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# A novel histidine kinase gene, *ZmHK9*, mediate drought tolerance through the regulation of stomatal development in *Arabidopsis*

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ABSTRACT

Plants have developed complex signaling networks to regulate biochemical and physiological acclimation, environmental signals were perceived and transmitted to cellular machinery to activate adaptive responses. Here, a novel drought responsive histidine kinase gene was identified and designated as *ZmHK9*. Under normal conditions, *ZmHK9* was predominantly expressed in roots, and the roots of *ZmHK9*-OX transgenic lines are markedly hypersensitive to ABA and ethylene, as compare to wild type. Consistent with its expression induced by PEG and exogenous ABA treatment, promoter sequence of this gene possessed drought and ABA responsive element. Moreover, the transgenic plants were much less affected by drought stress and recovered quickly after rewatering, stomatal complex size and stomatal density in the transgenic plants are significantly smaller and lower than those of the wild-type plants. In addition, ABA induced stomatal closure and the stomatal aperture of *ZmHK9*-OX lines was smaller than that of wild type. Collectively, it can be concluded that *ZmHK9* regulates root elongation, stomatal development and drought tolerance through ABA dependent signaling pathway in *Arabidopsis*.



## 1. Introduction

Drought has been a central topic of plant stress physiology because it significantly reduces plant productivity. During a long history of evolution, plants have developed complex signaling networks to regulate biochemical and physiological acclimation, and environmental signals were perceived and transmitted to cellular machinery to activate adaptive responses (Huai et al., 2009; Xiong et al., 2002). Recent advances in genomic technologies have provided high-throughput integrated approaches to investigate global gene expression responses to drought stress (Bartels and Sunkar, 2005; Chaves et al., 2003; Ishitani et al., 2004). Microarray profiling under drought stress has been carried out in different plant species such as *Arabidopsis* (Kawaguchi et al., 2004; Oono et al., 2003; Seki et al., 2002), rice (Rabbani et al., 2003), barley (Ozturk et al., 2002; Talamé et al., 2007) and wheat (Mohammadi et al., 2007). These studies identified differentially expressed transcripts of genes related to phenotypic and physiological traits, such as abscisic acid (ABA) synthesis and signaling, biosynthesis of osmoprotectants, water uptake and a myriad of transcription factors, which enabled to characterize the functions of these genes by physiological studies and breeding programs (Hayano-Kanashiro et al., 2009).

Signal transduction pathways are the link between the sensing mechanism and the genetic response. It starts with signal perception, followed by the generation of second messengers, which can modulate intracellular $Ca^{2+}$ levels, often initiating a protein phosphorylation cascade that activate target proteins involved in various cellular processes, such as proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Xiong et al., 2002). Molecular analysis has demonstrated the existence of both ABA-dependent and ABA-independent regulatory systems in the transcriptional regulatory networks under drought stress (Yamaguchi-Shinozaki and Shinozaki, 2006). Meanwhile, other plant hormones, such as salicylic acid (SA), ethylene (ET) and jasmonic acid (JA) have been shown to affect abiotic stress responses through interplay with ABA (De Vleesschauwer et al., 2010; Kangasjarvi et al., 2005). Recently, several plant protein kinases have been found to be activated by drought stress. For example, MKK4 has been characterized as the MAP kinase that mediates drought and cold stress and plant

*Abbreviation:* bp, base pair(s); cDNA, DNA complementary to RNA; CT, cycle threshold; DNA, deoxyribonucleic acid; SA, salicylic acid; ET, ethylene; RH, relative humid; JA, jasmonic acid; ABA, abscisic acid; CHASE, Cyclases/Histidine kinases Associated Sensory Extracellular; HK, histidine kinase; dap, days after pollination; DAG, days after germination; OX, overexpression; WT, wild type; ORF, open reading frame; PCR, polymerase chain reaction; UTR, untranslated region.

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response in alfalfa cell (Jonak et al., 1996). 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, whereas *AHK2, AHK3*, and *CRE1* act as negative regulators in stress responses in a cytokinin-dependent manner (Tran et al., 2007).

In maize (*Zea mays* L.), seven genes encoding histidine kinases were cloned and their expression profiles were analyzed (Muñiz et al., 2010). To the best of our knowledge, up to date, the relationship of histidine kinase and abiotic stresses in maize was not characterized in detail. Here, a novel drought-responsive histidine kinase gene in maize, *ZmHK9*, was cloned and transformed into *Arabidopsis* under the control of CaMV35S promoter. Our results indicated that overexpression of *ZmHK9* improved drought tolerance in the *ZmHK9*-OX transgenic *Arabidopsis* and this may be caused by less stomatal number and reduced stomatal complex size.

## 2. Materials and methods

### 2.1. Plant materials

Maize (*Zea mays* L.) inbred line Zong31 was used in this study. Plants were grown in greenhouse on a 16 h light/8 h dark cycle, maintaining a temperature of 29 °C and 25 °C for the light and dark cycles, respectively. Fourteen different tissues from Zong31 were spliced and frozen immediately in liquid nitrogen then stored at −80 °C for further use. For exogenous ABA and PEG treatment, the seedling roots of 8 days after germination (DAG) were submerged into a water solution supplemented with 50 μM trans-ABA (Sigma) and 20% PEG6000 (m/v), respectively. The treated seedlings were harvested at the given time periods, frozen immediately in liquid nitrogen, and stored at −80 °C.

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used as the wild type. Plants were grown in the greenhouse on soil at 20 °C under long-day conditions (16 h light/8 h dark). For *in vitro* seedling assays, seeds were surface-sterilized and cold treated at 4 °C for 3 d in the dark and then exposed to white light. Seedlings were grown at 20 °C on horizontal plates containing MS medium as modified by Kemper et al. (1992), 3% sucrose, and 0.9% agarose (Merck) unless otherwise specified.

### 2.2. Isolation, domain scan and promoter analysis of full-length cDNA of ZmHK9

A local implementation of NCBI BLASTX was used for sequence searching. All publicly known HK genes from *Arabidopsis* (*AHK1*, *AHK5*) were used in initial protein queries. The primer pair was designed in terms of the clone sequence (Gene ID: GRMZM2G025579). The two primer sequences were as follows: forward primer (1F): 5′-ATGTGATCGGAAAACCGTGCTGA-3′; and reverse primer (1R): 5′-CTTTACAATAATTGGATAGTCA-3′. Putative motifs of *ZmHK9* protein were predicted by SMART (Simple Modular Architecture Research Tool). Putative promoter sequences (2000 bp upstream the 5′UTR region) of *ZmHK9* were obtained from the draft maize genome sequence. Database of PlantCARE was used to detect one or more hormone-and stress-responsive elements.

### 2.3. Extraction of total RNA and reverse transcription

Total RNA was extracted using polysaccharide and polyphenol total RNA isolation kit (spin column) (Bioteke). The amount and quality of the total RNA were checked through electrophoresis in 1% agarose gel. The concentration of RNA was measured by spectrophotometer NanoDrop, ND1000 (Nano Drop Technologies). Equal amount of 2 μg total RNA was reverse transcribed to cDNA in 25 μl reaction using M-MLV reverse transcriptase reverse transcriptase (Promega Corporation, USA). Reverse transcription was performed for 60 min at 37 °C with a final denaturation step at 95 °C for 5 min. Aliquots of 2 μl of the obtained cDNA each were subjected to real-time quantitative PCR analysis.

### 2.4. Real-time quantitative PCR

*ZmHK9* gene-specific primers were used for quantitative PCR analysis, and *18s rRNA* was amplified as an endogenous control, cDNAs from three biological samples were used for analysis. The two primer sequences were as follows: sense primer: 5′-TCCTGTCTTGTCGACCATTTT-3′; and anti-sense primer: 5′-AAACCGATGTTGCTCATGAAT-3′. The reaction conditions were performed as follows: 94 °C for 4 min, followed by 40 cycles: 94 °C for 30 s, 66 °C/58 °C 30 s, 72 °C 30 s, and then 72 °C for 5 min. Quantification results were presented in terms of the cycle threshold (CT) value determined according to the manually adjusted baseline. Relative gene expressions in different samples were determined using the method described previously. Briefly, differences between the CT values of target gene and *18s rRNA* were calculated as $\Delta CT = CT_{target} - CT_{18s\ rRNA}$, and expression levels of target genes relative to *18s rRNA* were determined as $2^{-\Delta CT}$. For each sample, PCR was repeated three times, and the average values of $2^{-\Delta CT}$ were used to determine the differences of gene expression.

### 2.5. Plasmid construction and generation of transgenic lines

Total RNA prepared from roots of Zong31 was used as templates to synthesize the first strand of cDNA using oligo-dT primer. The coding region of *ZmHK9* gene was amplified from cDNA described above, and the resulting PCR fragments were cloned into a Gateway entry vector pDNOR221. After confirmation by DNA sequencing, the *ZmHK9* fragments were recombined into pB2GW7 destination vector so that the expression of *ZmHK9* could be driven by CaMV35S promoter. The resulting constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, which was then used for the transformation of

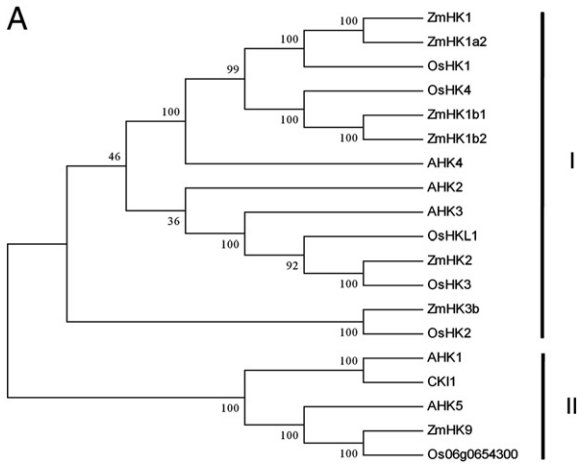


**Fig. 1.** Sequence analysis of *ZmHK9* gene. (A) Phylogenetic relationship of *ZmHK9* with *Arabidopsis* and rice HKs. Multiple sequence alignment was performed using MEGA4.1, and the phylogenetic tree was constructed following the Neighbor-Joining method using amino acid sequences of each HK protein. The accession numbers of selected HKs are listed as follows: *ZmHK1*, NP_001104859; *ZmHK1a2*, NP_001105857; *ZmHK1b1*, NP_001105858; *ZmHK1b2*, NP_001105913; *ZmHK2*, NP_001104866; *ZmHK3b*, NP_001104867; *ZmHK9*, GRMZM2G025579; *AHK1*, NP_565424; *AHK2*, NP_568532; *AHK3*, NP_564276; *AHK4*, NP_565277; *AHK5*, NP_196633; CKI1, NP_182265; OsHK4, NP_001051083; OsHK2, NP_001064436; OsHK3, NP_001045241; OsHK1, NP_001051083; OsHKL1, NP_001066724; Os06g0654300, NP_001058244. (B) Comparison of five HK amino acids in class II. The black boxes indicate identical residues, and gray boxes indicate residues conserved among the three proteins. HK: histidine kinase domain, RL: receiver-like domain and R: receiver domain.