Role of cytokinin responsive two-component system in ABA and osmotic stress signalings

Lam-Son Phan Tran,[1,*] Kazuo Shinozaki[2] and Kazuko Yamaguchi-Shinozaki[3]

[1]Signaling Pathway Research Unit; [2]Gene Discovery Research Group, RIKEN Plant Science Center; Tsurumi, Yokohama, Japan [3]Graduate School of Agricultural and Life Science; University of Tokyo; Tokyo, Japan; and Japan International Center of Agricultural Sciences; Tsukuba, Ibaraki Japan

The two-component signaling systems (TCSs), which mediate the histidine-aspartate signaling, control diverse biological processes of many organisms, including cell division, cell growth and proliferation and responses to environmental stimuli and growth regulators. We have provided in planta evidence that the cytokinin (CK) responsive TCS mediates abscisic acid (ABA) and osmotic stress responses. By using loss-of-function approach we have demonstrated that the three cytokinin (CK) receptor histidine kinases AHK2, AHK3 and AHK4/CRE1 act as negative regulators in ABA, drought and high salinity stress signalings. Genome-wide expression profiling of the stress-tolerant *<ahk2,3>* double mutant suggested that CK receptor kinases mediate osmotic stress response in both an ABA-dependent and ABA-independent manner. Additionally, we showed evidence for the role of CK in mediating stress responses, judging from the fact that AHK4 requires the CK to function as a negative regulator in osmotic stress response. Our results suggested that cross-talk exists among CK, ABA and osmotic stress signaling pathways, and that CK signaling and CK metabolism may play crucial roles not only in plant growth and development but also in osmotic stress signaling.

Water deficit and high salinity stress limit crop productivity worldwide. In response to these stresses, plants activate a number of defense mechanisms that function to increase tolerance to the adverse conditions.[1] Phosphorylation, which is mediated by two-component systems (TCSs) or histidine-to-aspartate (His-Asp) phosphorelays, is a key mechanism for stress signal transduction in a cell.[2] A great number of TCSs have been identified and characterized not only in many prokaryotic organisms but also in key plant species including *Arabidopsis thaliana*, rice (*Oryza sativa*) and *Lotus japonicus*.[3-7] Increasing evidence has indicated that the Arabidopsis TCS pathways are involved in response to environmental stimuli, ethylene signaling, light perception, circadian rhythm and cytokinin (CK)-dependent processes which include shoot and root development, vascular defferentiation and leaf senescence.[4,8-10] Genome-wide analysis supports the existence of eight histidine kinases (HKs) in Arabidopsis. Among the HKs, ETR1 and ERS1 act as ethylene receptor HKs, while others including AHK1/ATHK1, AHK2, AHK3, AHK4/CRE1, CKI1 and AHK5/CKI2 are non-ethylene receptors.[10] AHK1 has been shown to function as positive regulator in abscisic acid (ABA) and osmotic stress signalings in both ABA-dependent and ABA-independent pathways.[11,12] CKI1 is implicated in megagametophyte development, and together with AHK2 and AHK3 it is also required for vascular bundle formation in Arabidopsis.[13,14] AHK5 has been shown to be involved in root elongation through an ETR1-dependent ABA and ethylene signaling pathway.[15] AHK5 may also have function in mediating H_2O_2-dependent processes in stomatal guard cells.[16]

In Arabidopsis, CK signaling is mediated by a multi-step phosphorelay, which is comprised of sensor HKs (AHKs), phosphotransfers (AHPs) and response regulators (ARRs).[10,17] Analysis of *ahk2*, *ahk3* and *ahk4* single, double and triple mutants suggest that the AHK2, AHK3 and AHK4 function as CK receptor HKs, and act as positive regulators in CK signaling and plant growth.[18-20] Interestingly, AHK4 exhibits a dual function depending on the presence or absence of CK. In the presence of CK, AHK4 phosphorylates the AHP. Conversely, it removes phosphate from AHP in the absence of CK.[21] The five authentic AHPs (AHP1-AHP5) and a pseudo AHP (AHP6) are involved in mediating the transfer of the phosphoryl group from the AHKs to the ARRs.[17] Analysis of *ahp* multiple mutants indicate that most of the AHPs act as redundant, positive regulators of CK signaling and affect many aspects of plant development.[22] In contrast, the pseudo AHP6 plays an inhibitory role in CK signaling.[23] As for the ARRs, the typical ARRs are classified into either type-A (9 members) or type-B (11 members) or type-C (2 members).[10,17] The 11 type-B ARRs, which are not induced by CK, are transcription factors that contain receiver domain and a large C-terminal region harboring a Myb-like DNA-binding domain and a glutamine-rich domain. Analysis of multiple type-B *arr* mutants demonstrates that type-B ARRs act as positive regulators of CK signaling.[24] By contrast, most of type-A ARRs, which have short C-terminal domains and are rapidly transcriptionally upregulated by CK treatment, are partially redundant negative regulators of CK signaling.[10,17]

Since AHK1 was first discovered to function in osmotic stress response by its ability to complement the function of the yeast SLN1 histidine kinase in the <*sln1 sho1*> yeast double mutant, which is lethal under high-osmolarity conditions due to the disruption of both *SLN1* and *SHO1*,[25] we were interested in testing whether the cytokinin receptor AHK2, AHK3 and AHK4 have catalytic activity similar to those of AHK1 and SLN1 under high-salinity conditions. Therefore, we introduced the *AHK2*, *AHK3* and *AHK4* cDNAs into the <*sln1 sho1*> mutant for testing their possible function in complementation of SLN1 in high-osmolarity conditions.[25,26] Yeast transformants containing *AHK2*, *AHK3* grew as well as those having *AHK1* or *SLN1* on minimal medium containing 0.3 M NaCl. When *AHK4* was introduced into the yeast mutant, transformants could grow under high-salt concentration only in the presence of CK. Our results indicated that the CK receptor HKs were able to complement the function of SLN1 similar to AHK1, giving rise to high-osmolarity tolerance to the <*sln1 sho1*> mutant.

Next, to examine the potential functions of the CK receptor HKs in abiotic stress signaling, we initially analyzed the expression patterns of genes encoding CK receptor HKs under various stresses and hormone treatments. The transcripts of all three *AHK2*, *AHK3* and *AHK4* were rapidly induced by dehydration. 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 perhaps cold stress. These results suggested that these CK receptor HKs play an important role not only in CK response but also in stress response.

To further confirm the stress responsive role of CK receptor HKs in osmotic stress responses, we first compared the level of drought and salt stress tolerance of the *ahk2*, *ahk3* and *ahk4* single mutants, as well as the <*ahk2,3*> double mutant, to WT plants. The results showed a strong drought and salinity tolerance for both *ahk2* and *ahk3* single mutants. The <*ahk2,3*> mutant was even more tolerant to drought and salt stresses than the respective single ones, suggesting a combinatory function of AHK2 and AHK3 in osmotic stress signaling. Both the *ahk4* mutant and WT responded similarly to drought and salt stresses without CK. However, in the presence of CK, the *ahk4* mutant displayed a strong salt stress-tolerant phenotype. In the absence of CK, AHK4 is locked in its phosphatase form, exhibiting phosphatase activity instead of phosphorylation activity.[21] In the presence of CK, AHK4 changes to its HK form in a CK-dependent manner and can function as a negative regulator of stress signaling. To our knowledge, this is the first direct evidence to demonstrate the involvement of CK in stress signaling as a potential mediator.

These results collectively suggested that all of the AHK2, AHK3 and AHK4 HKs function in stress responses and that they act as negative regulators. Comparative genome-wide expression analysis of <*ahk2,3*> double mutant and WT plants identified many stress- and/or ABA-responsive genes which are upregulated in <*ahk2,3*> mutant. These results together with ABA-sensitive phenotype of the *ahk2*, *ahk3* and *ahk4* single mutants suggested that AHK2, AHK3 and AHK4 act as negative regulators in both ABA-dependent and ABA-independent pathways.

The fact that the CK receptor AHK2, AHK3 and AHK4 are involved in ABA and osmotic stress signalings as negative regulators strongly indicates that there are cross-talks among CK, ABA and stress signaling pathways. Moreover, our results indicate that CK mediates stress response, demonstrating that CK signaling and CK metabolism may play crucial roles not only in plant growth and development but also in abiotic stress signaling. Recent reports suggested that CKs may be an important signal traveling from roots to shoots, and ABA:CK ratios in xylem sap are important for stress signaling.[27,28] How does the CK affect plant stress response and how do stresses affect CK metabolism, i.e., how do the CK contents are changed under different stress conditions, analysis of the relationship between CK metabolism and stress responses may provide answers to these questions. Functional analysis of genes involved in CK metabolisms, such as genes encoding adenosine phosphate-isopentenyltransferases, which are involved in CK biosynthesis,[29] and genes encoding CK oxidases, which are involved in CK degradation,[30] in stress response will unravel the regulatory role of CK in stress signaling.

By analogy to the CK responsive signaling, we suggest that the stress signaling is mediated by the multistep His-Asp phosphorelay (**Fig. 1**). However, the functions of the AHPs and ARRs in stress response as well as their downstream genes of the phosphorelay remain still unknown. Investigation of various combinations of *ahp* and *arr* mutants may aid
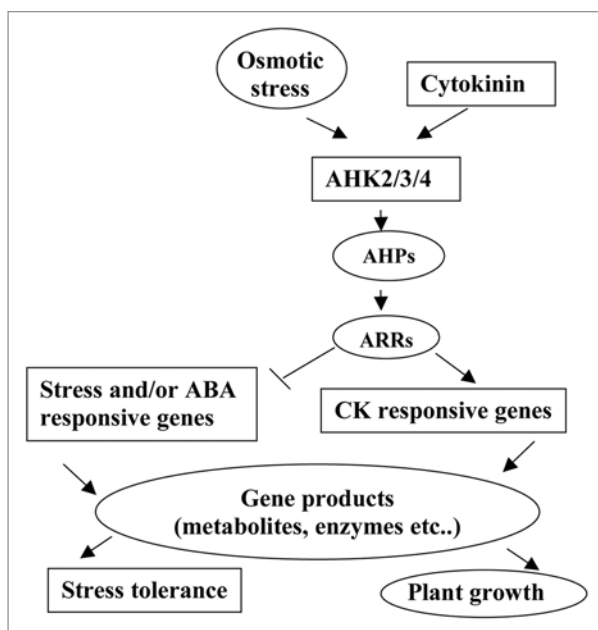
Figure 1. Signal tranduction network in stress responses and plant growth.

in identifying those *AHP* and *ARR* genes involved in stress signaling. Discovery of mechanisms of activation and the targets of the downstream components of the CK responsive phosphorelay in stress signaling is important and challenging goal for study of regulatory network of plant stress response and plant growth.

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