHK1 has an essential redundant role in regulating plant growth and development.

Function of AHK1 and Other NER HKs in ABA Signaling. Because osmotic stress and ABA signaling are closely related, we examined the ABA sensitivities of the *ahk1* mutants to determine whether the AHK1 has any function in ABA signaling. The germination rate of the *ahk1* mutants was higher in the presence of ABA (Fig. 4*A*), indicating that *ahk1* mutants were ABA-insensitive. Thus, AHK1 appears to function as a positive regulator in ABA signal transduction. We were interested to determine how the remaining stress-responsive NER HKs (Fig. 2) respond to ABA. We used a germination assay to analyze the ABA sensitivities for the *ahk2*, *ahk3*, and *cre1* mutants. Unlike the *ahk1* mutants, the *ahk2*, *ahk3*, and *cre1* mutants were strongly sensitive to ABA (Fig. 4). The *ahk3* mutant was most sensitive to ABA, and *cre1* was the least sensitive. However, the response of *cre1* was still significantly higher than WT plants (Fig. 4*A*). In contrast with AHK1, these data indicated that AHK2, AHK3, and CRE1 may act as negative regulators in ABA signaling.

***ahk1* Mutant Displays a Stress-Sensitive Phenotype.** Using a transgenic approach, we have previously shown that the drought-dependent overexpression of *AHK1* enhances drought tolerance. Thus, we were interested to determine how the *ahk1* mutant responds to drought stress. Under drought stress, fewer *ahk1* plants survived than WT plants, indicating a clear drought sensitivity for *ahk1* mutant (Fig. 5 *A–C*). In addition, the relative water content in the *ahk1* mutant plants decreased more rapidly than in WT (Fig. 5*D*). Moreover, the *ahk1* mutants were more sensitive to high-salinity stress than WT (Fig. 5 *E* and *F*). Collectively, these loss-of-function studies strengthen the results for the gain-of-function studies demonstrating that AHK1 functions as a positive regulator in osmotic stress signaling in *Arabidopsis*.

AHK1 Down-Regulates Many Stress- and ABA-Responsive Genes Under Drought Stress. To understand the entire transcriptional network of *AHK1*, we compared the expression profiles in the 2-week-old *ahk1* mutant with that of WT under unstressed conditions using an Agilent *Arabidopsis* 2 22K oligonucleotide array. A total of 16 and 9 genes were down- and up-regulated, respectively, with a ratio of >2 (SI Table 1 and SI Fig. 11). Most of the down-regulated genes were induced by either stresses or ABA (SI Table 1 and SI Fig. 11*B*). Thus, it was conceivable that expression of many stress-inducible genes would decrease in the *ahk1* mutant when exposed to stresses. Therefore, we performed microarray analyses to identify down-regulated genes during dehydration stress in *ahk1* mutant. Both the *ahk1* and WT plants were dehydrated for either 2.5 h or 9 h before the microarray experiments. Overall there were 190 and 120 genes down-regulated with a ratio of >2 in 2.5-h and 9-h dehydrated *ahk1* plants, respectively. Among the down-regulated genes were many stress- and ABA-responsive genes (SI Tables 2 and 3 and

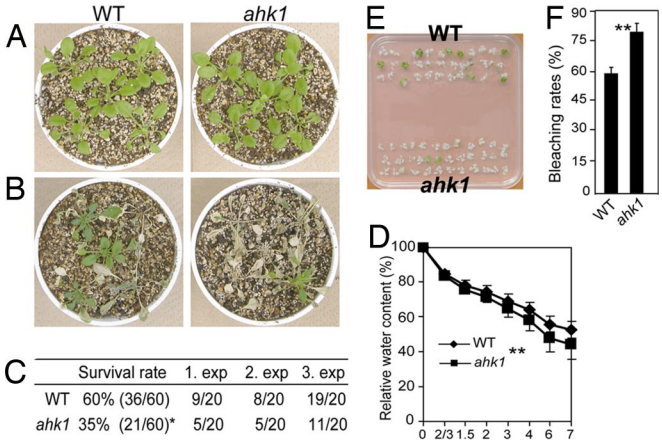

Fig. 5. Sensitivity of the *ahk1* mutant to drought and salt stresses. (*A*) Fully watered control plants. (*B*) Plants exposed to drought stress. Photos were taken 3 days after the stressed plants were rewatered. (*C*) Number of surviving plants from three independent stress experiments ($n = 20$). The percentage of survivors and the numbers of survivors per total number of tested plants are indicated. The asterisk indicates significantly lower survival rates than WT as determined by χ^2 test ($P < 0.01$). (*D*) Measurement of relative water content from 4-week-old soil-grown plants. Detached leaves were dehydrated, and the relative water content was measured at the indicated time points. Error bars represent standard deviation ($n = 10$). Asterisks indicate a significant difference as calculated by Student's *t* test ($P < 0.005$). (*E*) Evaluation of salinity stress in plate conditions. Plants were grown on GM plates for 10 days and were transferred on 0.5× Murashige and Skoog medium/200 mM NaCl medium for ≈8–9 days. (*F*) Bleaching rates and standard deviations (error bars) were calculated from three independent experiments ($n > 30$). Asterisks indicate significantly higher bleaching rates than WT as determined by χ^2 test ($P < 0.001$).

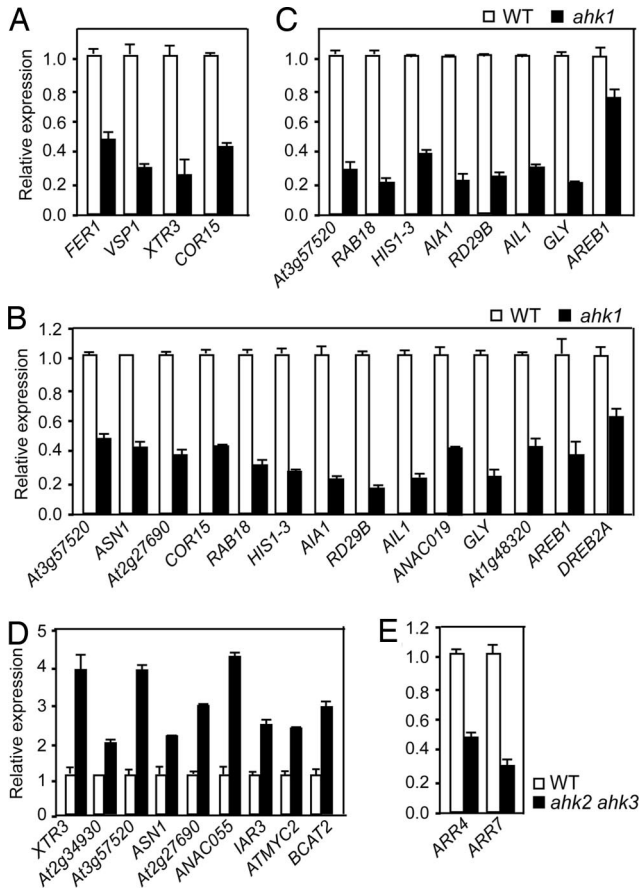

Fig. 6. Confirmation of microarray data by qRT-PCR analysis. (*A*) Down-regulated genes in the unstressed *ahk1* plant. (*B*) Down-regulated genes in *ahk1* plants after 2.5 h of dehydration stress. (*C*) Down-regulated genes after of 9 h of dehydration stress in *ahk1* plants. (*D*) Up-regulated genes in the unstressed *ahk2 ahk3* plants. (*E*) Down-regulated genes in the unstressed *ahk2 ahk3* plants. The relative expression level was normalized to 1 in the WT plant. Data represent the means and standard errors of three replicate reactions.

SI Figs. 12 and 13 *A* and *B*). Many of the down-regulated ABA-responsive genes are downstream targets of the ABA- and dehydration-responsive AREB1 transcription factor (TF) (21) (SI Tables 2 and 3). Other stress-responsive genes are downstream targets of other important TFs such as ANACs and DREB2A. Both the AREB1 and ANAC TFs play an essential role not only in osmotic stress, but also in ABA signaling (21, 22). Some representative downstream genes of these TFs were marked in SI Tables 2 and 3. qRT-PCR was performed to confirm the expression of several genes that were identified by the microarray analyses (Fig. 6 *A–C*). Because the 22K oligo array does not contain the oligo designed for *AREB1*, we examined the expression of *AREB1* in the dehydrated *ahk1* mutant plants by qRT-PCR (Fig. 6 *B* and *C*). Our results indicated that *AREB1* expression was significantly reduced during dehydration in the *ahk1* mutant, especially after 2.5 h of dehydration. Consequently, this would lead to the down-regulation of its downstream genes. These results collectively demonstrated that the stress-sensitive and ABA-tolerant phenotype of the *ahk1* mutant was due to the decreased expression of a set of genes that function in osmotic and ABA signal transduction pathways.

AHK2, AHK3, and CRE1 HKs Act as Negative Regulators in Osmotic Stress Responses. Our results demonstrated that the functions of AHK2, AHK3, and CRE1 in ABA signal transduction are opposite that of AHK1. Thus, we hypothesized that these CK receptor HKs may also play an opposite role in stress responses. To test this hypothesis, we first compared the level of drought and salt stress tolerance of the *ahk2*, *ahk3*, and *cre1* mutants, as well as the *ahk2 ahk3* double mutant, to WT plants. The results showed a strong drought and salinity tolerance for both *ahk2* and *ahk3* mutants (Fig. 7). The *ahk2 ahk3* double mutant was even more tolerant to drought and salt stresses than the respective single ones (Fig. 7), suggesting a combinatory function of AHK2 and AHK3 in osmotic stress signaling. Both the *cre1* mutant and WT responded similarly to drought and salt stresses without CK (Fig. 7). However, in the presence of CK, the *cre1* mutant displayed a strong salt stress-tolerant phenotype (Fig. 7 *F* and *G*). These results collectively suggested that all of the AHK2, AHK3, and CRE1 function in stress responses and that they act as negative regulators. Comparative genome-wide expression analysis of *ahk2 ahk3* double mutants and WT plants identified 40 genes that were up-regulated with a ratio of >2 in the *ahk2 ahk3* mutant during unstressed conditions (SI Table 4). The majority of these up-regulated genes are stress- and/or ABA-responsive (SI Table 4 and SI Fig. 14*A*). These data are in good accordance with the stress-tolerant and ABA-sensitive phenotype of the mutants. Several genes were chosen to confirm the reliability of the microarray data by qRT-PCR analysis (Fig. 6 *D* and *E*). Among the 48 genes that were down-regulated with a ratio of >2, two response regulators (ARR4 and ARR7) are worthy of mentioning (SI Table 4 and SI Fig. 14*B*). Interestingly, we found many auxin-responsive genes that were down-regulated in the *ahk2 ahk3* mutant, which may partially explain its dwarf phenotype (SI Table 4 and SI Fig. 14*B*).

Discussion

We previously demonstrated that AHK1 functions as an osmosensor in yeast (7). In this report we used both gain- and loss-of-

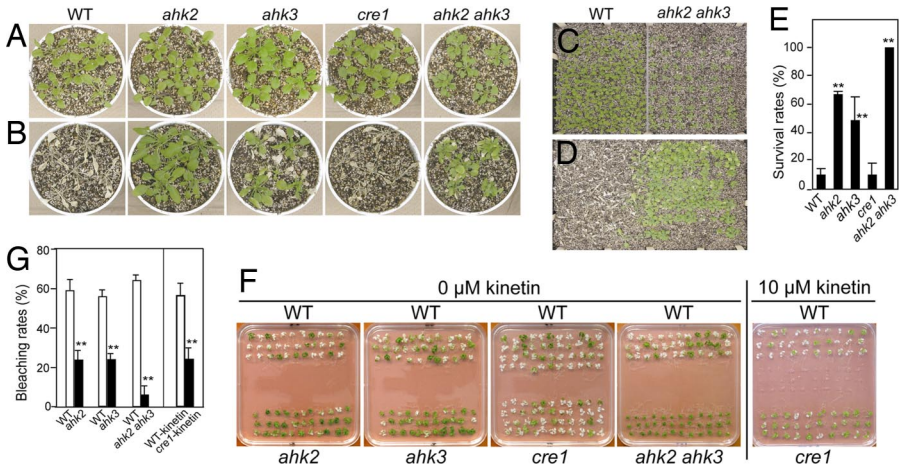

Fig. 7. Stress-tolerant phenotype of *ahk2*, *ahk3*, *cre1*, and *ahk2 ahk3* mutants. (*A*) Fully watered control plants. (*B*) Plants exposed to drought stress. Photos were taken 3 days after plants were rewatered. (*C*) Two-week-old WT and *ahk2 ahk3* mutant plants were transferred from GM plates to the same soil tray and grown for an additional week. (*D*) Drought stress was imposed by withholding water for 18 days and was followed by a period of rewatering for 3 days. (*E*) Survival rates and standard deviations (error bars) were calculated from three independent experiments ($n > 20$). Asterisks indicate significantly higher survival rates than WT plants as determined by χ^2 test ($P < 0.001$). (*F*) Evaluation of salinity stress in plate conditions. Plants were grown on GM plates and subsequently transferred on a 0.5× Murashige and Skoog medium plate containing 200 mM NaCl in the absence or presence of 10 μM kinetin. Observations were taken ≈8–9 days subsequent to the transfer. (*G*) Bleaching rates and standard deviations (error bars) calculated from three independent experiments ($n > 30$). Asterisks indicate significantly lower bleaching rates than WT as determined by χ^2 test ($P < 0.001$).

function approaches to clearly demonstrate that AHK1 can act as a positive regulator in osmotic stress signaling in *Arabidopsis* plants. Overexpression of *AHK1* using its native promoter increased *AHK1* expression under dehydration stress and consequently improved the drought tolerance of transgenic plants (Fig. 3). In agreement with the gain-of-function study, *ahk1* mutants were sensitive to both drought and salt stress (Fig. 5).

The relative water content of the *ahk1* mutant decreased more quickly than that of the WT plant (Fig. 5*D*). In addition, *AHK1* mRNA was readily expressed in leaves and increased after stress treatment (7). These data suggest that AHK1 may function in leaves. Because the *ahk1 ahk2 ahk3* triple mutant has smaller leaves and shorter inflorescences than the *ahk2 ahk3* double mutant, it is likely that AHK1 also functions in the shoot as well (SI Fig. 10). At the same time this result also demonstrates the essential role of AHK1 in plant growth and development, similar to AHK2, AHK3, and CRE1 (9, 10). The *ahk1* mutant showed an ABA-insensitive phenotype at the germination stage (Fig. 4) and exhibited drought stress sensitivity (Fig. 5). These results collectively support the hypothesis that AHK1 is an important positive regulator of the ABA signal transduction pathway.

Microarray analysis of the *ahk1* knockdown mutant under both unstressed and stressed conditions revealed a down-regulation of many stress- and/or ABA-inducible genes. Among these genes were important TFs that function in both ABA-dependent and ABA-independent pathways, such as AREB1, ANAC, and DREB2A (SI Tables 1–3 and SI Figs. 11–13) (21–25). These results suggest that AHK1 functions upstream of these important TFs in osmotic stress signaling pathways. The transcriptome analysis results also indicate that AHK1 positively regulates many ABA-inducible genes and controls stress responses in *Arabidopsis* through both ABA-dependent and ABA-independent signaling pathways.

Similar to AHK1, the function of SLNI was complemented in yeast by AHK2, AHK3, CRE1, CKI1, and CKI2, thereby indicating that they have HK activity (Fig. 1). Among three CK receptor HKs, only CRE1 required CK for its activation in yeast (Fig. 1). It has been previously reported that the *sln1* yeast mutant carrying either AHK2 or AHK3 could grow faster with CK than without it (20), implying that all AHK2, AHK3, and CRE1 are involved in CK signaling. These data are consistent with reports regarding their *in planta* functions. Loss-of-function studies demonstrated that AHK2, AHK3, and CRE1 function as CK receptors in CK signaling (9, 10).

In contrast with the *ahk1* mutant, germination assays demonstrated that the *ahk2*, *ahk3*, and *cre1* mutants are hypersensitive to ABA (Fig. 4). These results suggest that the CK receptor HKs function as negative regulators in ABA signal transduction. CRE1 was functional in the absence of exogenous CK in the assays. Thus, CRE1 might be activated by endogenous CK, which was increasingly synthesized upon seed imbibition in the embryonic axis of germinating seeds and decreased with seedling development (26). It is possible that the increase of CK content might antagonistically regulate ABA synthesis, inhibit ABA action in germinating seeds through the CK receptor HKs, and consequently alleviate the inhibition effect of ABA on seed germination. A detailed functional analysis of these HKs in ABA signaling mutants may provide us further information concerning their involvement in regulating the interactions between CK and ABA signaling pathways.

The expression of *AHK2*, *AHK3*, and *CRE1* was induced by stresses (Fig. 2), suggesting that these CK receptor HKs play an important role not only in CK response but also in stress response. Indeed, the *cre1* mutant displays a salt-tolerant phenotype in the presence of exogenous CK. Furthermore, the *ahk2*, *ahk3* single, and *ahk2 ahk3* double mutants showed strong stress tolerance against both drought and high-salinity stresses even without CK (Fig. 7). In the absence of CK, CRE1 is locked in its phosphatase form, exhibiting phosphatase activity instead of phosphorylation activity (20). In the presence of CK, CRE1 changes to its HK form in a CK-dependent manner and can function as a negative regulator of stress signaling. Consequently, CK makes the WT more sensitive to salt stress than the *cre1* mutant. Hence, to our knowledge, this is the first direct evidence to demonstrate the involvement of CK, perhaps as a negative regulator, in stress signaling. AHK2 and AHK3 was able to increase the *ARR5* transcript in the presence of CK, indicating that CK enhanced HK activity of AHK2 and AHK3 in *Arabidopsis* (10). This suggests the important role of CK for stimulating the functions of AHK2 and AHK3 in stress responses as well. In agreement with these observations, the WT plants appeared paler with the addition of exogenous CK (Fig. 7). Our results collectively showed evidence that, whereas AHK1 functions

as a positive regulator, AHK2, AHK3, and CRE1 act as negative regulators in stress responses in a CK-dependent manner.

Microarray analysis has been critical for understanding the diversity of these HKs and has provided a biological insight into the nature of signaling. Under normal conditions, many stress- and/or ABA-responsive genes, including *ANAC055* and *ATMYC2*, were up-regulated in the *ahk2 ahk3* double mutant (SI Table 4, Fig. 6*D*, and SI Fig. 14*A*). Importantly, overexpression of these genes significantly improves drought tolerance and/or ABA sensitivity of *Arabidopsis* plants (25, 27). These data strongly support the hypothesis that CK receptor HKs function as negative regulators in ABA signaling, through which these HKs negatively control osmotic stress responses. In addition, the knockout of *ahk2* and *ahk3* suppressed the expression of the type-A ARR4 and ARR7 genes (SI Table 4 and Fig. 6*E*). Because HKs direct the phosphorelay through response regulators, these data suggest that ARR4 and ARR7 may potentially function in ABA and stress signalings. Similar to CK signaling (28), the type-A ARRs, being repressors, might provide a negative feedback regulation of stress signaling. Thus, suppression of the type-A ARR expression might alleviate the effect of negative feedback regulation, allowing resetting and/or fine-tuning of the physiological state of the cell.

In conclusion, AHK1 functions in osmotic stress signaling, germination, and plant growth as a positive regulator in plants. In contrast, CK receptor HKs (AHK2, AHK3, and CRE1) are also involved in the same processes, except in plant growth, as negative regulators. Overall, it is apparent that cross-talk may exist among CK, ABA, and stress signaling pathways.

Materials and Methods

Plant Materials and Generation of Transgenic Plants. *Arabidopsis* plants were grown, transformed, and treated as previously described (25). The 1,780-bp *AHK1* promoter fragment upstream of the initiation codon and the *AHK1* ORF were cloned into the pGreenII0229 vector (www.pgreen.ac.uk).

Screening for T-DNA Insertion Mutants. The *ahk1* T-DNA insertion mutant in the Col-0 ecotype (accession no. SALK_000977) was obtained from the Salk Institute (http://signal.salk.edu). The *ahk1* Ds-insertional mutant in the Nossen ecotype (DS11-4433-1) was obtained from RIKEN (www.brc.riken.go.jp/inf/en/index.shtml). Primers that were used to screen homozygous mutants are listed in SI Table 5. The set of *ahk2*, *ahk3*, and *cre1* single and multiple mutants in the Col ecotype were obtained from ref. 9. A series of multiple *ahk1*, *ahk2*, *ahk3*, and *cre1* mutants in the same backgrounds were constructed by genetic crosses and screened by using primers listed in either SI Table 5 or ref. 9.

Complementation Analysis of the Yeast *sln1 sho1* Double Mutant. The cDNAs encoding the NER HKs and the ER HKs were cloned into YEpGAP vector. The resulting plasmids were introduced into *sln1 sho1* mutant and examined as previously described (7).

Transcriptional Analyses. Total RNA was extracted with TRIzol Reagent according to the supplier's instructions (Invitrogen). RNA gel blot hybridization was performed as previously described (25). cDNA synthesis and qRT-PCR were carried out according to ref. 24. The primer pairs that were used in qRT-PCR and prepared specific probes are listed in SI Table 5.

Microarray Analysis and Data Mining. Total RNA was isolated from plants and used for the preparation of Cy5- and Cy3-labeled cDNA probes. The reproducibility of the microarray analysis was assessed by incorporating a dye swap into the experimental plan. Furthermore, only genes showing a signal intensity $>$1,000 in at least one experiment were analyzed further. Microarray analysis and data mining were carried out as previously described (23). Responsiveness of genes to different hormones was analyzed by using Genevestigator (www.genevestigator.ethz.ch).

Stress Tolerance Test. Drought stress tolerance of transgenic or mutant plants was conducted as described (25). For salt stress tolerance, 10- to 11-day-old plants were transferred onto 0.5$\times$ Murashige and Skoog plates containing 200 mM NaCl. The plates were maintained at 22°C under a 16-h light/8-h dark cycle until visual symptoms were observed.

Analysis of Plant–Water Relations. Relative water loss was determined as previously described (21).

Germination Assay. Seeds were sterilized and plated on GM medium containing 1% sucrose and 0 or 1 μM ABA. After 4 days of stratification at 4°C in the dark, plates were incubated at 22°C under a 16-h light/8-h dark cycle. The appearance of cotyledons was considered to be germination.

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