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ORIGINAL ARTICLE

Effect of Abiotic Stresses on Histidine kinases Gene Expression in Zea mays L. cv. SC. 704

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UV-B radiation and osmotic stress (like drought and salinity) have a significant effect on physiology, morphology, biochemistry and molecular biology. To cope with such stimuli, plants must be able to effectively sense, respond to and adapt to changes in their biological activities. Hence, signal transduction pathways play important role in response to environmental stimuli. In this study, the expression of three Histidine Kinases including *ZmHK1*, *ZmHK2* and *ZmHK3a* was studied in maize plants exposed to 8 days drought, salinity and UV-B stresses applying transcript approach. The semi-quantitative RT-PCR analyses of *ZmHKs* showed up-regulation of *ZmHK1* and *ZmHK3a* genes after 8 days exposure to applied stresses except salinity in leaves, although, their regulation was more prominent during drought stress. Astonishingly, exposure to these stresses showed down-regulation of all genes in maize roots. However, the *ZmHK1* behavior was quite different from two other homologues and showed up-regulation in combined stresses. We suggest that ZmHK1 and ZmHK3a, as cytokinin transmembrane receptors, sense osmolarity changes in cells caused by dehydration. Our data supports the involvement of ZmHK homologues under these stresses in maize and provides a gene expression dynamics during the stress which will be valuable for further studies of the molecular mechanisms of stress tolerance in maize.

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Key words: drought stress; RT-PCR; salinity stress; UV-B radiation; ZmHKs gene expression

Continuous climate changes may induce several stress factors such as drought, high temperature, salinity and enhanced UV-B radiation, simultaneously (Verslues et al., 2006). In nature, the plant encounters stress combinations concurrently or at different times through the growing season (Tester et al., 2005), which must represent an integrated response to them (Knight and Knight, 2001). In the field of plant biology which it's goal is the study for life activities at molecular level (mainly, DNA and protein macromolecules), interactions at various biostimuli interface at different scales (Casati and Walbot, 2004). Molecular responses to such environmental stresses have been studied for a long time which established a complex network in signal transduction pathway that sense, transduce and control the environmental stimuli (Shinozaki et al.,

2003). These transduction networks occupy a central place in higher plants, usually acting in concert with other signals, to regulate cellular processes such as division, elongation and differentiation (Casati and Walbot, 2004). Many responses of plants to water stress are influenced by cytokinin (CK) which affects plant growth, development, and senescence (Badenoch-Jones et al., 2006; Pospisilova et al., 2000). Under this condition, the content of endogenous CK mostly decreases which heightenthe response of shoot to increase ABA concentration. CKs play important roles in several aspects of plant growth, metabolism and development as well as responses to adverse environmental factors. CK titers change at low and high temperature, drought, water deprivation, excess salinity, changes in nutrient solutions, pathogen infection, wounding, high metal concentration and herbicide treatment (Hare et al., 1997; Atanasova et al., 2004; Atanasova et al., 2005). Plants sense and respond to CKs by a signal transcription pathway called two-component system. The two-component Histidin kinases (HK) or two-component system (TCS) is known to play an important role in the regulation of prokaryotic and lower eukaryotic cellular responses to environmental stimuli (Stock et al., 2000; Urao et al., 2000a, 2000b). The existence of the first bacterial-type HK in plants was reported by Chang et al. (1993). In bacterial systems, Env Z protein (an osmosensor), consists of a membrane-localized HK that senses the input signal and a response regulator (RR) that contains a conserved regulatory domain (Pareek et al., 2009). The TCS signalling pathway has been shown to be present in several plant species, including Arabidopsis (Hwang and Sheen, 2001; Grefen and Harter, 2004) and rice (*Oriza sativa* L.; Pareek et al., 2006). HKs or two-component systems are used by bacteria, fungi and plants to sense environmental factors, such as presence of ligands, osmotic and oxidative conditions or pathogenic factors (Stock et al., 2000; Mizuno, 2005; Nemecek et al., 2006). In Arabidopsis genome 11 genes have been known to encode HK-like protein including *ETR1*, *ETR2*, *EIN4*, *ERS1*, *ERS2*, *AtHK1*, *AtHK2*, *AtHK3*, *AtHK4*, *CKI1* and *CKI2* (Aoyama and Oka, 2003; Inoue et al., 2001; Urao et al., 1999; Yamada et al., 2001; Suzuki et al., 2001; Chang et al., 1993). The important role of AtHK1 protein as osmotic stress adaptor was already shown since the *AtHK1* mutant water loss was higher than wild type plants. The mutant also was affected by wilting phenotype (Hao et al., 2004). Moreover, gain-of-function and loss-of-function approaches represent the gene as a positive regulator of drought, salt and ABA responses (Tran et al., 2007). Chen et al. (2009) reported that expression of *ATKH1* confers high tolerance of salinity and water deficit tin *Lycium barbarum* L.. So far, 8 HK transmembrane protein including ZmHK1, ZmHK1a2, ZmHk1b1, ZmHK1b1', ZmHK1b2, ZmHK2, ZmHK3a and ZmHK3b were identified in maize (www.maizegdb.org). Homology analyses showed that *ZmHK1*, *ZmHK2*, and *ZmHK3a*/*b* were closely related to *AtHK4*, *AtHK3* and *AtHK2*, in both the HK domain and the input domain, respectively (Yonekura-Sakakibara et al., 2004). The mechanisms by which environmental changes affect CKs are still not clear, but their adaptive function are undoubted (Vaseva et al., 2006). Identification of HKs as cytokinin receptors suggested that phosphorelay from sensor HKs to response regulators may play a role in cytokinin signaling. Yonekura-Sakakibara et al. (2004) showed that at least three ZmHKs act as cytokinin-responsive HKs in maize. They also found that the ZmHKs have

different ligand preferences and are not strictly tissue-specific, which redundantly expressed in most tissues (Yonekura-Sakakibara et al., 2004). The genes of HK, HPt and RR have been identified in maize, but their functions have not been well understood (Asakura et al., 2003; Yonekura-Sakakibara et al., 2004). To fully understand the mechanisms of cytokinin signalling and evolution of cytokinin-signalling genes, it is necessary to examine genes of monocotyledonous plants and compare them with those of dicotyledonous plants such as *Arabidopsis thaliana* (Ito and Kurata, 2007).

After wheat and rice, maize is a third important crop in the world and its importance in food production and economics is clear (Salvador et al., 1997). Expression pattern of maize cytokinin receptors to long term application of these stresses have not been reported up to now. Therefore, we aimed to study the 3 *ZmHKs* gene expression dynamics during drought and salinity as well as UV-B radiation in maize plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of maize (*Zea mays* L. cv. SC. 704) were obtained from Urmia Agriculture Research Centre. Seeds were graded and the big uniform shaped ones selected. Seeds were surface sterilized with 2% sodium hypochlorite for 10 min then washed with sterile distilled water three times. Following vernalization, seeds were placed in growth chamber and transferred to the plastic pot containing a soil mixture of sand (30%) and compost (70%). Plant experienced the following growth conditions for 21 days: the light regime of 16/8 h light/dark with 80% humidity and 22°C temperature. 21 day-old-maize plants were divided into 6 groups and undergone following treatment for 8 days:

Control(C), plants were watered regularly and grown normally till the end of experiment period; ***Salinity(S),*** plants were watered with 25 mM NaCl in first day of treatment. Gradual increases of NaCl (25 mM) were added every day for 4 days, up to final concentration of 100 mM; ***Drought (D),*** plants were prevented from watering by the last day of treatment; ***UV-B Radiation (UV),*** plants were subjected to UV-B radiation with lamps (Philips 30W LF-215M. France) installed 45 cm above the plants. Maize plants were irradiated 10 min in the first day of treatment and then time of plant exposure to this radiation increased every day by 10 min to reach the final dose obtained in 40 min; ***Salinity and UV-B Radiation (UV+S),*** plants were treated with both salinity and UV-B radiation, simultaneously; ***Drought and UV-B Radiation (UV+D),*** plants were treated with both drought and UV-B radiation, simultaneously.

The young leaves were harvested 8 days after the beginning of the treatmentsfrom each plant and transferred to liquid nitrogen for further analyses.

Estimation of Relative Water Content (RWC)

To estimate the relative water content, Smart and Bingham (1974) method was used. Leaves were excised before down, weighed fresh (FW) and placed in distilled water in the dark for 24 hours to re-hydrate. The following morning, leaf turgid weight (TW) was measured. Leaves were dried at 80°C for 48 hours and dry weight (DW) was determined. The RWC was calculated following this formula:

$$RWC = [(TW - FW)/ (TW- DW)] \times 100$$

Extraction of RNA From Plant Tissue

RNA concentration was measured by spectrophotometer for each sample. Approximately

100 mg of leaf tissue was sampled and placed in to 1.5ml eppendorf tube and immediately transferred into liquid nitrogen. Plant materials were ground to powder by mortar and pestle. Total RNA was extracted according to the method described for Trizol. 400 µl of Trizol ® was added to the frozen plant tissue, and sample vortexed for a few seconds to homogenize. A further 600 µl of Trizol ® was added to the sample. Following vortexing for 15 sec, samples were incubated at room temperature (RT) for 5 min to permit dissociation of nucleoprotein complexes. 200 µl of chloroform was added and shaken by hand for 15 sec. The sample was centrifuged for 15 min at 4°C. After Centrifugation (12000 x *g*), the aqueous phase was transferred to an eppendorf containing 500 µl of isopropyl alcohol for precipitation. This step was followed by 15 min incubation on ice. Centrifugation at 4°C for 10 min pelleted the RNA. The RNA pellet was washed by addition of 1ml 70% ethanol and centrifuged for five minutes at 7500 *rcf*. The pellet was air-dried and dissolved in 44 µl of RNase freeH_2O.

RT-PCR Reaction

First-strand cDNA was synthesized in a 12 µl reaction system (Fermentas) containing1 µl oligodT and 11µl total RNA (1µg) at 65ºC for 5 min followed by addition of 2 µl dNTP,1µl M-MLV reverse transcriptase (Fermentas), 1 µl RNase inhibitor and 4 µl reaction buffer at 42°C for 60 min.

PCRs were conducted in 25 µl volumes containing 12.5 µl master mix, 50 pmol of and reverse primers and 2 µl cDNA. The reactions were initiated by 94°C for 5 min, followed by 30 cycles of: 94°C 1 min, 60°C 1 min and 72°C 1 min and a final extension at 72°C for 5 min. The intensity of PCR amplified bands was visualized under UV and measured using Image J 1.41 software. The primers are listed in Table 1.

Statistical analysis

The experiment was conducted by completely randomized design with three replications for sampling and statistical analysis was performed using SPSS 19 program. The data represent means calculated from three replicates. The analysis of variance procedure (ANOVA) was used to compare the effect of these stresses to control and statistical significance was set at $p < 0.05$.

RESULTS

Relative Water Content (RWC)

In this research, 8 days exposure to stress conditions caused a decrease in RWC for alltreated plant. According to our data, decreasing in RWC was more significant under drought condition (34.66%). Plants exposed to salinity showed 11.33% reduction of RWC. However, leaf tissues of UV-B treated plants showed almost 4.3% RWC decline which was higher than two other stressed treated plants. Plants under simultaneous stress conditions showed a decrease of about 6.33% and 22.33% for UV+S and UV+D, respectively. Data comparison revealed a synergistic manner between UV-B radiation and salinity as well as drought stress (Fig.1).

Phylogenetic Relationship of ZmHK Genes

Up to now, 8 Histidine kinase transmembrane protein including ZmHK1, ZmHK1a2, ZmHk1b1, ZmHK1b1', ZmHK1b2, ZmHK2, ZmHK3a and ZmHK3b were detected in maize (www.maizegdb.org). The relationship among these Histidine kinases has been drawn based on amino acid composition in figure 2.

Expression Pattern of ZmHK1 Under Various Stresses

The result of *ZmHK1* gene expression indicated that this gene had been up regulated in all treatments. However, its expression was more significant in LD in comparison to LC leaves. This indication represents the effect of these stresses on the *ZmHK1* induction. On the contrary, *ZmHK1* gene expression induced in maize roots of RUV+S and RUV+D. No prominent induction of ZmHK1 expression was observed in other stress treated roots in comparison to RC (Fig.3).

Expression Pattern of ZmHK2 Under Various Stresses

The RT-PCR result of all stressed leaves indicated no significant difference in comparison to LC leaves. Interestingly, *ZmHK2* expression had been decreased in roots of RUV, RD and RUV+S stress treated maize compared to RC roots. Down-regulation of RUV+D and RS were not prominent than the others. This indication showed the effect of these stresses on the *ZmHK2* suppression in root system (Fig.4).

Expression Pattern of ZmHK3a Under Various Stresses

The RT-PCR result for *ZmHK3a* gene expression indicated that the *ZmHK3a* had been up regulated more significantly in the LD in comparison to LUV, LUV+S and LUV+D leaves. On the contrary, *ZmHK3a* expression decreased in LS leaves in comparison to LC leaves. The mRNA level of this gene had even been dropped to below control levelin roots; however RUV+D gene expression reduction was not significant (Fig.5).

Table 1: Primers used in semi-quantitative RT-PCR experiment.

Primer name	Sequence
ZMHK1-FWD	5′-AAGTAGGAGCTTGACAGAGGCACTA-3′
ZMHK1-REV	5′-GTACAGGTCTCCCATCTACCAA-3′
ZMHK2-FWD	5′-AGCATTGGGTGGGATAGATAAACT-3′
ZMHK2-REV	5′-GAAGGCTGCCCAGTGTTGAA-3′
ZMHK3a-FWD	5′-TGGAATCAGTGGCGTGAATG-3′
ZMHK3a-REV	5′-GCTCCCCAAAAAAGCAGATAGA-3′
UbQ 10-FWD	5′-CCACTTGGTGCTGCGTCTTAG-3′

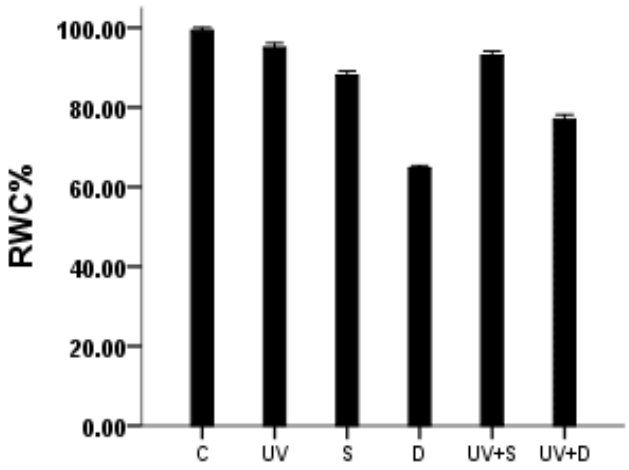

Figure 1: Percentage of relative water content (RWC) in maize leaves plants. Error bars are representative of standard deviation.

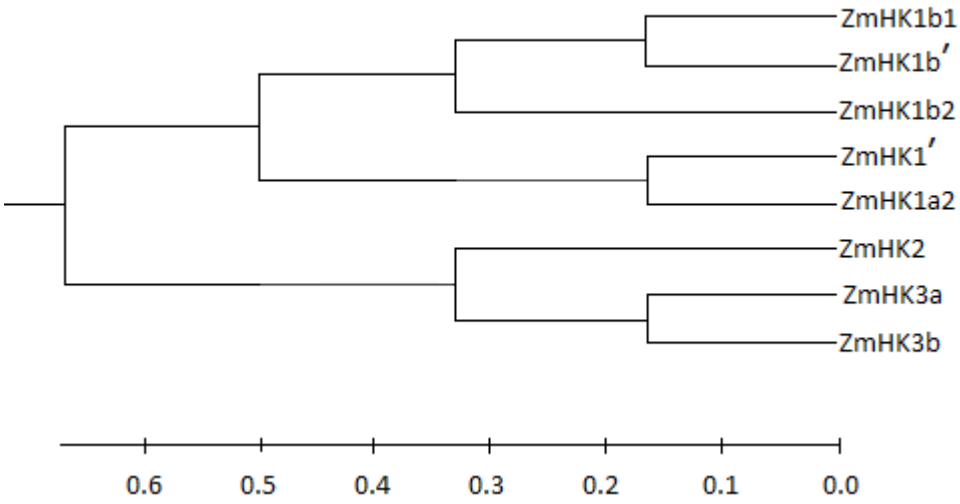

Figure 2: Relationship among ZmHKs (Zea mays Histidine kinases) based on amino acids alignment. The analyses were performed using Mega 4 program.

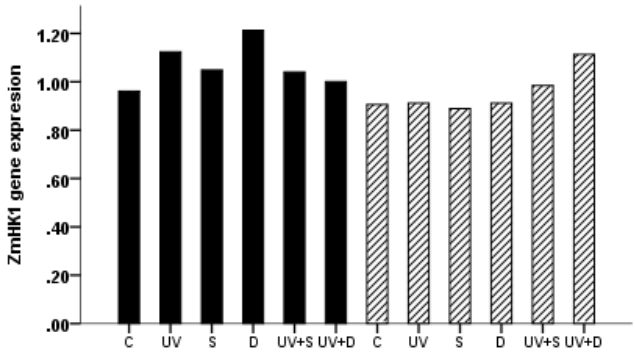

Figure 3: Relative mRNA expression of *ZmHK1* in *Zea mays* L. cv. 704 leaves (black column) and roots (hatched column). The values were normalized with ubQ_{10} level. The values are from three independent experiments. Error bars are representative of standard deviation.

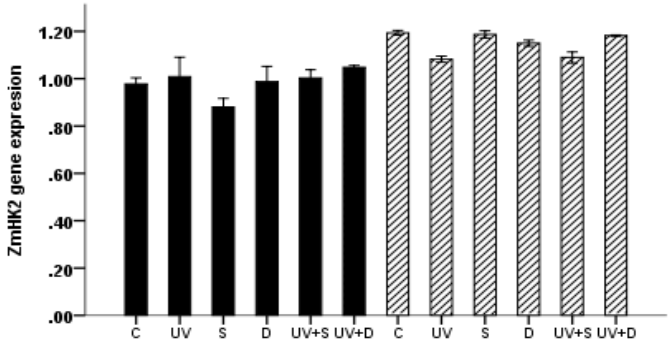

Figure 4: Relative mRNA expression of *ZmHK2* in Zea mays L. cv. 704 leaves (black column) and roots (hatched column).The values were normalized with ubQ10 level. The values are from three independent experiments. Error bars are representative of standard deviation.

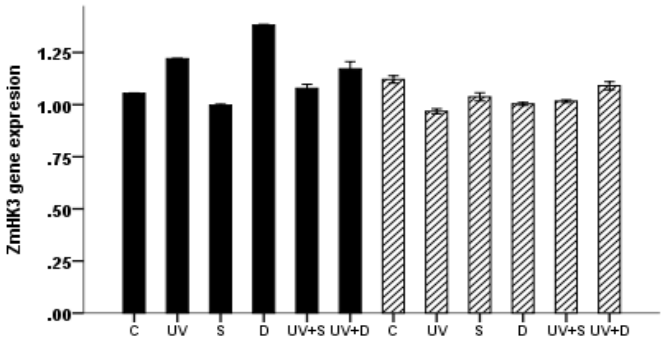

Figure 5: Relative mRNA expression of *ZmHK3a* in Zea mays L. cv. 704 leaves (black column) and roots (hatched column).The values were normalized with ubQ10 level. The values are from three independent experiments. Error bars are representative of standard deviation.

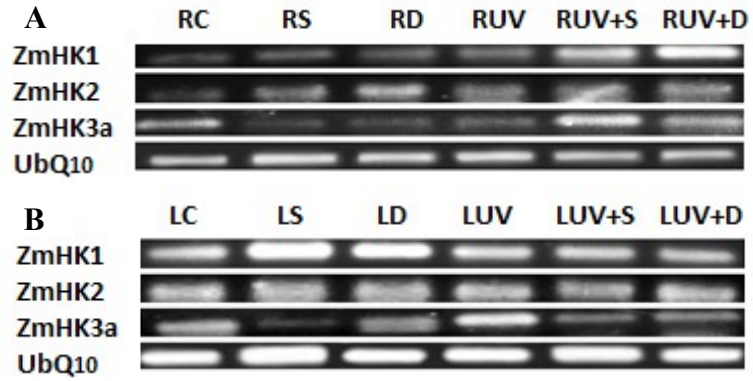

Figure 6: Gel graph of *ZmHKs* gene expression in maize leaves (A) and roots (B). Maize plants exposed to various stresses for 8 days and their mRNA levels analyzed by RT-PCR.

DISCUSSION

The RWC is mostly used to realize the plants water status. Water status has a close relationship with several physiological parameters such as turgor, growth, stomatal conductivity, transpiration, respiration and photosynthesis (Kramer and boyeer, 1995). The ability of plants to survive in severe water deficits depends on their ability to limit water loss through the leaf epidermis after minimizing the aperture by stomata (El-Jaafari, 2000). Our results showed a decrease for RWC in maize treated plants with more decrease under drought condition. We observed a high level of RWC in leaves of plants treated with UV-B radiation (fig. 1). This can be attributed to different mechanism implicated to different stress conditions. Moreover, rapid induction of osmolytes and stress proteins are responsible for high level of RWC in plants treated with UV-B. It's suggested that UV-B regulate the effect of drought by rapid induction of dehydrine proteins and compatible osmolytes in Arabidopsis (Schmidt et al., 2000).

Several Histidine kinase-encoding genes have been cloned in eukaryotes. *Escherichia coli* and yeast sense the osmotic stress by EnvZ and SLN1 osmosensors, respectively (Maeda et al., 1994; Mizuno, 1998). In Arabidopsis, AtHK1 has been located in plasma membrane and acts as an osmosensor which detects water stress and initiates downstream responses in early vegetative stages of plant growth (Urao et al., 1999). Urao et al., (1999) found that *ATHK1* mRNA increased

markedly under high salinity and low temperature. Moreover, Tran et al., (2007) reported the increase of *AtHK2* expression after ABA and NaCl treatments. The expression of *AtHK3a* was up-regulated under cold and high salinity. Examine of the *ZmHK1* and *ZmHK3a* transcript level of leaves, revealed up-regulation of these maize homologues after 8 days water deficit conditions. These results suggest that a long change in osmolarity may trigger the induction of the *ZmHK1 and ZmHK3a* genes (fig. 3 & 5). However, expression of *ZmHK2* and *ZmHK3a* in leaves treated with salinity showed slight reduction. According to our obtained results, the higher expression of *ZmHK1* and *ZmHK3a* had a clear correlation with RWC%. We found that plants with lower RWC had a higher increase in their genes expression. These results suggest that the expression of the *ZmHK1* and *ZmHK3a* genes is transcriptionally regulated in response to changes in external osmolarity, as plants under more stressful situations, had more induction level of these genes. However the response pattern of LS was different for *ZmHK2* and *ZmHK3a*. Although the expression level of *ZmHK1* and *ZmHK3a* induced after treatment with these stresses, the mRNA level of ZmHK2 had no significant changes compared to control. There are much evidence that CK plant hormone is predominantly associated with stimulation of cell division, control of plant growth, development, chloroplast differentiation, leaf senescence, nutrient signaling and stress responses (Mok, 1994; Forde, 2002). The expression of various genes is modulated by this hormone and numerous cytokinin-regulated genes have been described (Schmu¨ lling et al., 1997; Rashotte et al., 2003). For example, AtHK2, AtHK3 and AtHK4 transcription, which serve as CK receptors, was already observed within 10 min of dehydration (Tran et al., 2007). Roots are a major site of cytokinin synthesis that can be affected by its translocation to different parts of the plant via the xylem and water supply (Tian et al., 1995; Hirose et al., 2008).

In maize, at least 3 HK (ZmHK1, ZmHK2 and ZmHK3a) were found to be cytokinin-responsive (Asakura et al., 2003; Sakakibara et al., 1998). In *Arabidopsis thaliana* Under stress conditions, apparently the transcriptional regulation of genes for such signal transducers confer higher *AtHK*s expression in plant organs, including roots, leaves, stems and flowers tested, while the *AtHK2* signal was relatively weak (Ueguchi etal., 2001). Roots represent the interface between plants and their soil environment. They are involved in nutrient acquisition and responses to environmental conditions, such as gravity, light, water, and temperature (Jackson, 1997). Several ion transporter genes, which are involved in nutrient uptake, are expressed predominantly in roots (Lauter et al., 1996; Muchhal et al., 1996). Urao et al. (1999) found that the *Arabidopsis thaliana* Histidine kinase 1(*AtHK1)* mRNA is more abundant in roots than in other organs, suggesting a functional importance of AtHK1 in root tissue. According to our RT-PCR findings, the mRNA levels of ZmHK2 and ZmHK3a in maize roots were abundant, too.

We suggest that *ZmHK1 and ZmHK3a* sense changes in osmolarity in cells caused by dehydration and subsequently transduce the stress signals to the nucleus through a protein phosphorylation cascade, probably a MAPK cascade (Urao et al., 1999). To our knowledge no report has been recorded on the effect of water deficit on *ZmHKs* expression in maize. Our data support the involvement of *ZmHK* homologues under stresses in maize and provides a gene expression dynamics

during dehydration period and will be valuable for further study of the molecular mechanisms of stress tolerance in maize.

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