

# Overexpression of *ZmMAPK1* enhances drought and heat stress in transgenic *Arabidopsis thaliana*

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**Abstract** Mitogen-activated protein kinase (MAPK) signal transduction cascades play a crucial role in the response to extracellular stimuli in eukaryotes. A number of MAPK family genes have been isolated in plants, but the maize MAPK genes have been little studied. Here, we studied the role of maize MAP kinase 1 (*ZmMAPK1*) using gene expression, protein subcellular localization, transformation in *Arabidopsis*, expression patterns of the stress-responsive genes and physiological parameter analysis. Our physiological parameter analysis suggested that over-expression *ZmMAPK1* can increase proline content and decrease malondialdehyde content under drought, and prevent chlorophyll loss and the production of scavenger reactive oxygen species under heat stress. The resistance characteristics of the over-expression of *ZmMAPK1* were associated with a significant increase in survival rate. These results suggest that *ZmMAPK1* plays a positive role in response to drought and heat stress in *Arabidopsis*, and provide new insights into the mechanisms of action of MAPK in response to abiotic stress in plants.

**Keywords** Maize · Mitogen-activated protein kinase 1 · Drought stress · Heat stress · Transgenic plants

## Abbreviations

ABA	Abscisic acid
CaMV	Cauliflower mosaic virus
MDA	Malondialdehyde
ROS	Reactive oxygen species
MAPK	Mitogen-activated protein kinase
ORF	Open reading frame
SOD	Superoxide dismutase
GFP	Green fluorescent protein

## Introduction

Maize (*Zea mays* L.) is an economically important grain crop, whose growth and yield are severely inhibited under various stress conditions (Zhang et al. 2014). One of the universal signal pathways involved in the response to external stimuli is the mitogen-activated protein kinase (MAPK) cascade (MAPK Group 2002). MAPK signaling networks are found in all eukaryotic organisms and regulate fundamental aspects of biology, including not only the initiation of developmental pathways, but also responses to abiotic and biotic stresses, such as drought, heat stress, reactive oxygen species (ROS), ultraviolet (UV) light, hormones, and pathogenic attack (Thomas 1992; Lampard et al. 2009; Pitzschke and Hirt 2009).

MAPKs are activated by a highly conserved mechanism of sequential phosphorylation events in which a MAPK kinase kinase (MAPKKK) activates a MAPK kinase (MAPKK) that then activates a MAPK by phosphorylation of tyrosine and threonine residues in the TxY (TEY or TDY) motif (Herskowitz 1995; Fu et al. 2002; Lei

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Liuji Wu and Xiaofeng Zu have contributed equally to this work.

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et al. 2014). The MAPKs can be divided into four groups (A–D): the TEY subtype can be classified into three groups A, B and C, whereas the TDY subtype forms a more distant group D (MAPK Group 2002). Activated MAPK can phosphorylate a variety of substrates including transcription factors, cytoskeleton-associated proteins and other protein kinases (Nakagami et al. 2005; Pitzschke and Hirt 2006; Zhang et al. 2010). Since the first plant MAPK gene, *MsERK1*, was discovered in alfalfa (Duerr et al. 1993), further MAPK genes have been identified and explored using both genetic and biochemical approaches. Recently, several putative MAPKs genes have been isolated from different plant species, such as *Arabidopsis*, tobacco, rice and maize (Kosetsu et al. 2010; Pan et al. 2012; Liu et al. 2013b).

At present, 19 MAPKs have been identified in maize, but only a few MAPKs genes have been characterized (Wei et al. 2014). *ZmMPK3* accumulated markedly and rapidly when maize seedlings were subjected to cold, drought, UV light, salinity, heavy metal or mechanical wounding (Wang et al. 2010). Overexpression of *ZmMPK4* in transgenic tobacco resulted in increased tolerance to low temperature stress (Zhou et al. 2012b). *ZmMPK5* is required for NADPH oxidase-mediated self-propagation of apoplastic hydrogen peroxide ( $H_2O_2$ ) in brassinosteroid-induced antioxidant defense systems in leaves of maize plants, and is also activated by abscisic acid (ABA) and cold (Berberich et al. 1999; Lin et al. 2009; Zhang et al. 2010). *ZmMPK6* is able to interact with 14-3-3 proteins (Lalle et al. 2005). *ZmMPK7* is responsible for the removal of ROS under ABA and  $H_2O_2$  stress (Zong et al. 2009). *ZmMAPK17* is transcriptionally regulated by multiple stresses, and its overexpression in tobacco produced enhanced resistance to low temperatures and viral infection (Pan et al. 2012).

In the present study, we isolated and characterized a maize MAPK gene, designated *ZmMAPK1*. Overexpression of *ZmMAPK1* in *Arabidopsis thaliana* increased resistance to drought and heat. Physiological parameter analysis indicated that transgenic plants overexpression *ZmMAPK1* showed a significant change compared with control lines.

## Materials and methods

### Plant materials and growth conditions

Seedlings of maize (*Zea mays* L.) inbred line B73, from Henan Agricultural University, China, were grown in Hoagland's solution (pH = 6.0) in a greenhouse at 22/28 °C (night/day), with photosynthetically active radiation (PAR) of 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , and a photoperiod of 14/10 h (day/night). Leaves of seedlings at the V4 development stage (when the fourth leaf has a visible "collar" at the base of the leaf) were collected and used for investigations.

High-temperature and drought-stress treatment seedlings were grown in nutrient soil in the same greenhouse.

*Arabidopsis thaliana* (ecotype Columbia, Col-0) was used for experiments involving transformation and various abiotic stress conditions (Luo et al. 2013). *Arabidopsis* seedlings from surface sterilized seed, were grown in a chamber at 22/20 °C (night/day), with PAR of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , 60 % relative humidity, and a photoperiod of 16/8 h (day/night) in half Murashige and Skoog (MS) medium with salt and vitamins containing 3 % sucrose and 0.8 % (w/v) agar for 10 d, then transplanted into nutrient soil. The seeds used in the experiment were harvested at the same stage, and stored in the same way, to ensure that they all had the same vitality.

### Isolation of total RNA and DNA

Total RNA was isolated from untreated maize leaves at the V4 development stage by using an RNeasy Plant mini kit (Qiagen, Valencia, CA, USA) according to the instructions supplied by the manufacturer. Approximately 2  $\mu\text{g}$  of total RNA was used as a template for first-strand cDNA synthesis using the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Genomic DNA was isolated by the cetyl-trimethyl ammonium bromide method.

### Isolation of the maize MAPK1 gene

A blast search with rice MAPK1 sequences as a query revealed good homology with the products of the predicted maize gene. To isolate the full-length cDNA of this maize gene, RT-PCR was performed using a cDNA amplification kit (Takara, Tokyo, Japan) based on in silico predictions. The specific primers of this cDNA (*ZmMAPK1F* and *ZmMAPK1R*) were designed according to the relevant sequences and were shown in supporting information Table S1. PCR product was purified and subcloned into the pMD19-T vector (Takara, Tokyo, Japan) for sequencing (Wu et al. 2011a).

### Phylogenetic analysis

The amino acid sequences of the plant MAPK proteins were retrieved from GenBank. A phylogenetic tree was constructed based on the sequence alignment. Protein sequences were obtained from maize, rice, *Nicotiana tabacum* and *Arabidopsis*. These proteins were used to construct a phylogenetic tree using the Neighbor-Joining method and a bootstrap test based on MEGA 5.2 software.

### Quantitative real-time PCR analyses

To analyze levels of *ZmMAPK1* gene expression in maize leaves, quantitative real-time PCR (qRT-PCR) was

performed with RNA samples harvested at the indicated times after exposure to various stresses. The transcript levels of stress-related genes in  $T_3$  *A. thaliana* (transgenic lines and wild type) before and after stress treatment were also analyzed (Wang et al. 2014), such as the *ABI*, *ABA2*, *ABA3*, *ABI1*, *ABI2*, *NCED3*, *ACS5* and *ICS1*. Each PCR reaction contained 7.5  $\mu$ L SYBR Premix Ex Taq (TaKaRa), 0.5  $\mu$ L of each primer (10  $\mu$ M), 5.6  $\mu$ L water, 0.4  $\mu$ L of passive reference Dye III and 0.5  $\mu$ L of each reverse-transcribed cDNA product. PCR was performed in 96-well optical reaction plates using iQ5 (Bio-Rad, Richmond, CA, USA) after pre-incubation for 3 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 56 °C for 15 s, and extension at 72 °C for 30 s, following the manufacturer's instructions. The maize 18S ribosome RNA (rRNA) and *Arabidopsis ACTIN2* genes were used as internal controls to normalize the relative gene expression levels in maize and *Arabidopsis*, respectively. Technical triplicates were performed for each biological replicate, and the average values were used for quantification (the PCR primers are listed in supporting information Table S2). RT-PCR was performed using SYBR and run on an Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

### Subcellular localization of the ZmMAPK1 protein

To investigate the subcellular localization of ZmMAPK1, the entire coding sequence without the stop codon was cloned into the NcoI and SpeI sites of the pCAMBIA1302 vector to yield the final plasmid 35S:ZmMAPK1:GFP. The open reading frame (ORF) of ZmMAPK1 was amplified by PCR with NcoI and SpeI linker primers (ZmMAPK1F + NcoI and ZmMAPK1R + SpeI, the underlined nucleotide sequence indicates the NcoI and SpeI restriction enzyme cutting site in supporting information Table S1). The recombinant fusion ZmMAPK1-green fluorescent protein (GFP) fused plasmid was introduced into onion epidermal cells by particle bombardment. After transformation, tissues were incubated on MS agar medium in the dark at 23 °C for 16 h. Transformed onion cells were observed by confocal microscopy (Olympus, Tokyo, Japan). This experiment was performed in triplicate with identical results.

### Vector construction and *Arabidopsis* transformation

The coding sequence of the *ZmMAPK1* gene was amplified using primers (ZmMAPK1F' + NcoI and ZmMAPK1R' + SpeI), and cloned into a binary vector pCAMBIA1304 under control of the cauliflower mosaic virus (CaMV) 35S promoter. The resulting vector was

mobilized into *Agrobacterium tumefaciens* strain GV3101. Transformation of *Arabidopsis* plants was carried out by the floral dip method (Bechtold and Pelletier 1998). Transgenic plants were first screened on half MURASHIGE and SKOOG medium supplemented with 50 mg/L hygromycin. Seeds from each  $T_1$  plant were individually collected. Selected  $T_2$  plants were propagated, and homozygous overexpression lines were confirmed by RT-PCR analysis.  $T_3$  progeny homozygotes were obtained for further analysis.

### Plant abiotic stress treatments

To prepare the V4 development stage maize seedlings for qRT-PCR to analyze *ZmMAPK1* gene expression levels under different abiotic stresses, they were incubated with Hoagland's solution containing 350 mM NaCl, 10 mM  $H_2O_2$ , 20 % PEG6000 (w/v). A 37 °C high temperature and drought stress treatment was applied to the soil. Each experiment was performed three times with each replicate containing 30 seedlings.

*Arabidopsis thaliana* homozygous  $T_3$  seedlings of the transgenic overexpression lines and WT seedlings were used for stress treatments. For the water-deficit treatment, watering was withheld from three-week-old seedlings for 14 d and then the plants were re-watered. For the salt-tolerance assay, the three-week-old seedlings were treated with 250 mM NaCl for 7 d. For the heat-stress treatment assay, the three-week-old seedlings were exposed to 37 °C for 3 d, and then returned to the normal growth environment (Liu et al. 2013a; Lv et al. 2011). All the treatments were performed in triplicate. The phenotypic and statistical aspects of the survival rates of the plants were observed one week after each treatment.

### Physiological characterization of $T_3$ *ZmMAPK1* transgenic plants

#### Quantification of proline content

Three-week-old *Arabidopsis* WT and transgenic lines under drought, heat-stress or salt-stress conditions were used for measurement of the different physiological indices under different treatments. All experiments were performed in triplicate.

*Arabidopsis* leaf samples (0.1 g) for the measurement of proline were treated with 3 % (w/v) sulphosalicylic acid followed by boiling for 1 h. The amounts of proline were measured with ninhydrin, which was detected at 520 nm, and the standard liquid proline was used as a reference (Bates et al. 1973). Approximately 0.3 g of plant material was homogenized in 6 mL of 3 % aqueous sulfosalicylic acid and the homogenate filtered

through. A total of 2 mL of filtrate was reacted with 2 mL of ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100 °C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene, mixed vigorously with a test tube stirrer for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:  $[(\mu\text{g Pro ml}^{-1} \times \text{ml toluene})/115.5 \mu\text{g } \mu\text{mol}^{-1}]/[(\text{g sample})/5] = \mu\text{mol Pro g}^{-1}$  fresh weight material.

#### Measurement of malondialdehyde content

Malondialdehyde (MDA) content was assayed according to Lv (2007). About 0.1 g of maize leaf was used and absorbance values at 450, 532, and 600 nm were determined with a spectrometer (Perkin-Elmer Lambda 25, Boston, MA, USA). The concentration of MDA was calculated using the following formula:  $C (\mu\text{mol/L}) = 6.45 (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \text{OD}_{450}$ .

#### Determination of hydrogen peroxide

The *Arabidopsis* seedlings were grown at 37 °C for 3 d. Then, after recovering under normal conditions for 1 d, their phenotype was observed and leaf samples were harvested for determination. The hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) content after heat stress was measured as described by Zhou et al. (2012a).

#### Water-loss measurements

For the rate of water loss assay, detached leaves (from three-week-old plants) of transgenic *Arabidopsis* lines and WT plants were placed in a growth chamber. Their fresh weight (FW) was recorded immediately and then every 30 min for 8 h until it failed to decrease. The proportion of water lost was calculated as  $(\text{initial fresh weight} - \text{final fresh weight})/\text{initial fresh weight} \times 100 \%$  (Yoo et al. 2010).

#### Chlorophyll measurement

Total chlorophyll content was measured with a chlorophyll meter (SPAD 502 Plus; Konica Minolta Sensing) as described previously (Abe et al. 2012; Tian et al. 2013). Measurements of chlorophyll content under stress conditions were performed after applying a treatment of 37 °C for 3 days. Transgenic *Arabidopsis* and WT type leaves had to be fully expanded at the time of measurement. Thirty

plants of each line were used for the chlorophyll content assays.

#### Statistical analyses

To determine the statistical significance of stress treatments in various genotypes, we performed one-way ANOVAs using Dunnett's *t* test with SPSS 17.0 statistics software (SPSS Corp., Chicago, IL, USA). The difference was considered significant if  $P < 0.05$ .

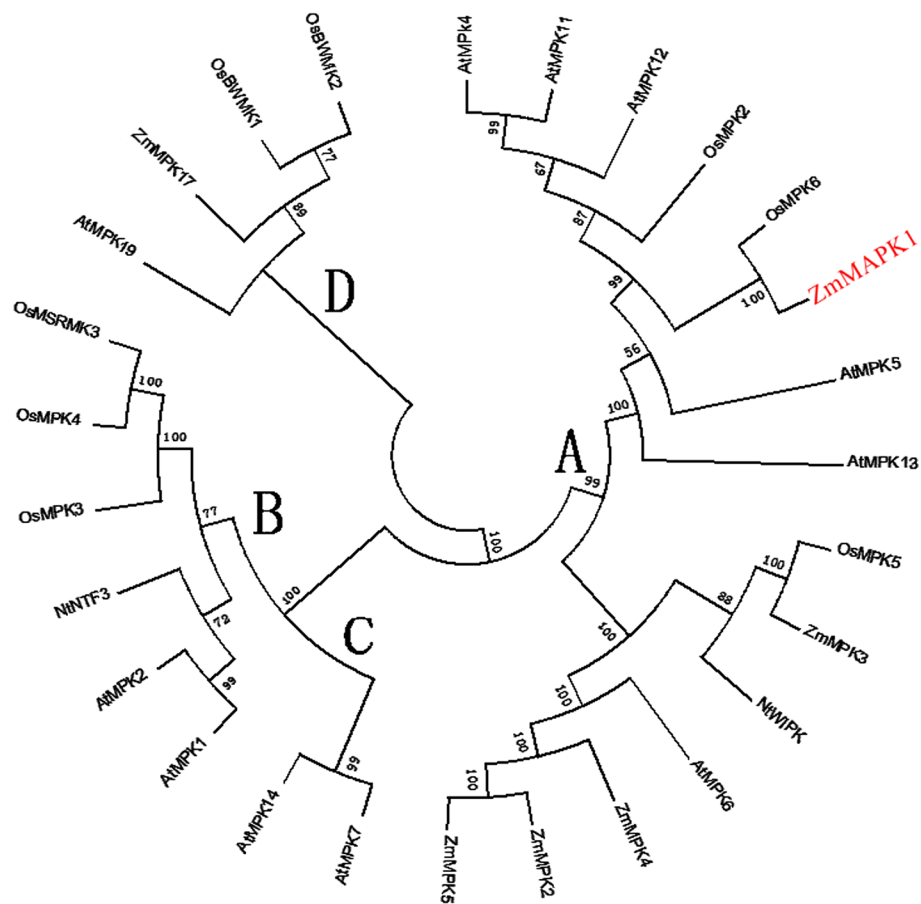
## Results

### Cloning and sequence analysis of *ZmMAPK1*

A 1871 bp full-length cDNA sequence was obtained by in silico cloning and sequencing in the *Z. mays* B73 inbred line and has been submitted to GenBank (accession No. KM386659). The CDS contains an 1125 bp open reading frame (ORF), which encodes a protein of 375 amino acids with a predicted molecular weight of about 42.44 kDa and a PI of 6.51. The amino acid sequence of *ZmMAPK1* exhibits a molecular structure typical of plant MAPKs. The *ZmMAPK1* protein contains 11 subdomains of protein kinases with serine/threonine specificity that is conserved in eukaryotic organisms (Figure S1). The TEY motif is also conserved in the *ZmMAPK1* protein sequence, including the threonine and tyrosine residues whose phosphorylation is necessary for MAPK activation. To elucidate the phylogenetic relationships among MAPK proteins in various species, phylogenetic analysis was performed using MEGA 5.2 (Fig. 1). *ZmMAPK1* is homologous to *Oryza sativa* *OsMPK6* and *A.thaliana* *AtMPK4*, both of which are classified as group A MAPKs.

### Subcellular localization of *ZmMAPK1*

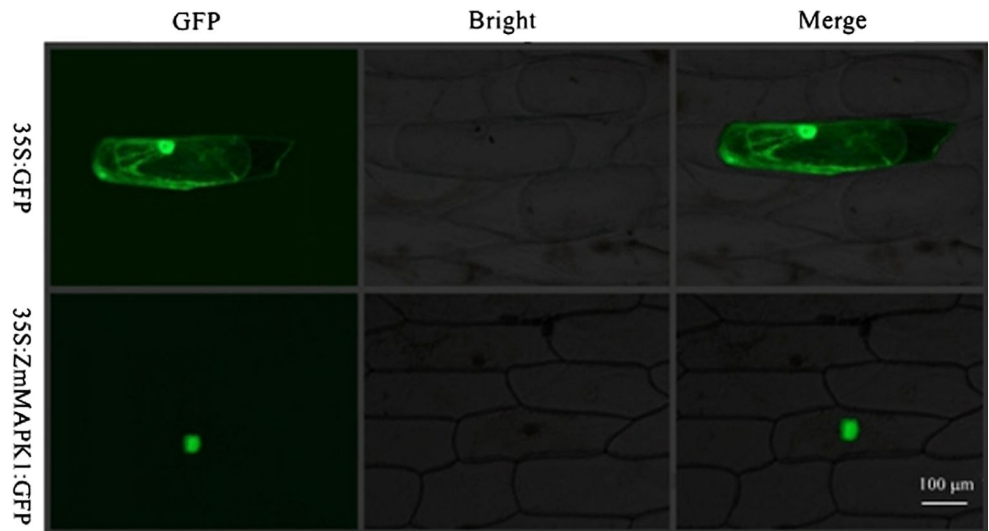
Previous studies have shown that different MAPKs have different subcellular localizations, including the plasma membrane, nucleus, cytoplasm, or protoplasts (Zong et al. 2009; Kosetsu et al. 2010; Shen et al. 2010; Zeng et al. 2011; Pan et al. 2012). In silico analysis, we used the softberry database (<http://linux1.softberry.com/berry.phtml>) to predict that *ZmMAPK1* was localized in the nucleus (Figure S2). To confirm the subcellular localization, the ORF of the *ZmMAPK1* was fused in frame to the GFP marker gene under the control of the CaMV 35S promoter. The 35S: GFP control exhibited GFP signals evenly in the plasma membrane, cytoplasm and nucleus, whereas the 35S: *ZmMAPK1*: GFP fusion protein emitted green fluorescence specifically localized in the nucleus (Fig. 2). This result was consistent with the prediction.



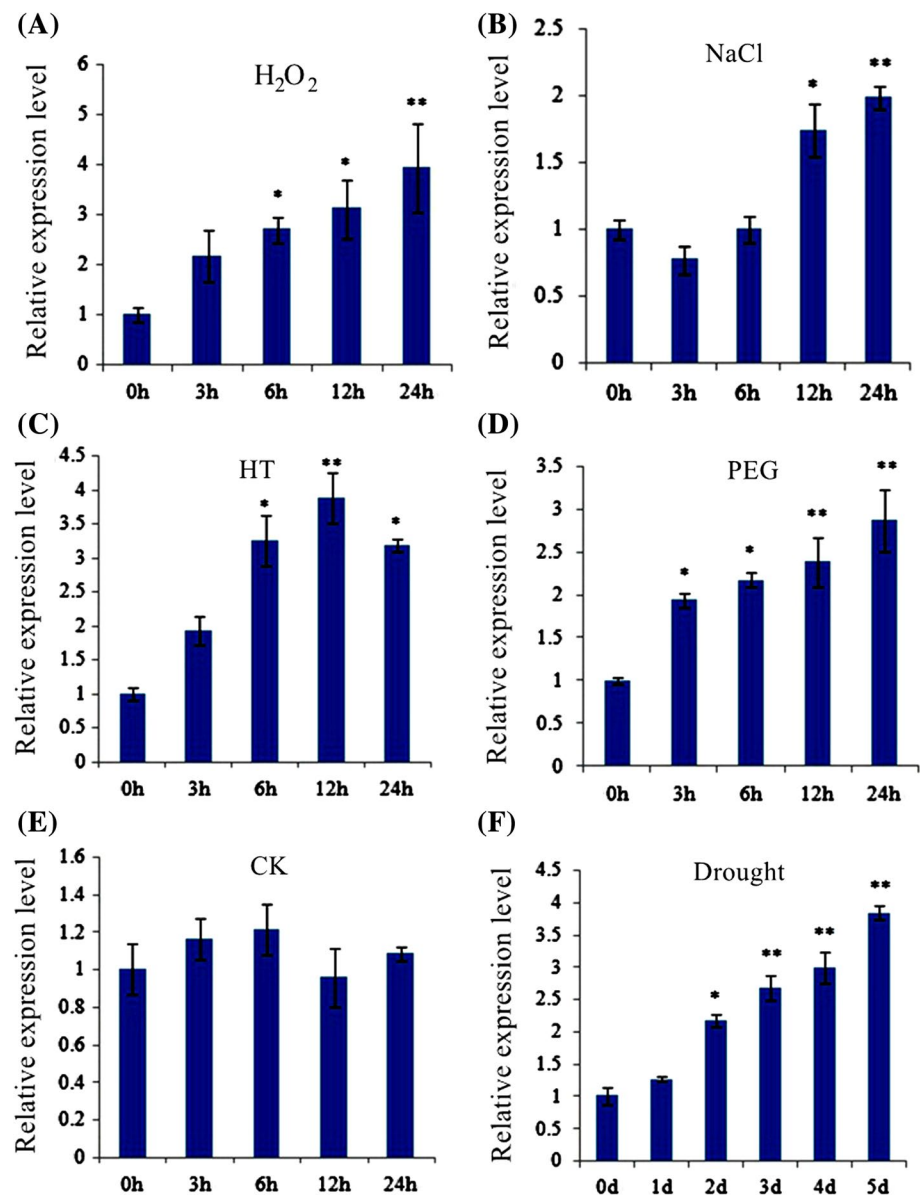
**Fig. 1** Phylogenetic analysis of *ZmMAPK1* with other MAPKS from *Arabidopsis*, rice, maize, and *Nicotiana tabacum* using the amino-acid sequence alignment function in the MEGA 5.2 program. Bootstrap analysis was performed using 1000 replicates to evaluate the reliability of the various phylogenetic groups A, B, C and D. GenBank Accession numbers: *ZmMAPK1* KM386659, *ZmMPK2* NM\_001111373.1, *ZmMPK3* EU130900.1, *ZmMPK4* AB016801.1, *ZmMPK5* AB016802.1, *ZmMAPK17* NM\_001154688.1, *AtMPK1* NP\_172492.1, *AtMPK2* NP\_564746.1, *AtMPK4* NP\_192046.1, *AtMPK5*

NP\_567378.4, *AtMPK6* NP\_181907.1, *AtMPK7* NP\_179409.1, *AtMPK9* NP\_974331.1, *AtMPK10* NP\_191538.1, *AtMPK11* NP\_001117210.1, *AtMPK12* NP\_182131.2, *AtMPK13* NP\_001030990.1, *AtMPK14* NP\_195363.1, *AtMPK19* NP\_188090.2, *OsMPK2* AAS79349.1, *OsMPK3* AAG40581.1, *OsMPK4* CAB61889.1, *OSMPK5* AF479883, *OsMPK6* EF174189.1, *OsMPK18* AAT39148, *OsBWMK1* AAD52659.1, *OsBWMK2* AF177392.1, *OsBIMK2* NP\_001045815.1, *OsMSRMK3* CAD54741.1, *NiNTF3* CAA49592.1, *NiWIPK* BAA09600.1

**Fig. 2** The subcellular localization of *ZmMAPK1*. Transient expression of 35S: GFP, 35S: *ZmMAPK1*: GFP in onion epidermal cells was produced via particle bombardment. The fluorescence was visualized using laser scanning confocal microscopy under bright and fluorescence fields. *Bar* = 100  $\mu$ m



**Fig. 3** Expression patterns of *ZmMAPK1* in maize seedling leaves under different abiotic stresses were detected with qRT-PCR. V4 stage maize seedlings were exposed to different treatments: **a** 10 mM  $H_2O_2$ , **b** 350 mM NaCl, **c** 37 °C high temperature, **d** 20 % PEG6000 (w/v), **e** control (no treatments), **f** drought stress in soil



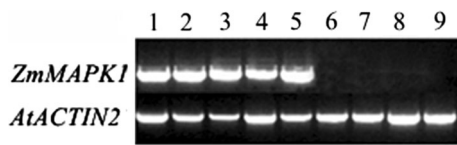
### Expression patterns of *ZmMAPK1* in maize leaves exposed to abiotic stresses and signaling molecules

To investigate the expression profile of *ZmMAPK1*, we performed quantitative real time PCR (qRT-PCR) to analyze changes in maize leaves at the V4 development stage under various stresses. Exogenous application of 10 mM  $H_2O_2$  significantly increased the transcription of *ZmMAPK1* in the following 24 h, and reached a peak at 24 h (Fig. 3a). After treatment with 250 mM NaCl, the transcript level of *ZmMAPK1* was slightly reduced at 3 h, but then increased to a maximum at 24 h; there was about a two-fold change in expression level (Fig. 3b). This pattern was similar to that of *ZmMAPK5* in maize under salt stress (Zhang et al. 2014). Treatment with 20 % PEG6000 increased *ZmMAPK1* expression in

maize seedling leaves, and high temperature (37 °C) led to an increase in expression after 12 h of treatment (Fig. 3c, d). There was no significant change in *ZmMAPK1* transcript levels with water treatment (CK) during the 24 h cycle (Fig. 3e). Drought stress, applied by withholding watering of the soil for 5 d until the maize seedling leaves had significant ‘rolling’, increased *ZmMAPK1* transcription in the following 5 d (Fig. 3f). Both the 20 % PEG6000 and drought stress results indicate *ZmMAPK1* may play a positive role in the plant’s response to drought stress.

### Identification of transgenic *Arabidopsis*

To investigate the effect of *ZmMAPK1* in response to multiple stresses in *Arabidopsis*, transgenic plants containing



**Fig. 4** RT-PCR analysis of *ZmMAPK1* overexpression in *Arabidopsis*. The transcript levels of *ZmMAPK1* in different overexpression lines and pCAMBIA1304 vector control lines analyzed by RT-PCR. Total RNA extracted from leaf samples collected from T<sub>2</sub> *Arabidopsis* plants grown under normal conditions. The *Arabidopsis* ACTIN2 gene was selected as an internal control. 1 overexpression line 1; 2 overexpression line 2; 3 overexpression line 3; 4 overexpression line 4; 5 overexpression line 5; 6 pCAMBIA1304 vector control line 1; 7 pCAMBIA1304 vector control line 2; 8 pCAMBIA1304 vector control line 3; 9 wild type

*ZmMAPK1* under the control of the CaMV35S promoter in the pCAMBIA1304 vector were generated, and the pCAMBIA1304 vector was also used for transformation in *Arabidopsis* as a control. Independent transgenic lines were obtained by homomycin-resistance selection and confirmed by semi-quantitative RT-PCR. The RT-PCR analysis revealed that the *ZmMAPK1* gene was expressed normally in the transgenic lines, but no expression was detected in the pCAMBIA1304 vector control lines and WT plants (Fig. 4). The homozygous T<sub>3</sub> generation of three independent overexpression lines, namely OE-1, OE-3, OE-5, and the control lines CL-1, CL-2, CL-3 were used for further analysis.

### Overexpression of *ZmMAPK1* improves tolerance to drought and heat stress in *Arabidopsis*

Since there is much evidence for cross-talk between signaling pathways regulating the response to abiotic stress (Jin et al. 2011; Frey et al. 2012; Wang et al. 2013), transgenic *Arabidopsis* plants overexpression the *ZmMAPK1* gene were generated. Three-week-old *A. thaliana* homozygous T<sub>3</sub> seedlings of the transgenic lines and WT plants were used for drought, heat and salt stress experiments. After 14 d drought stress, the transgenic *ZmMAPK1* lines had a higher drought tolerance than the pCAMBIA1304 vector control lines and WT plants (Fig. 5a, b), as shown in Fig. 5b, the pCAMBIA1304 vector control lines and WT plants became severely wilted and impaired after dehydration stress. However, the transgenic *ZmMAPK1* lines showed more open, green leaves (Fig. 5c). Following the dehydration treatment, the plants were re-watered for a week to determine their survival rate. The *ZmMAPK1* transgenic lines had a significantly higher survival rate than the control lines and WT plants after the water deficit (Fig. 5d). The data indicate that overexpression of *ZmMAPK1* enhances *Arabidopsis* drought stress tolerance. The drought-tolerance phenotype of the overexpression

*ZmMAPK1* plants was further evaluated by measuring water loss from detached leaves. We found that the detached leaves of the *ZmMAPK1* plants lost water more slowly than those of the control lines (Fig. 6).

For the heat stress treatment assay, we observed that the phenotype of the overexpression lines showed a notable tolerance after treatment with 37 °C for 3 d. All the leaves of the control line were rolled while those of the transgenic lines were not (Fig. 7a, b). The survival rate of the *ZmMAPK1* lines was significantly higher than that of the control lines after they were transferred to normal growth conditions for one week (Fig. 7c). These results suggest that overexpression of *ZmMAPK1* also has a positive effect on heat stress tolerance.

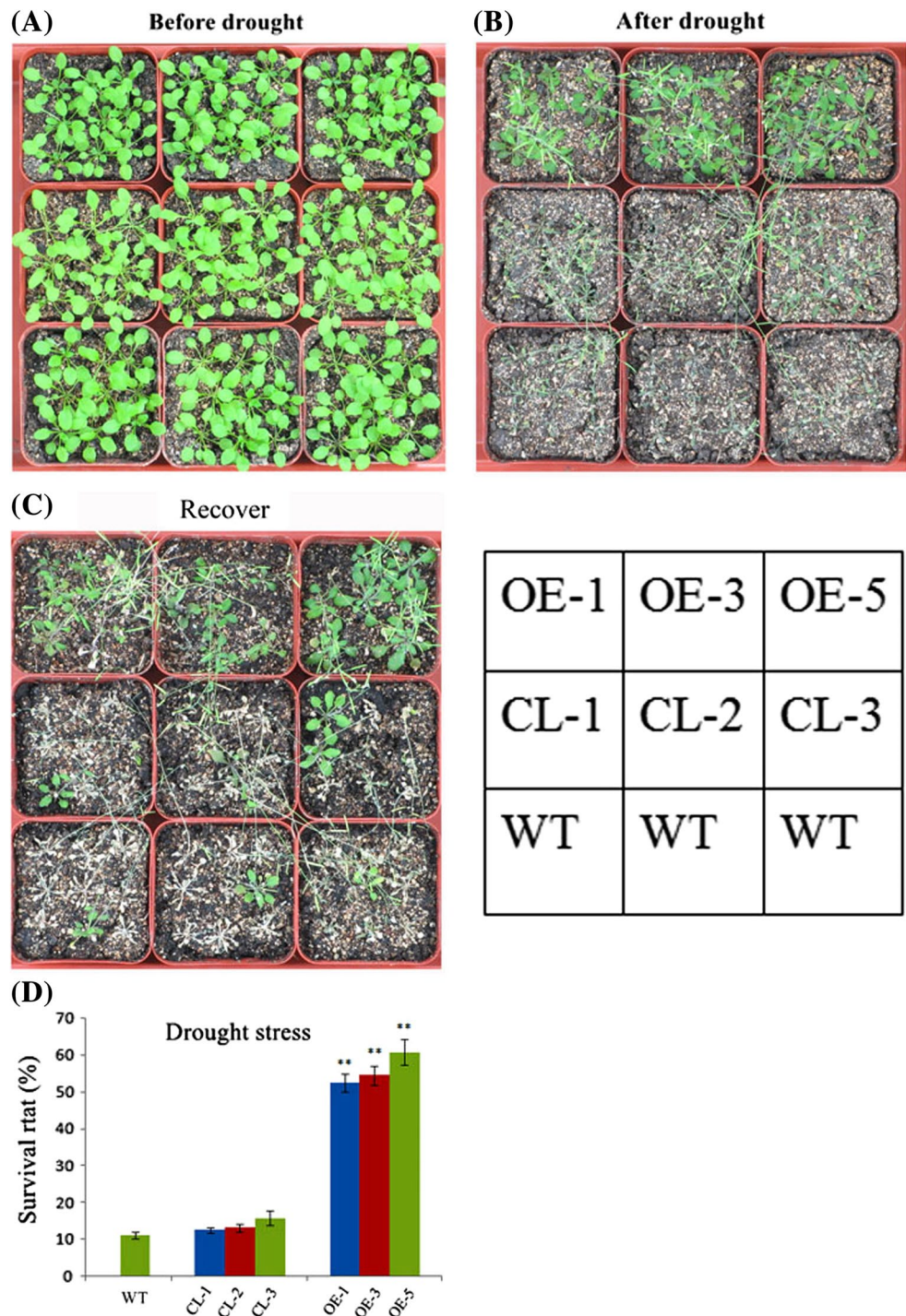
As for the salt-tolerance assay, after the transgenic and WT *Arabidopsis* seedlings were treated with 250 mM NaCl for 7 d, almost all of the transgenic and WT *Arabidopsis* seedlings had a similar appearance (data not shown), and the survival rates were not significantly different.

### Overexpression *ZmMAPK1* altered expression patterns of stress responsive genes

The induction of numerous stress-inducible marker genes is a hallmark of stress adaptation in plants (Zhu et al. 1997; Bao et al. 2014). To identify the possible genes involved in the *ZmMAPK1*-mediated drought stress responsive pathway, we performed qRT-PCR to examine the expression profiles of the ABA biosynthesis or ABA signaling regulators responsive genes under drought stress treatment, such as *ABA1*, *ABA3*, *ABII* and *ABI2*. Sample were analyzed following the 0, 1, 3, 6 d and recovered (RE) with water for one week. The qRT-PCR analyses demonstrated that the *ABA1*, *ABA3*, *ABII* were significantly induced by drought stress treatment, these results imply that they were acting as the positive factor in regulating the expression of these stress-responsive genes (Fig. 8a, b, c), while the *ABI2* gene was slightly changed compare with the control lines (Fig. 8d).

To elucidate the mechanisms underlying heat stress treatment, we also characterized the expression of some genes related with heat stress. It has been reported that some plant hormones, such as ABA, SA and the ethylene biosynthetic gene, is involved in heat response signaling (Larkindale et al. 2005; Lv et al. 2011). The genes known to be involved in plant heat response, such as 9-cis-epoxycarotenoid dioxygenase 3 (*NCED3*), *ABA2*, 1-aminocyclopropane-1-carboxylate synthase 5 (*ACS5*) and isochorismate synthase 1 (*ICS1*) were chosen. The expression level of the genes were measured at five stages, namely 0, 1, 2, 3 d and recover to normal growth conditions for one week (RE). As shown in Fig. 8e, f, the transcript levels of *NCED3* and *ACS5* were significantly increased after heat stress for

**Fig. 5** Phenotypes of *ZmMPAK1* transgenic plants under drought stress. Drought tolerance assay of the *ZmMAPK1* overexpression (OE) lines, OE-1, OE-3, OE-5 plants; pCAMBIA1304 vector control lines (CL), CL-1, CL-2, CL-3; and wild type (WT) plants. **a** The three-week-old plants were grown in nutrient soil before drought, deprived of water for 2 weeks (**b**), and then recovered with water (**c**). Similar results were observed in three independent experiments. **d** Survival rate of the plants in (**c**) under drought stress. The data presented are the mean  $\pm$  SD ( $n = 81$ ); Asterisks indicate significant differences compared with control line values ( $*P < 0.05$ ;  $**P < 0.01$ ). Similar results were observed in three independent experiments



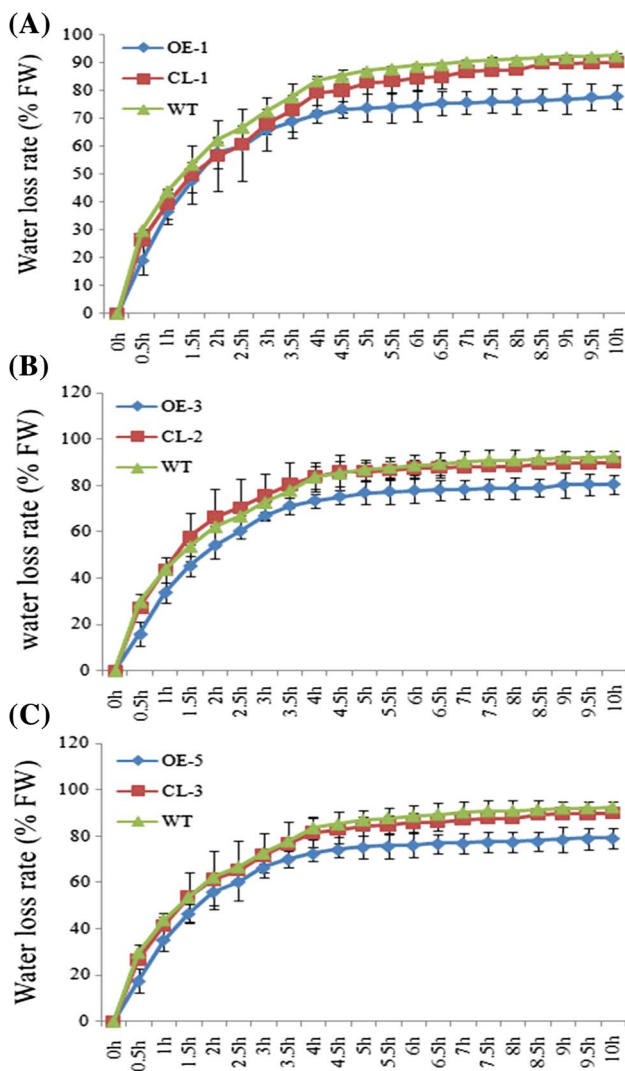
3 days. That's because the *AtNCED3* is an important gene in ABA biosynthesis, and the *AtACS5* is a member of ACS family, which has been implicated in the plant response to high temperature (Wang et al. 2005). The expression of the SA biosynthetic gene *ICS1* and ABA biosynthesis gene *ABA2* were suppressed during the processes of the heat stress treatment, but the control line was more suppressed in heat stress stage (Fig. 8g, h). These results indicated that

the overexpression of *ZmMAPK1* in Arabidopsis resulted in an alteration in the expression of stress-responsive genes.

#### Determination of MDA, proline, and chlorophyll content, and reactive oxygen species accumulation

The different stress tolerances of overexpression transgenic plants have been correlated with changes in proline





**Fig. 6** Rate of water loss from detached leaves of transgenic and control plants. **a** Rate of water loss from detached leaves of the OE-1, CL-1 and WT plants. **b** Rate of water loss from detached leaves of the OE-3, CL-2 and WT plants. **c** Rate of water loss from detached leaves of the OE-5, CL-3 and WT plants. The data presented are the mean  $\pm$  SD of three replicates ( $n = 27$ ); Asterisks indicate significant differences compared with control line values (\* $P < 0.05$ ; \*\* $P < 0.01$ )

and MDA contents (Sharma et al. 2011; Cheng et al. 2013). Previous studies showed increases the MDA content in plants subjected to drought stress (Lim and Lee 2014). To investigate the effect of drought and heat stress on lipid peroxidation, we measured the MDA content in transgenic and control plants. The MDA level in overexpression lines 1, 3 and 5 was lower than those in control lines and WT plants after drought and heat stress (Fig. 9a, b). To determine whether *ZmMAPK1* overexpression affected proline accumulation, the proline content in overexpression and control lines was measured. Under drought stress, proline activity was higher in *ZmMAPK1* overexpression transgenic plants,

but activity in the control line and WT line was similar. As shown in Fig. 9d, under heat stress, the proline content in the *ZmMAPK1* overexpression transgenic plants were also higher than that in the control lines.

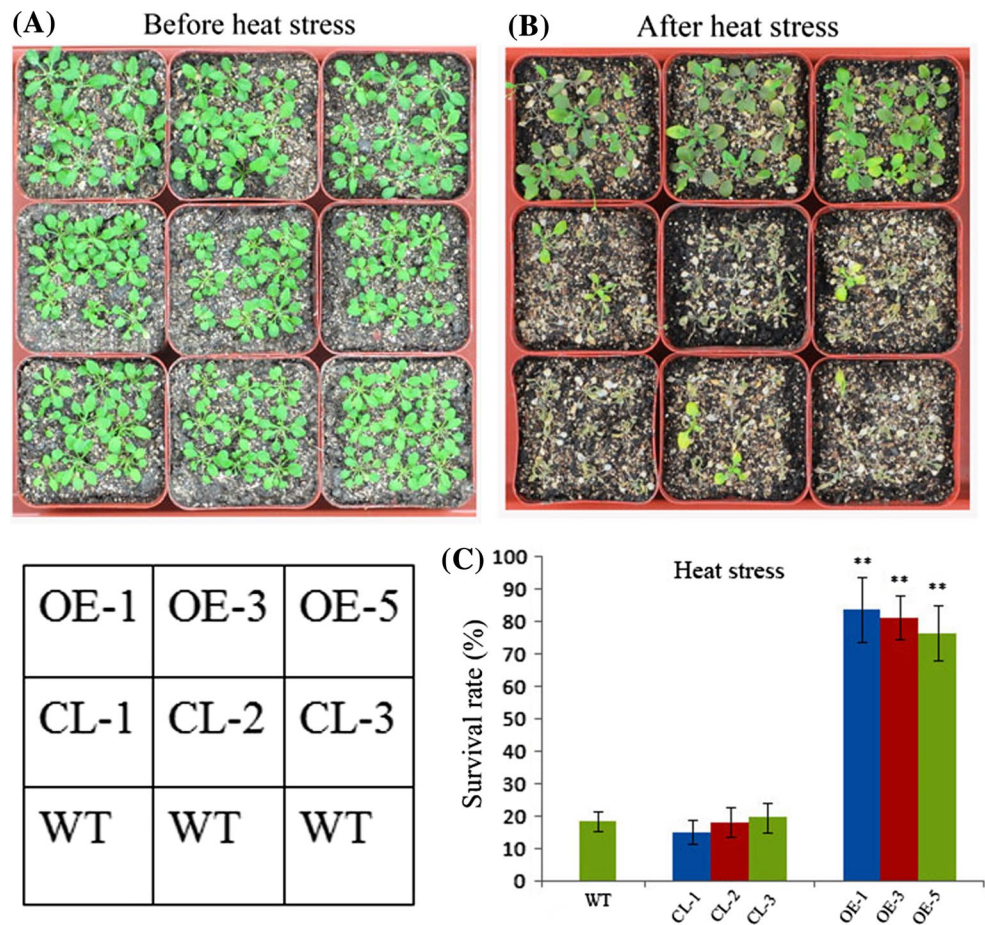
Many studies have indicated that various abiotic stresses can result in damage to plants via oxidative stress involving the generation of ROS (Zhu 2002; Zhou et al. 2012a; Cai et al. 2014). We quantified the amount of  $H_2O_2$  among the overexpression and control lines after 2 weeks of drought and growth at 37 °C for 3 d, respectively. The amount of  $H_2O_2$  in all the transgenic lines and the WT plants increased after drought treatment, but the increase in the overexpression lines was smaller (Fig. 9e). There was no significant difference in  $H_2O_2$  content among WT, overexpression and control lines under normal conditions ( $P < 0.05$ ). The  $H_2O_2$  content after heat stress was similar to that after drought stress (Fig. 9f), the results suggest that low  $H_2O_2$  concentrations were beneficial for the drought and heat stress response in the overexpression transgenic lines.

To test whether endogenous chlorophyll content is involved in the heat stress response, we analyzed the chlorophyll content in *Arabidopsis* leaves after heat stress. After 37 °C heat stress, total chlorophyll content was examined in the transgenic line and the WT plants. Clear differences in the chlorophyll contents in overexpression lines were evident compared with the control line and the WT plants (Fig. 9g). No significant difference in chlorophyll content was found between the control line and the WT plants. The survival rates of the overexpression lines were higher than that of the control line (Fig. 7c). The results showed that overexpression of *ZmMAPK1* can increase the chlorophyll content in *Arabidopsis* for resistance to heat stress.

## Discussion

Plants have developed sophisticated signaling pathways to deal with environmental stress (Ning et al. 2010). Here, we have identified a MAPK1 homologue in maize that we named *ZmMAPK1*. The database search and sequence analysis suggest that *ZmMAPK1* contains 11 conserved sub-domains and belongs to group A of the plant MAPKs. It is well known that MAPK cascades play essential roles in the activation of plant defense signal pathways in response to various abiotic stresses (Fu et al. 2002; Colcombet and Hirt 2008; Rodriguez et al. 2010; Zhang et al. 2012; Ding et al. 2013). The group A MAPKs in maize has been characterized in terms of their expression patterns under stress. Overexpression of *ZmMPPK4* in transgenic tobacco resulted in increased tolerance to low temperature stress by the accumulation of fewer ROS, and activated more relative gene expression (Zhou et al. 2012b). In this research, we examined the response of *ZmMAPK1* to drought, high

**Fig. 7** Phenotypes of *ZmMAPK1* transgenic plants under heat stress. Heat tolerance assay of the *ZmMAPK1* overexpression (OE) lines, OE-1, OE-3, and OE-5; pCAMBIA1304 vector control lines (CL), CL-1, CL-2, CL-3; and wild type (WT) plants. **a** The three-week-old transgenic and control plants were grown in nutrient soil before heat stress was applied. **b** The seedlings were kept at 37 °C for 3 d, and then transferred to normal growth conditions for one week. Similar results were observed in three independent experiments. **c** Survival rate of the plants in (b) after heat stress. The data presented are the mean  $\pm$  SD ( $n = 27$ ); Asterisks indicate significant differences compared with control line values ( $*P < 0.05$ ;  $**P < 0.01$ ). Similar results were observed in three independent experiments

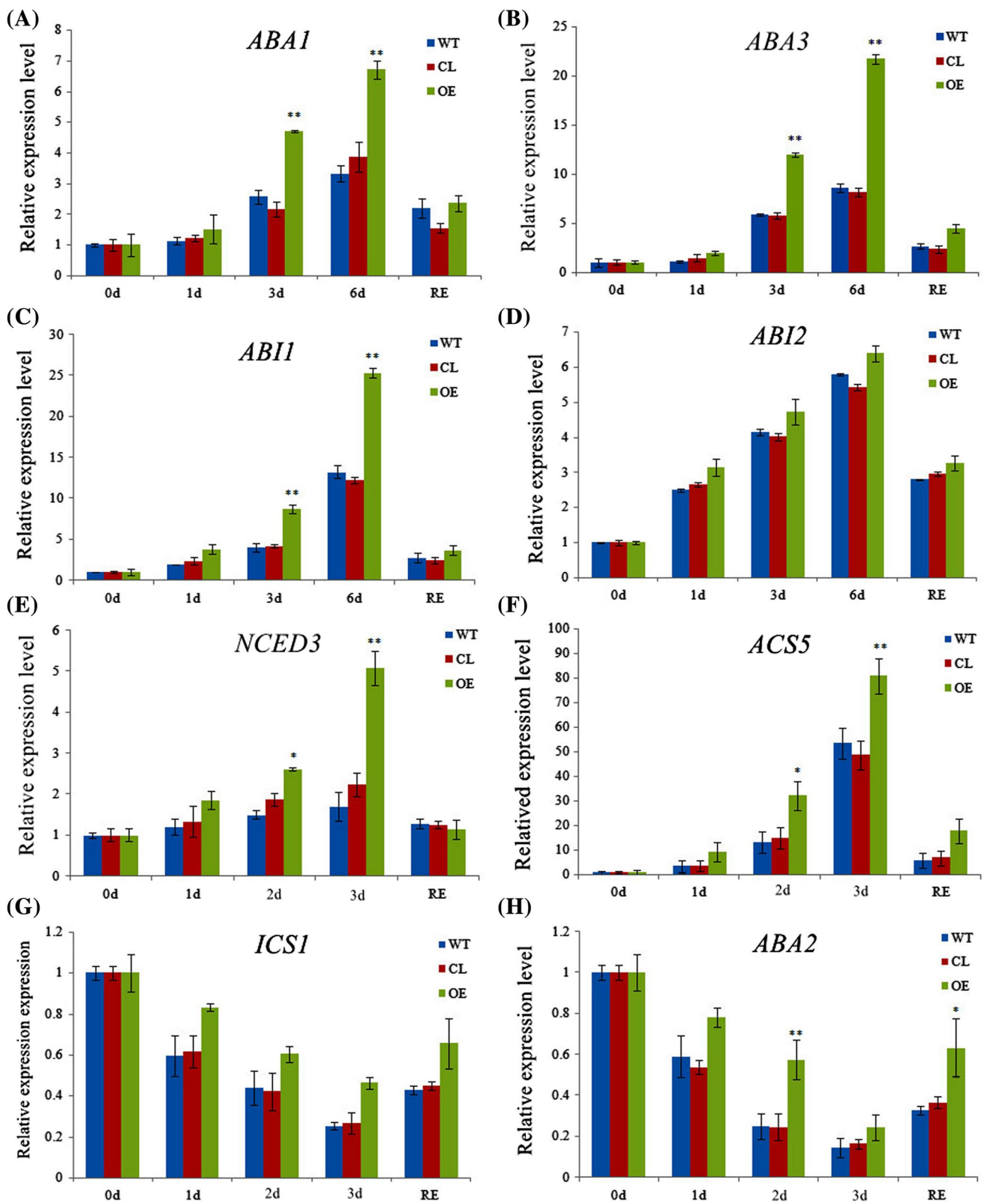


temperature, PEG, and sodium chloride. The observed changes in expression level of *ZmMAPK1* in response to different stress stimuli were similar to those of group A MAPKs in maize. Therefore, we infer that *ZmMAPK1* may have a similar function in resistance to various stresses. We also provided genetic and chemical evidence that *ZmMAPK1* plays an important role in response to abiotic stress (Figs. 3, 9).

MAPKs can be induced by various environmental stresses (Colcombet and Hirt 2008; Andreasson and Ellis 2010; Wu et al. 2011b), so the regulation of the large superfamily gene mechanism is complex. In our study, the relative expression levels of *ZmMAPK1* increased prominently in the few days before the leaf phenotype exhibited withering, and the gene expression level was about 3.8 fold higher after drought treatment (Fig. 3f). From the PEG6000 treatment of seedlings, we know that *ZmMAPK1* expression increased during the following 24 h (Fig. 3d). All the results indicate that *ZmMAPK1* was involved in drought stress. ABA is a major phytohormone in mediating inhibition of water loss from drought stressed leaves (Lim et al. 2014), the phytohormones may contribute to decrease water loss from the overexpression plants. As the *ABA1* and *ABA3* are

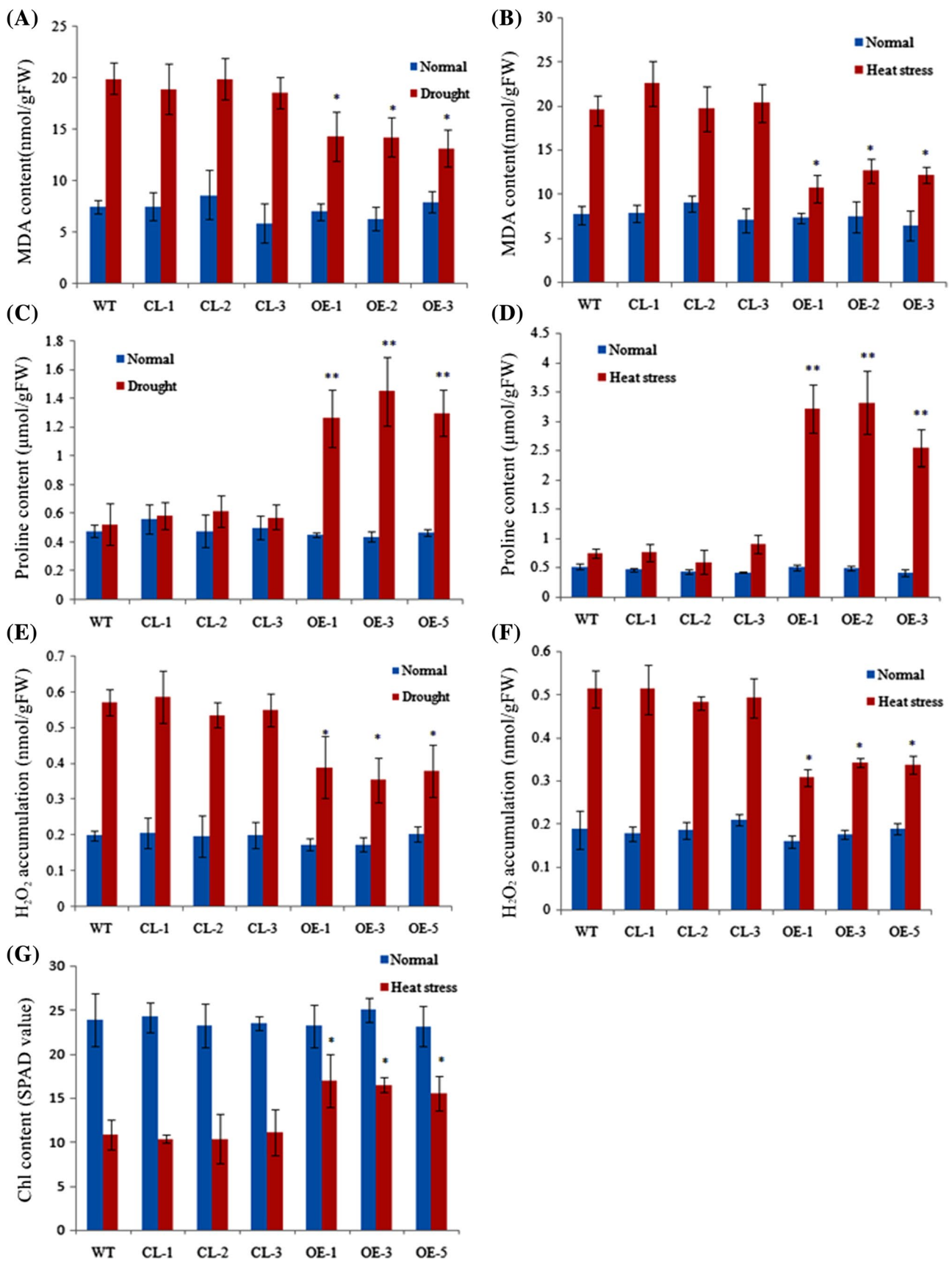
the major ABA biosynthetic genes, the results showed that they were significantly expressed in the OE lines (Fig. 8a, b). The *ABII* is the ABA signaling regulators gene which was also had a great effect on the ABA biosynthesis in the plants, which plays a pivotal role in coordinating the adaptive response to abiotic stress (Hartung et al. 2005). Proline is also an important osmoprotectant that protects cells from damage under different abiotic stress conditions as previous report. Overexpression of *ZmMAPK1* in *Arabidopsis* resulted in higher survival rates than in control lines under drought stress (Fig. 5d), and the resistance to drought was significantly different (Fig. 5a, b, c). Therefore, we conclude that *ZmMAPK1* has an important effect on resistance to drought stress.

It has been previously reported that heat stress is often combined with drought stress (Macková et al. 2013) and heat stress may cause changes in the content of ROS, MDA and proline (Larkindale and Knight 2002; Lv et al. 2011). Oxidative stress is the main driver of heat stress in plants, and the more  $H_2O_2$  accumulates in the cell membranes, the greater the danger to plant development, especially in the delay of plant growth (Guan et al. 2013). The results showed that *ZmMAPK1* is significantly induced



**Fig. 8** Expression levels of stress-responsive genes by qRT-PCR in overexpression line (OE), control line (CL) and wild type (WT). **a, b, c** and **d** are under the drought stress treatment; **e, f, g** and **h** are under heat stress treatment. Gene-specific primers were used for detection of relative transcript levels of stress-responsive genes, the raw data

were normalized using *ACTIN2* as an internal reference. Each value is the mean  $\pm$  SE ( $n = 27$ ); Asterisks indicate significant differences compared with control line values (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Similar results were observed in three independent experiments



◀ **Fig. 9** Determination of the MDA, free proline, H<sub>2</sub>O<sub>2</sub> and chlorophyll content in plants of the T<sub>3</sub> *ZmMAPK1* overexpression lines, OE-1, OE-3, and OE-5; pCAMBIA1304 vector control lines (CL), CL-1, CL-2, CL-3; and wild type (WT) plants. **a** Quantification of the MDA concentration in the overexpression lines, control lines and WT plants after 2 weeks drought stress. **b** Quantification of the MDA concentration in the overexpression lines, control lines and WT plants after 37 °C heat stress for 3 days. **c** Concentration of the free proline content in the overexpression lines, control lines and WT plants after 2 weeks drought stress. **d** Concentration of the free proline content in the overexpression lines, control lines and WT after 37 °C heat stress for 3 days. **e** Quantification of H<sub>2</sub>O<sub>2</sub> content regulated by overexpression lines, control lines and WT plants under drought treatment for 2 weeks. **f** Quantification of H<sub>2</sub>O<sub>2</sub> content regulated by overexpression lines, control lines and WT after 37 °C heat stress for 3 days. **g** Changes in chlorophyll (Chl) content in overexpression lines, control lines and WT plants after 37 °C heat stress for 3 days. Values are the mean ± SE (n > 80); Asterisks indicate significant differences compared with control line values (\*P < 0.05; \*\*P < 0.01). Similar results were observed in three independent experiments

by exposure to 37 °C (Fig. 3c), and highly expressed after heat stress for 12 h in the leaves, which suggests that *ZmMAPK1* also plays a positive role in resistance to heat stress. Overexpression of the *ZmMAPK1* gene in *Arabidopsis* resulted in increased survival rates after heat stress, and the heat stress related genes has a significant changed (Fig. 8e, f). The survival rates of overexpression lines were about four times those of the controls. Compared with the control lines, the result shows that the transgenic plants had a higher tolerance under heat stress (Fig. 7).

In this study, we measured different physiological indices. Environmental stresses often cause physiological changes in plants, so such indices are used to evaluate abiotic stress tolerance and resistance in crops (Tian et al. 2013). ROS is the main product of heat stress, and MAPKs are the key players in ROS signaling (Pitzschke and Hirt 2009; Zhang et al. 2010). Because elevated H<sub>2</sub>O<sub>2</sub> concentrations in plants can accelerate cell death and decrease drought tolerance and thermotolerance (Choi et al. 2007), we investigated the ROS level in the overexpression lines. The result showed that total ROS accumulation was increased after heat treatment, but the overexpression lines had lower levels than the controls (Fig. 9f). Proline is related to drought resistance and may more closely reflect the balance between osmotic stress and resistance. In this study, the rate of water loss of detached leaves from the *ZmMAPK1* overexpression lines was slower than in the control line (Fig. 6), and the survival rates were higher than in controls (Fig. 5d). These results indicated that the overexpression transgenic lines had higher water retention ability. Chlorophyll content is widely used in heat tolerance assays, and is a determining factor for the accumulation of biomass. In our study, changes in chlorophyll accumulation were lower than normal, although it was higher in

the transgenic line than in the control and WT plants under heat stress (Fig. 9g).

Water deficit and heat stresses induce various physiological responses in plants, especially the production of MDA. MDA is the main compound produced in plants under drought and heat stress, which can damage membranes and result in cell death (Levine et al. 1994, Liu et al. 2013a). The higher the content of MDA, the higher is the danger to plant survival under environmental stress. MDA levels in transgenic lines 1, 3 and 5 were obviously lower than those in controls and WT plants after drought stress (Fig. 9a, b). Cell membrane stability is affected by lipid peroxidation caused by ROS accumulation under stress conditions (Sudhakar et al. 2001), and heat stress-induced H<sub>2</sub>O<sub>2</sub> is required for effective expression of heat shock genes in *Arabidopsis* (Volkov et al. 2006). As shown here, the ROS content was lower in the overexpression transgenic plants than in control and WT plants after drought and heat stress (Fig. 9e, f). Physiological parameters in plants are important for growth and development. The altered physiological indices may promote plant survival in conditions of environmental stress, and they help to maintain cell membrane stability by reducing ROS. This effect is partly due to the increased activities of some anti-oxidant enzymes such as superoxide dismutase (SOD), an interpretation consistent with previous studies.

In summary, a MAPK1 homologue (*ZmMAPK1*) has been identified and characterized in maize. Morphological and physiological evidence strongly demonstrated that the transgenic *ZmMAPK1* plants acquired enhanced tolerances to drought and heat stresses. The enhanced stress tolerance was partly confirmed by higher plant survival rates, proline content, and lower MDA accumulation in the plants. Further studies are needed to understand the function of the MAPK pathway under drought and heat related stresses.

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