Biochemical and Biophysical Research Communications 443 (2014) 357–362

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Overexpression of tomato *SpMPK3* gene in *Arabidopsis* enhances the osmotic tolerance

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ARTICLE INFO

Article history:
Received 1 November 2013
Available online 23 November 2013

Keywords:
Tomato
SpMPK3
Osmotic stress
Arabidopsis

ABSTRACT

Mitogen-activated protein kinase (MAPK) class plays diverse roles in plant response to abiotic stresses. *SpMPK3*, a serine/threonine protein kinase containing a Thr-Glu-Tyr (TEY) activation domain, has been reported to function against bacterial, fungus and insect attack in tomatoes. But, its roles in abiotic stress response are poorly experienced. Herein, the experiment we have conducted demonstrates that there is a substantial increase of *SpMPK3* expression in tomato leaves by drought, salt and cold stress. Transgenic *Arabidopsis* plants overexpressing *SpMPK3* showed improved seed germination and antioxidant capacity under osmotic stress, but decreased seed germination with ABA treatment compared to wile-type. In addition, *SpMPK3* overexpression enhanced the transcription levels of ABA inducible genes—*RD29A*, *RAB18* and *RD22*, and transcription factor genes—*ZAT6*, *ZAT10* and *MYB44*, in transgenic *Arabidopsis* compared with wild-type in response to ABA or salt stress. These results suggest that *SpMPK3* is a positive regulator in response to osmotic stress in *Arabidopsis* at germination and development stage.

1. Introduction

The cultivated tomato, *Solanum lycopersicum*, as the second most consumed vegetable is widely grown around the world and constitutes a major agricultural industry (FAOSTAT 2011; http://faostat.fao.org). However, various biotic and abiotic stresses impair the quality and yield of tomatoes by hindering the growth and development of the seedlings. Many signaling pathways are involved in mediating the plants' responses to stresses. Mitogen-activated protein kinase (MAPK) cascade, the foundation as the core of the signal transduction networks, plays crucial roles in the ability of plants to tolerate and adapt to stresses by regulating stress signal transduction and the expression of relevant genes.

The MAPK signaling pathway is a three-tiered phosphorelay cascade consisting of MAPKs (MPKs), which are activated by MAPK kinases (MPKK or MKKs), which in turn are activated by MAPKK kinases (MAPKKKs). As the last component of cascade to be activated, MAPKs can phosphorylate specific serine/threonine residues on the target protein, thereby regulating a variety of cellular activities [1,2]. In tomato, 16 *MPK* genes are identified and generally grouped into four subfamilies according to their sequences and structures [3]. Previous studies showed that MAPKs members in subfamily A, namely *MPK1*, *MPK2* and *MPK3* could act on biotic stress. *SlMPK1*, *-2*, and *-3* were investigated to be involved in *Cf-4*-mediated hypersensitive response (HR) and resistance of tomato to *Cladosporium fulvum* [4]. Also, Mayrose and Melech-Bonfil reported that *SlMPK2* and *SlMPK3* participated in defense response to *Xanthomonas campestris pv. Vesicatoria* [5,6]. Besides, *SlMPK1*, *-2* and *-3* were functioned against systemin-mediated insect herbivores by regulating jasmonic acid (JA) biosynthesis and the expression of JA-dependent defense genes as well as *Mi-1*-mediated resistance to aphids [7,8]. Plants trigger MAPK cascades upon biotic stress, but also when challenged by abiotic stresses. Nie et al. found that *SlMPK1* and *SlMPK2* also be involved in brassinosteroid-mediated oxidative and heat stresses [9]. Additional studies suggest that abscisic acid (ABA)-mediated responses, including antioxidant defense, guard cell signaling and seed germination involve with MAPK cascades [10]. The combination of these findings prompted us to wonder whether or not *SlMPKs* act on abiotic stresses, such as drought, salinity and cold that closely related to ABA signaling. Therefore, this study investigates the involvement of a *Solanum pimpineliforlium MPK SpMPK3* in ABA-related abiotic stresses, which remains to be established in tomato.

Here, we report that *SpMPK3* is activated by drought, salt and cold stress in tomatoes. Furthermore, the genetic transformation experiments showed that *SpMPK3* may positively regulate the osmotic tolerance and ABA sensitivity at germination and development stage in *Arabidopsis*.

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0006-291X/$ - see front matter © 2014 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bbrc.2013.11.061

2. Materials and methods

2.1. Plant materials and growth conditions

S. pimpineliforlium L03708 and *S. lycopersicum* L. cv. M82 was used to study the expression patterns of *SpMPK3* gene. The plants were grown in a growth chamber with a 16 h-light and 8 h-dark photoperiod at 25 °C.

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia seeds were soaked in a 1% sodium hypochlorite for 15 min and rinsed 5 times with sterilized water, then placed at 4 °C in the dark for 2 days, and germinated on plates containing one-half strength MS medium, supplemented with 0.75% phytoagar without sugar (pH 5.7) in a growth chamber at 22 °C with a 8-h-light/16-h-dark cycle. 10- to 12-day-old seedlings were transplanted to soil and grown in a growth chamber with a 16-h-light/8-h-dark cycle at 22 °C.

2.2. Construction of SpMPK3-overexpressing transgenic plants

To generate *SpMPK3*-overexpressing plants, the full-length coding sequences of *SpMPK3* were amplified from cDNAs by polymerase chain reaction (PCR) using a pair of specific primers set with *BamH*I and *Sac*I, respectively (Table S1), and cloned into the pMD-18T vector. *SpMPK3* were generated from pMD-18T-*SpMPK3* after sequenced and recombined into pVBG2307 destination vector digested with *BamH*I/*Sac*I. The recombinant constructs were electroporated into cells of *Agrobacterium tumefaciens* GV3101 and transformed into *Arabidopsis* using an *A. tumefaciens*-mediated floral dip method [11]. The seeds were selected on half-strength MS plates containing 100 mg/L kanamycin. The plants were examined by genomic PCR and reverse transcriptase PCR (RT-PCR) analysis. Homozygous T2 lines were obtained by self-crossing and were used for the experiments.

2.3. Stress treatment

For osmotic stress treatment, tomato seeds were sown in the cell tray filled with perlite and supplied 1/2 Hoagland nutrient solution every 2 days. 35-day-old plants were treated with 20% (w/v) polyethylene glycol (PEG) and 300 mM sodium chloride (NaCl), respectively. For cold stress treatment, tomato seeds were sown in the cells tray with a mix [grass charcoal/perlite (3/1, zv/v)] medium. 35-day-old plants were placed at 4 °C for cold treatment. Leaves from stress-treated plants were collected at 0, 1, 2, 6, 12, 24, 48 and 72 h after treatment, immediately frozen in liquid nitrogen and kept at −80 °C.

Light-grown, 3-week-old wild-type and transgenic *Arabidopsis* plants overexpressing *SpMPK3* were subjected to 150 mM NaCl and sprayed with 100 μM ABA (containing 0.5% Tween-20), respectively. After incubation for 5 h, leaves from mock and stress-treated plants were collected and frozen immediately in liquid nitrogen and kept at −80 °C for the expression analysis of stress-related genes.

All treatments were performed and analyzed triplicate in separate experiments.

2.4. Germination of SpMPK3-overexpressing Arabidopsis plants

Germination assays were carried out with 100 seeds in triplication. Surface-sterilized seeds 2 days after imbibition of wild-type and *SpMPK3-OE* were grown on half-strength MS medium containing different concentration of NaCl (0, 100 and 200 mM) or ABA (0, 0.3 and 0.5 μM), respectively, at 22 °C with a 8-h-light/16-h-dark photoperiod. The percentages of radicle emergence were determined after 7 days.

2.5. Histochemical detection of H_2O_2 and O_2^- radical

The O_2^- and H_2O_2 level was measured by nitroblue tetrazolium (NBT) staining and 3,3-diaminobenzidine (DAB) staining, respectively [12,13]. Light-grown, leaves of 3-week-old WT and *SpMPK3-OE* plants were treated with 150 mM NaCl and 100 μM ABA for 2 h, respectively. For NBT staining, leaves were incubated in HEPES buffer (pH 7.5) containing 6 mM NBT for 2 h. For DAB staining, leaves were incubated with 1 mg/mL DAB solution for 5 h. NBT or DAB-stained samples were boiled in 97% (v/v) ethanol to remove chlorophyll. The presence of O_2^- and H_2O_2 in leaves is visualized as a dark blue and dark brown color, respectively.

2.6. RNA isolation and real-time qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and then treated with DNase I (Fermentas, Glen Burnie, MD) to clean out DNA. cDNA synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit (Takara, Dalian, China) was used for real-time qPCR. Real-time qPCR was performed using SYBR Premix Ex Taq II (Takara, Dalian, China) on iQ5 Real-Time PCR Detection System (BIO-RAD Corp., Hercules, California, USA). The PCR cycling conditions were as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 20 s. Melting curve was routinely performed after 40 cycles to verify primer specificity. The $2^{-\triangle\triangle Ct}$ method was applied to calculate the fold change in the expression of each gene [14]. *S. pimpinellifolium* elongation factor 1−α (*EF*1α) mRNA level and *Arabidopsis Eukaryotic translation initiation factor 4A-1* (*eIF4A*) was used as internal control for normalization [15]. All primer sequences are given in Table S1.

2.7. Statistical analysis

R 2.15.2, open-source software, is used for the statistical analysis. The data were subjected to univariate ANOVA analysis of variance followed by a *post hoc* test. Values were computed as the means ± SD of three or more independent experiments.

3. Results and discussion

3.1. Expression analysis of SpMPK3 in response to drought, salt and cold stress in tomato

MAPK cascades, the center of signal transduction network, positively regulate plant to tolerate various environmental stresses. To analyze response of *SpMPK3* gene to abiotic stresses, L03708 and M82 tomato plants were exposed to 20% PEG, 300 mM NaCl and 4 °C temperature, respectively. The abundance of *SpMPK3* transcripts was analyzed by quantitative RT-PCR. As shown in (Fig. 1), the transcription levels of *SpMPK3* in L03708 were higher than that of M82 under each stress. When the plants were subjected to PEG solution, a rapid accumulation of *SpMPK3* transcript in L03708 was observed within 1 h (3-folds), maximized at 24 h (3.5-folds), and kept at 1.4-fold at 72 h. Interestingly, there was a slight down-regulation at 12 h. While in M82, the highest transcription level was detected at 2 h (1.5-fold), and then gradually declined to background level beyond with longer treatment. The expression patterns of *SpMPK3* were similar in L03708 and M82 plants under salt stress, which were increased first and then decreased gradually. *SpMPK3* transcription levels maximized at 6 h with a 4.5-fold increase in L03708, and a 2.0-fold increase in M82 plants. With cold treatment, although the expression patterns were similar, the expression levels were markedly different in the two materials. The highest transcription level was detected at 12 h when the

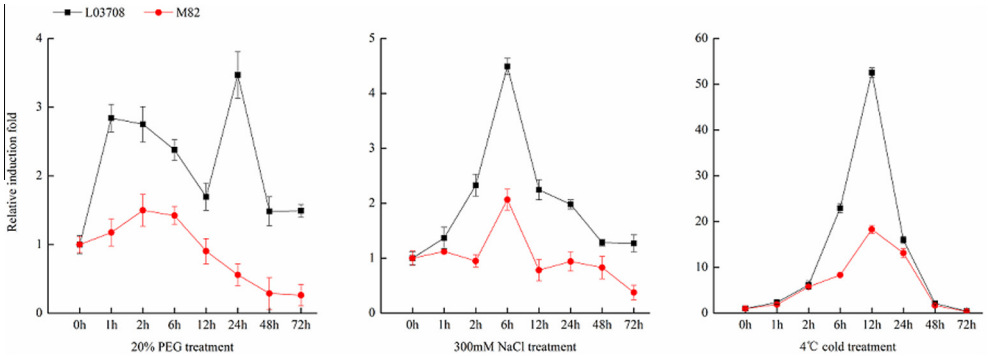

Fig. 1. Expression analysis of *SpMPK3* under drought, salt and 4 °C cold stress. The relative transcription levels of *SpMPK3* were calculated in various treated leaves in comparison to that in controls (0 h) defined as 1. Error bars represent standard deviation for three independent replicates.

transcription level of *SpMPK3* was induced to 80-fold in L03708 and 20-fold in M82 compared to control (0 h). And then, it gradually declined to background with longer treatment. In agreement with these results, the results of previous studies have suggested that the expression of *Arabidopsis AtMPK3*, with high homology to the *SpMPK3*, could be enhanced by high salinity and drought stresses [16]. The increases of *SpMPK3* expression level in response to osmotic and cold stress suggest that *SpMPK3* may be involved in basal tolerance of osmotic and cold stress in tomatoes.

3.2. SpMPK3 overexpression enhanced osmotic tolerance in transgenic Arabidopsis

In view of the potential role of *SpMPK3* in response to abiotic stress, a gain-of-function approach was employed to dissect the functional role of *SpMPK3*. *Arabidopsis* transgenic plants overexpressing *SpMPK3* under the control of *CaMV* 35S promoter were generated. Two homozygous lines (line 13 and line 15) in which *SpMPK3* was strongly expressed were selected for further study.

3.2.1. SpMPK3 overexpression conferred osmotic tolerance and ABA sensitivity in transgenic Arabidopsis at germination stage

Previous research has identified that several *MPKs* could function in seed germination under abiotic stresses [10]. To evaluate the possible roles of *SpMPK3* in the defense response of tomato seeds against various abiotic stresses, germination assays were performed. 100 seeds were placed on half-strength solid MS medium supplemented with 10% PEG and different concentrations of NaCl (100 mM or 200 mM), respectively. After 7 days of stratification, germination rates were examined as a percentage of radicle emergences. As shown in (Fig. 2), germination rate was higher of *SpMPK3-OE* seeds under osmotic stress compared with WT, and almost the same with each other under normal conditions. Nearly 92% *SpMPK3-OE* seeds germinated with the PEG stress, and WT seeds < 10% germinated. In response to 100 mM NaCl, *SpMPK3-OE* seeds showed higher germination rate—96% compared with WT seeds, whose germination rate significantly reduced to 31%. When the concentration of NaCl increased to 200 mM, the germination rate of *SpMPK3-OE* seeds still kept at 94%, while significantly reduced to 15% of WT seeds. The data presented here demonstrate that *SpMPK3* plays a positive role in response to osmotic stress at germination stage.

As a stress hormone, ABA plays a key role in the regulation of many physiological processes in response to stresses. Seed germination is just such an example, and is sensitively inhibited by ABA [17]. *MPKs* being considered as the mediator of ABA signaling, may involve in many aspects of ABA-regulated biological activities [10]. To investigate whether *SpMPK3* has any effects on ABA-inhabitant seed germination, 100 seeds were plated on half-strength solid MS medium supplemented with different concentrations (0.3 and 0.5 μM) of ABA. After 7 days of stratification, germination rates were determined as a percentage of radicle emergences. WT and *SpMPK3-OE* seeds fully germinated on half-strength MS medium without ABA. On the ABA-containing growth medium, although germination rates of WT seeds were concomitantly reduced as concentrations of ABA increased, 85% of WT seeds germinated at 7 days in the presence of 0.5 μM ABA (Fig. 2B, line 3). In contrast, *SpMPK3-OE* transgenic seeds exhibited hypersensitivity to ABA. More than 80% of the transgenic seeds were unable to germinate in the presence of 0.3 μM ABA concentration, and less than 5% of the transgenic seeds germinated with 0.5 μM ABA (Fig. 2). Previous findings have shown that *MPKs* overexpression reduced germination rate of *Arabidopsis* seeds when they exposed to ABA [18]. This experiment also provides the evidence that *SpMPK3* may be a positive regulator in response to ABA in tomato.

3.2.2. SpMPK3 overexpression enhanced antioxidant responses of transgenic Arabidopsis at development stage

Most abiotic stresses evoking an increased production of reactive oxygen species (ROS) has been demonstrated to cause oxidative damage to plants, and the O_2^- and H_2O_2 are believed to be the most important components [19]. Kim showed that mkk4 mutants appeared to be sensitive to osmotic stresses with an increased ROS production in *Arabidopsis* [20]. As it is reported, *AtMKK1-MPK6* and *AtMKK4-MPK3* are two important MAPK cascades in response to abiotic tolerance in *Arabidopsis* [10]. Herein, the stress-induced ROS production was investigated in *SpMPK3-OE Arabidopsis* plants under salt and ABA treatments by histochemical staining. As shown in (Fig. 3A), basal levels with low production of O_2^- (NBT staining) and H_2O_2 (DAB staining) were detected in control plants (CK). However, the leaves of WT plants treated with NaCl and ABA were stained extensively, whereas those of *SpMPK3-OE* plants showed light staining. In addition, the percentage of NBT and DAB staining in leaves revealed that the productions of O_2^- and H_2O_2 were markedly decreased in *SpMPK3-OE* plants compared with WT plants under salt and ABA stress (Fig. 3B). As a whole, these results demonstrate that *SpMPK3* may be involved in salt and ABA-promoted ROS production.

3.3. Levels of stress-responsive genes and transcription factor genes are up-regulated in transgenic plants relative to wild-type

Osmotic stress increases the cellular level of the phytohormone ABA and the increased ABA in turn promotes the expression of ABA-dependent stress-responsive genes, such as *RD29A*, *RAB18* and *RD22* which can act as the putative target of *MPKs* or marker genes of the endogenous ABA level [21,22]. Additionally, transcription factors (TFs) as transcriptional regulators in plants can

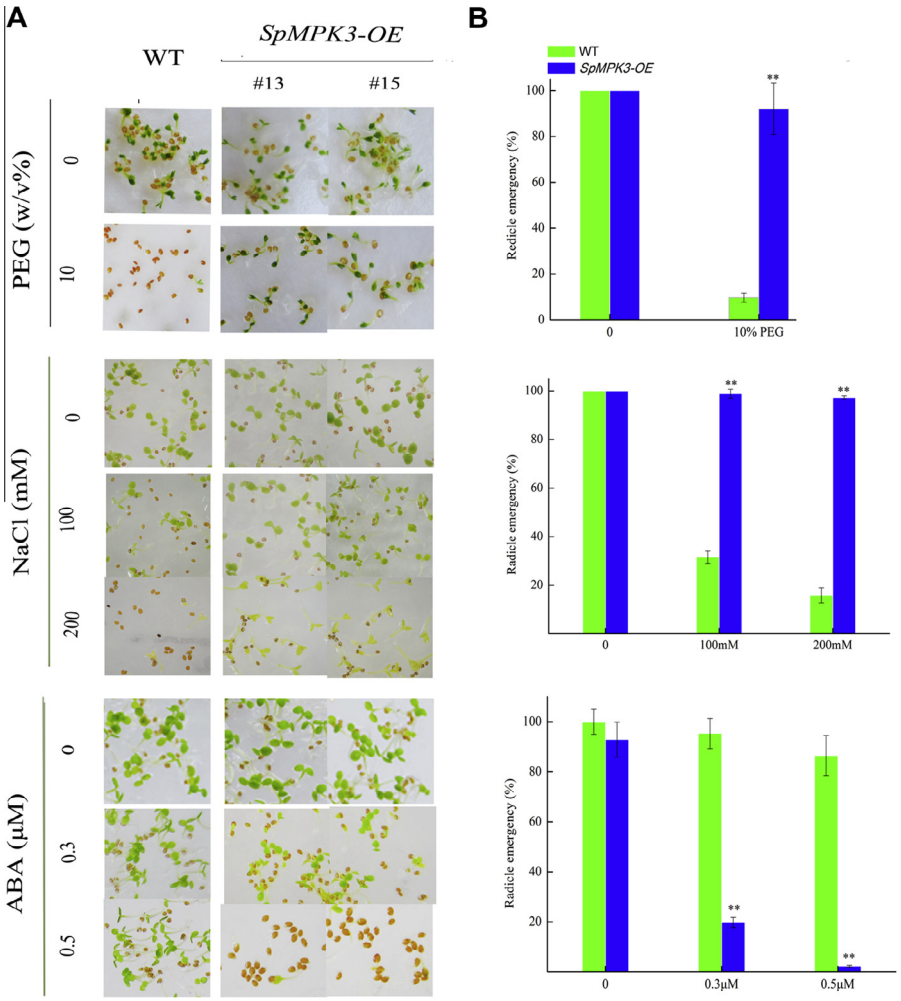

Fig. 2. Germination of WT and *SpMPK3-OE* seeds under osmotic and ABA stress. (A) Phenotypic analysis of WT and *SpMPK3-OE* seeds. (B) The calculation of seed germination rates. Error bars represent standard deviation for three independent replicates. Double asterisks indicate significant difference at $P < 0.0001$.

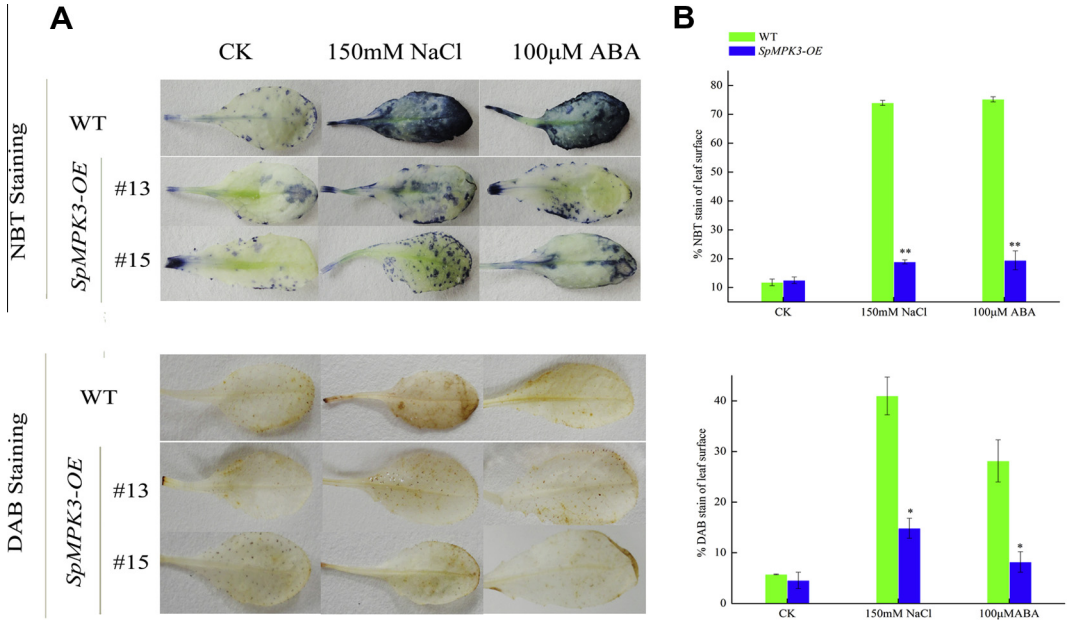

Fig. 3. Superoxide accumulations in leaves of 3-week-old seedlings under 150 mM NaCl and 100 μM ABA treatment for 2 h. (A) Detection of O_2^- and H_2O_2 production by NBT and DAB staining, respectively. (B) The quantity of O_2^- and H_2O_2 in leaves. The data represent means of 6 leaves from each of transgenic line and WT plants, respectively. Error bars represent the standard deviation. $^*P < 0.05$; $^{**}P < 0.01$.

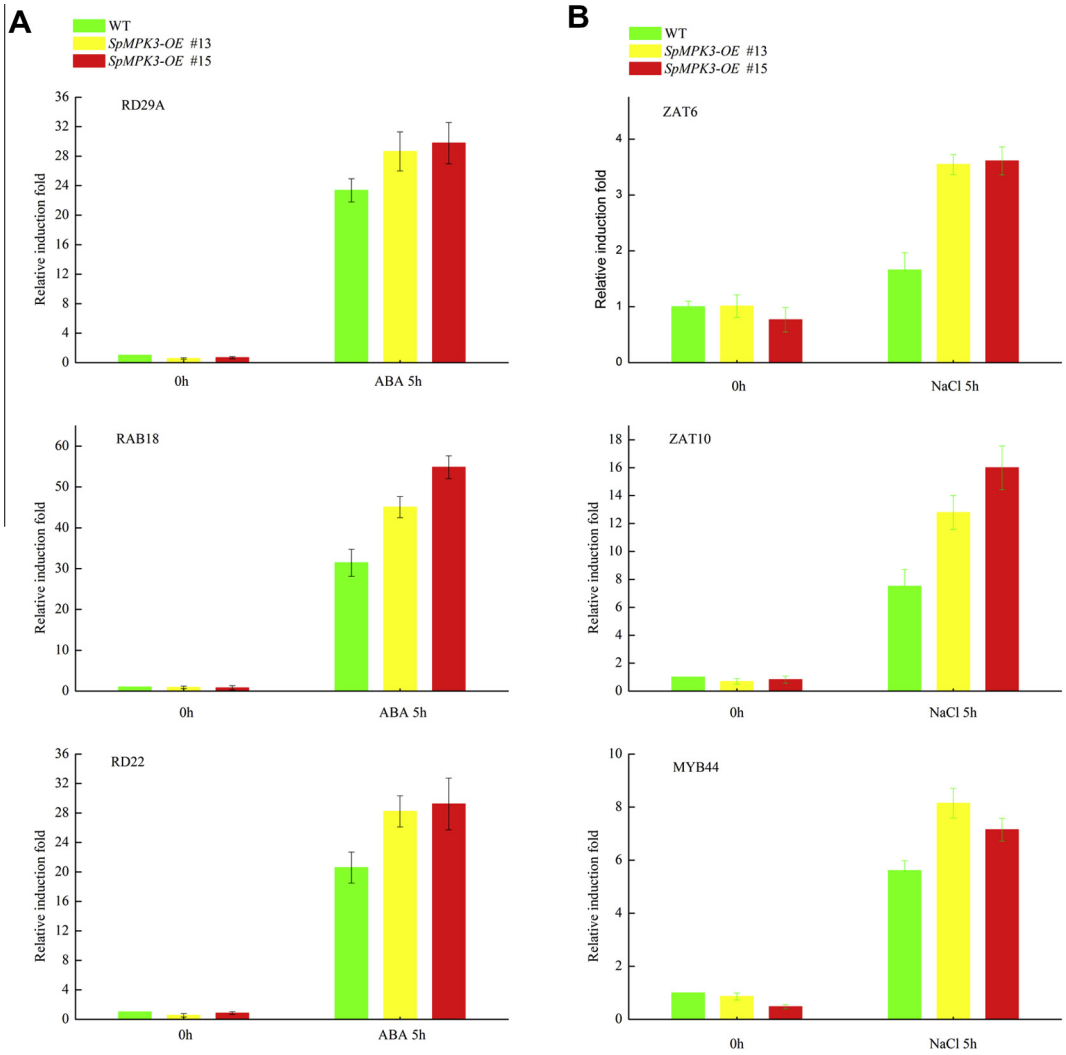

Fig. 4. Real-time qRT-PCR analyses of ABA and osmotic induction stress-inducible genes. Light-grown, 3-week-old wild-type and *SpMPK3* transgenic plants were treated with 100 μM ABA and 150 mM NaCl for 5 h, respectively. Total RNA was obtained from treated plants and analyzed by qRT-PCR using the gene-specific primers listed in Table S1. Error bars represent standard deviation for three independent replicates.

function in abiotic tolerance as well [23]. The *ZAT6*, *ZAT10*, and *MYB44*, can act as the substrate of *MPKs* or act on the *MPK* signaling pathway in response to various abiotic stresses including salt, drought and cold in *Arabidopsis* [24–26]. Since our results showed that *SpMPK3* is positively correlated with both ABA sensitivity and osmotic tolerance at germination and development stage, to clarify whether *SpMPK3* affects the expression profiles of stress-responsive genes, the expression patterns of *RD29A*, *RAB18* and *RD22*, and MPKs-targeted transcription factors *ZAT6*, *ZAT10* and *MYB44* were monitored in WT and *SpMPK3-OE* transgenic plants under ABA and NaCl treatment, respectively. Light-grown, 3-week-old WT and *SpMPK3-OE* plants were treated with ABA (100 μM) and NaCl (150 mM) for 5 h, respectively. Total leaf RNAs were analyzed by real-time qRT-PCR using each gene-specific primers (Table S1). The degree of ABA induction of *RD29A*, *RAB18* and *RD22* were higher in *SpMPK3-OE* plants than in WT plants, with the biggest difference in *RAB18* expression. The expression of *RAB18* was induced by ABA to 60-fold in *SpMPK3-OE* plants, which was two times higher than that in WT plants. The expression of *RD29A* and *RD22* was induced by ABA to approximately 29-fold in *SpMPK3-OE* plants, which was higher than that of WT plant with a 20-fold increase. The data presented here indicate that *SpMPK3* may function in these ABA-responsive genes (Fig. 4A). In addition, *ZAT6*, *ZAT10* and *MYB44* were also up-regulated in *SpMPK3-OE* plants as compared with WT plants. The magnitude of *ZAT6* and *ZAT10* was two times higher in *SpMPK3-OE* plants than in WT plants after NaCl treatment. The transcription level of *MYB44* was up-regulated 1.5 times higher in *SpMPK3-OE* plants than that of WT plants by NaCl. This study provides evidence that *SpMPK3* may act positively on regulating ABA induction genes *RD29A*, *RAB18* and *RD22* and stress responsive transcription factors genes *ZAT6*, *ZAT10* and *MYB44* to tolerate various stresses in *Arabidopsis*.

In conclusion, the transcription levels of *SpMPK3* rapidly increased in tomato plants exposed to drought, salt and cold conditions. In addition, *SpMPK3* overexpression in *Arabidopsis* enhanced osmotic tolerance and ABA sensitivity of transgenic seeds, and confers transgenic plants antioxidant capability when plants were subjected to osmotic stress. However, due to the diverse roles of *MPKs* in plant development, further studies are required to elucidate the physiological importance of *SpMPK3* in osmotic tolerance.

Acknowledgments

We are grateful to ABRC for providing *Arabidopsis* seed stocks. We owe gratitude to AVRDC-The World Vegetable Center and

Tomato Genetic Research Center for supplying the experimental material. This work is funded by the "the Twelfth Five-Year-Plan" of National Science and Technology Project in rural areas (#2011BAD12B03-03-5), and the Science and Technology Coordination and Innovation Project of Shaanxi Province (#2011KTCL02-03).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.061.

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