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ORIGINAL ARTICLE

Grape *VvMAPK9* positively regulates salt tolerance in *Arabidopsis* and grape callus through regulating the antioxidative system

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Abstract

Mitogen-activated protein kinase pathways are involved in plant resistance to a variety of adverse environmental processes, and their downstream component MAPKs play an important role in this process. However, the function of MAPKs in abiotic stresses is still far from being clear in grape (*Vitis vinifera* L.). Here, we isolated a novel group B MAPK gene (*VvMAPK9*) from grape, which is induced by different abiotic stresses such as salt, drought and high temperature (42 °C). Overexpressing *VvMAPK9* in *Arabidopsis thaliana* significantly enhanced the tolerance to salt stress. Compared with wild type plants, the transgenic lines exhibited higher germination rate and longer root length as well better growth status under salt stress. In addition, overexpression of *VvMAPK9* in grape callus also increased the salt stress tolerance and enhanced the callus's ability to scavenge reactive oxygen species (ROS), which correlated with higher activity of ROS-related antioxidant enzymes. These results indicate that *VvMAPK9* may positively regulate salt stress by regulating the antioxidative system.

Key message

Grape *VvMAPK9* positively regulates salt tolerance in *Arabidopsis* and grape callus through regulating the antioxidative system

Keywords Grape · MAPK · Salt stress · Antioxidant

Introduction

Plants are constantly exposed to a variety of abiotic stress throughout their life cycle, including high salinity, drought and extreme temperatures (Qin et al. 2011; Tuteja 2007). In order to cope with these stresses, plants have changed their physiological structure, morphology and evolved different signaling pathways to sense and transmit various signals (Bohnert et al. 1995). Among these signaling pathways, mitogen-activated protein kinase (MAPK) cascade pathways are highly conserved and involved in environmental stress resistance such as high salinity and drought (Danquah et al. 2014; Sun et al. 2015). A typical MAPK cascade is composed of three kinases: MAPK kinase kinase (MAPKKK or MEKK), MAPK kinase (MAPKK or MEK) and MAPK, forming the MAPKKK-MAPKK-MAPK signaling pathway. Currently, many MAPKs have been identified from different plants, there are 20 MAPKs in *Arabidopsis*, 17 in rice, 21 in poplar, and 14 in grapevine. According to amino acid sequence similarity, MAPKs are divided into four subfamilies (A–D group). Group A, B and C all have a common

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phosphorylation motif TEY in their active loop, while group D MAPKs contains a TDY motif (Jonak et al. 1999; Hamel et al. 2006; MAPK Group 2002). In addition, groups A, B and C possess a conserved C-terminal docking domain, whereas it could not be found in the sequence of group D (MAPK Group 2002).

It is well documented that plant MAPK protein kinases are involved in different abiotic stresses, such as salt, drought, cold and high temperature. For example, *OsMAPK5* played a positive regulatory role in high salinity, drought and low temperature stress in rice (Xiong and Yang 2003). In *Arabidopsis*, *AtMAPK3* and *AtMAPK6* enhanced the ability of plants to resist salt stress and oxidative stress (Zhou et al. 2017; Pérez-Salamó et al. 2014), and the MEKK18-MKK3-MPK1/2/7/14 cascade pathway was involved in osmotic stress and ABA signaling (Danquah et al. 2015; Li et al. 2017). In *Zea mays*, *ZmMAPK1* have been reported to participate in both drought and high temperature stresses (Wu et al. 2015). Moreover, *Durum wheat TMKP1* phosphatase enhanced salt tolerance of plants (Zaidi et al. 2016). All these results suggested that the MAPK genes have important application values in the improvement of stress tolerance in crops.

Abiotic stresses always resulted in the rapid production of reactive oxygen species (ROS), particularly H_2O_2 and $O_2^{\cdot -}$. It is well known that ROS, at low concentration, are important signaling molecules, while high concentrations of ROS may result in oxidative stress and cause irreversible damage to biological organisms (Kovtun et al. 2000). Therefore, moderate the accumulation of ROS is critical to regulate many biological processes of plants. Previous studies reported that MAPK cascades are involved in the maintaining of ROS homeostasis. In *Arabidopsis*, the MEKK1-MKK2-MPK4/6 cascade is known to participate in the regulation of ROS production under abiotic stress (Teige et al. 2004; Xing et al. 2008), and *AtMAPK8* can negatively regulate the ROS accumulation (Takahashi et al. 2011). In *Nicotiana benthamiana*, NPK1-MEK1-NTF6 cascade was reported to enhance the ROS accumulation by promoting the expression of *NbRbohb*, which increased the plant tolerance to environmental stresses (Asai et al. 2008). Recent study reported that under salt stress the *PdMAPK3/6* were activated to negatively regulate the ROS production to reduce the oxidative damage in *Populus* (Lu et al. 2020). Taken together, the cross-talk between ROS and the MAPK cascade in the signal transduction network is very complex, and further studies are required to clarify these mechanisms.

Grape (*Vitis vinifera* L.) is one of the important economically fruit crops in the world. However, grape is constantly exposed to a variety of environment stresses during growth and development stages, which severely inhibit its growth, yield and economic value. Among these abiotic stresses, soil salinization is the main limiting factor that seriously restricts the development of grape industry. Developing salinity-tolerant grape varieties is considered as one of the most effective ways for increasing the yield of grape in high saline soil. However, it is difficult to breed highly halotolerant grapevine varieties by traditional breeding methods, whereas genetic engineering is an economic and more effective strategy on screening and introducing salinity–tolerant varieties. Therefore, it is necessary to reveal salt tolerance mechanisms and search for salt resistant genes. The important function of MAPK in abiotic stresses in plants has been revealed, but studies were mainly concentrated on the model plants, less on fruit trees, especially grapevine. In this study, a group B MAPK gene, *VvMAPK9* from grape was isolated and its expression pattern under abiotic stress was analyzed. Then, the function of *VvMAPK9* was investigated by overexpressing it in *Arabidopsis* and grape callus. This study would not only enrich our understanding of MAPK signaling in grape, but also provide the theoretical foundation for the application of *VvMAPK9* in grapevine rootstock breeding.

Materials and methods

Plant materials and stress treatments

The issue culture seedlings of grape rootstock A35 were cultured in MS solid medium supplemented with 0.2 mM indolebutyric acid (IBA) at 25 °C with a 16 h light/8 h dark cycle. 2-month-old grape seedlings were treated with 100 μM abscisic acid (ABA), 200 mM mannitol, 200 mM NaCl, and high temperature (42 °C), respectively. For tissue-specific expression analyses, young leaves, mature leaves, petioles, stems and roots were harvested from the same plants, frozen in liquid nitrogen, and stored at − 80 °C.

'*Crimson seedless*' grape calluses were cultured as previous described (Xu et al. 2019), which were used for gene transformation and salt tolerance assay. The grape callus was cultured on MS medium supplemented with 0.59 g/L 2-(N-Morpholino) ethanesulfonic acid, 10 mg/L picloram, and 2.2 mg/L thidiazuron, at 25 °C under dark conditions.

Arabidopsis thaliana were used for gene transformation and salt tolerance assay. The leaves of *Nicotiana benthamiana* seedlings were used for transient gene expression. *Arabidopsis thaliana* and *Nicotiana benthamiana* were all planted in plastic pots filled with vermiculite under a greenhouse conditions at 22 °C with a 16 h light/8 h dark photoperiod.

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA of grape was isolated by an improved cetyltrimethyl ammonium bromide (CTAB) method, and total

RNA of *Arabidopsis thaliana* was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (Vazyme, Nanjing, China). According to the supplier's instructions, the qRT-PCR was performed using the SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) in the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The grape β-actin and the *N. benthamiana* β-actin gene were used as the internal reference. All primers used in this study are listed in Table S1.

Isolation of the *VvMAPK9* open reading frame sequence

The open reading frame (ORF) of *VvMAPK9* was isolated by PCR amplification with the specific primers VvMPK9-F and VvMPK9-R (Table S1) that was designed and synthesized by Biosune Biotechnological Company, Shanghai, China. The PCR products were purified and combined with pMD19-T vector (TaKaRa, Dalian, China) and then transformed into *E. coli* cells (DH5α) for sequencing (Biosune Biotechnological Company, Shanghai, China).

Subcellular localization of VvMAPK9

The ORF sequence of *VvMAPK9* was fused to the N-terminus of the green fluorescent protein (GFP) gene controlled by the *35S* promoter. Cells of *Agrobacterium tumefaciens* GV3101 containing the recombinant plasmid cultured overnight were collected and resuspended in osmotic solution (10 mM MES, 10 mM $MgCl_2$, and 150 mM acetosyringone), and injected into leaves from 1-month-old *N. benthamiana* seedlings after placed in the dark for 3 h. After 2–3 days of transformation, the fluorescent signal was detected by a confocal microscope (LSM 510 META, Carl Zeiss). Leaves expressing the *35S-GFP* construct were used as a control (Shi et al. 2011).

Transformation of *VvMAPK9* into grape callus and *Arabidopsis thaliana*

The full-length cDNA of *VvMAPK9* was inserted into the binary vector PBI121 controlled by the *35S* promoter. Then, the recombinant plasmid was introduced into *A. tumefaciens* GV3101 strain and transformed into *Arabidopsis thaliana* using a floral dip method as previously described (Clough and Bent 1998). The transgenic seedlings were selected on 1/2 MS agar medium containing 50 mg/L kanamycin, and homozygous lines were screened and further confirmed by PCR and qRT-PCR. Subsequently, three homozygous transgenic lines (OE1, OE2, and OE3) were selected for further studies.

The grape callus with *VvMAPK9* overexpression were obtained as described by Xu et al (2019). Firstly, the recombinant plasmid was introduced into *Agrobacterium* strain LBA4404. Secondly, grape calluses were put in the *Agrobacterium* suspension for 20 min, blotted dry using sterile filter paper and cultured on solid MS medium with 100 μM acetosyringone in darkness at 25 °C. After 2 days, the calluses were screened on the MS medium with 100 mg/L kanamycin and 300 mg/L cefalexin. 2 months later, most of the calluses had died, and the surviving callus were subcultured on screening medium at 4-week intervals until the callus no longer turns black and dies. Finally, the transgenic callus were confirmed by qRT-PCR.

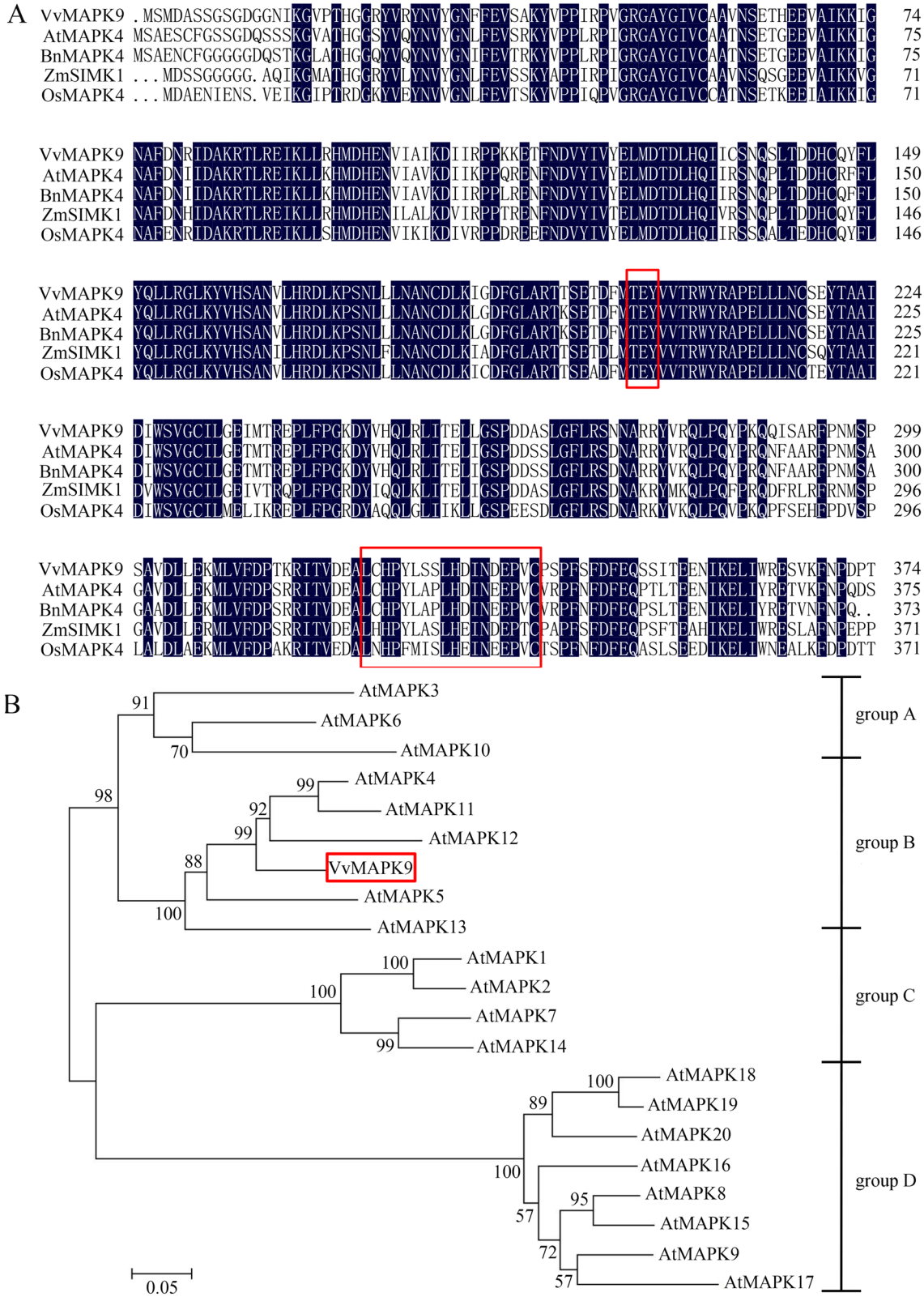
Salt tolerance assays

In *Arabidopsis*, wild type (WT) and transgenic *Arabidopsis* seeds (T3 generation) were disinfected and sowed on 1/2 MS medium containing different concentrations of NaCl for seed germination and root length analysis. Seed germination was monitored every 12 h, and root length was measured after 7 days of vertical culture. In addition, 2-week-old WT and transgenic *Arabidopsis* seedlings were irrigated with 200 mM NaCl solution, and the control seedlings were irrigated with water. The plant growth status was observed every day. After 2 weeks of salt stress treatment, the plants were photographed. Each experiment was performed at least three independent biological replicates.

For the salt assay of grape callus, the same size callus were inoculated on MS medium containing 150 or 200 mM NaCl, and photographed after 10 days treatments. The relative electric conductivity was measured as described by Zhou and Leul (1998). Total protein concentrations were quantified with the BCA Protein Assay Kit (Suzhou Kerming Biotechnology co. LTD, China). The activities of anti-$O_2{\cdot}^-$, superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were determined by spectrophotometry according to the instruction of the corresponding assay kit (Suzhou Kerming Biotechnology co. LTD, China), respectively. The expression levels of antioxidant enzymes genes were determined by qRT-PCR. Each experiment was conducted at least three times.

Bioinformatic and statistics analysis

Amino acid sequences of other plants MAPK genes were retrieved from GenBank (http://www.ncbi.gov/Genbank). Amino acid sequence alignments were done using the DNAMAN5.2.2 (https://www.lynnon.com). The phylogenetic tree was constructed by the Neighbor-Joining (NJ) method using MEGA 4 software (https://www.megasoftware.net/mega4/mega.html). The promoter sequence of *VvMAPK9* were performed using PlantCARE datebase (http://bioinforma


A
VvMAPK9
AtMAPK4
BnMAPK4
ZmSIMK1
OsMAPK4
B
AtMAPK3
AtMAPK6
AtMAPK10
AtMAPK4
AtMAPK11
AtMAPK12
VvMAPK9
AtMAPK5
AtMAPK13
AtMAPK1
AtMAPK2
AtMAPK7
AtMAPK14
AtMAPK18
AtMAPK19
AtMAPK20
AtMAPK16
AtMAPK8
AtMAPK15
AtMAPK9
AtMAPK17
group A
group B
group C
group D
0.05

◂**Fig. 1** Phylogenetic tree analysis and sequence alignment. **A** Alignment of the amino acid sequences of *Vitis vinifera* (VvMAPK9, XP_002278860.1) with *Arabidopsis thaliana* (AtMPK4, NP_192046), *Brassica napus* (BnMAPK4, ABB69023), *Zea mays* (ZmSIMK1, NP_001105239.2) and *Oryza sativa* (OsMAPK4, BAC99508.1). Identical amino acids are shaded in black. The phosphorylated TEY motif and the CD domain are marked by red frame. **B** Phylogenetic analysis of VvMAPK9 and *Arabidopsis thaliana* MAPK proteins. The neighbour-joining phylogenetic tree was constructed using MEGA 4.0. The numbers above or below the branches indicate the bootstrap values (> 50%) from 500 replicates

tics.psb.ugent.be/webtools/plantcare/html/). Statistical significance was analyzed using Duncan's multiple range tests with analysis of variance (ANOVA), and calculations were performed with SPSS Statistics.

Results

Sequence analysis of *VvMAPK9*

The full-length ORF sequence of *VvMAPK9* (XP_002278860.1) is 1128 bp, encoding a 375 amino acid peptide with a predicted molecular weight of 42.615 kD and an isoelectric point of 6.24. Multiple sequence alignments with other plant MAPKs demonstrated that VvMAPK9 contains a conserved phosphorylation motif TEY in the active loop and a CD domain in the C-terminal, which shares high homology (76.80–84.31%) with other group B MAPKs, such as AtMAPK4, BnMAPK4, ZmSIMK1 and OsMAPK4 (Fig. 1A). The Phylogenetic analysis revealed that *VvMAPK9* displayed high similarity to the members of the MAPK group B (Fig. 1B), as previously reported (Çakır and Kılıçkaya 2015). These results indicated that VvMAPK9 is a member of MAPK group B.

Subcellular localization

To investigate the localization of VvMAPK9, two constructs, 35S::GFP and 35S::VvMAPK9:GFP (Fig. 2A), were transferred individually into the epidermal cells of tobacco leaves by *Agrobacterium* GV3101 mediated transient transformation. As shown in Fig. 2B, the fluorescence signals of both VvMAPK9:GFP fusion protein and 35S::GFP were detected in the cytoplasm and nucleus by the laser confocal microscope, which indicated that VvMAPK9 protein may function in the nucleus and cytoplasm.

Expression pattern analysis in different tissues of grapevine

In order to identify the organ-specific expression pattern of *VvMAPK9* in grape, qRT-PCR was used. The RNA of 2-month-old grape seedlings from tissue culture was extracted for qRT-PCR. The results showed that the expression level of *VvMAPK9* was mainly expressed in young leaves, mature leaves and roots, but relatively low in petiole and stem (Fig. 3), which suggested that the expression of *VvMAPK9* was tissue specific.

Expression of *VvMAPK9* under different abiotic stresses

To investigate the potential functions of *VvMAPK9* under different abiotic stresses, 2-month-old grape tissue culture seedlings were exposed to various abiotic stresses and the expression profile of *VvMAPK9* was examined by qRT-PCR. As shown in Fig. 4A, the expression of *VvMAPK9* increased significantly under salt treatment and peaked after 8 h. After drought simulated by mannitol treatment, the expression of *VvMAPK9* increased at first and then decreased, and the peak appeared at 12 h (Fig. 4B). Under heat treatment, the transcription level of *VvMAPK9* dramatically increased and reached a peak at 4 h (Fig. 4C). In addition, the *VvMAPK9* transcription had a significant rise under ABA treatment, and expression peak appeared at 3 h (Fig. 4D). All these results indicated that *VvMAPK9* seem to be involved in responses to a variety of abiotic stresses.

Analyses of *cis*-elements in promoter sequence of *VvMAPK9*

To further investigate the mechanism that the *VvMAPK9* responds to abiotic stresses, 2000 bp upstream sequence of the *VvMAPK9* was analysed by the PlantCARE database. Many putative *cis*-acting elements were predicted in the promoter sequence of *VvMAPK9*, which are related to abiotic and biotic stress responses and light responsiveness (Table 1). Specifically, stress response element (STRE) is involved in responses to osmotic stress. Ethylene responsive element (ERE), MYB and MYC elements are participated in responses to drought stress. W-box (combined with WRKY transcription factor binding site) is related to inducer, injury and pathogen responses. In addition, some of these *cis*-elements have been shown to be involved in low temperature and salicylic acid responsiveness.

Overexpression of *VvMAPK9* in *Arabidopsis* enhanced salt tolerance

To investigate the role of *VvMAPK9* in abiotic stress resistance in plants, *VvMAPK9* was overexpressed in *Arabidopsis*. Three transgenic lines (OE1, OE2, and OE3) with different expression levels of *VvMAPK9* were selected for further experiments. Under non-stressful conditions, the seed germination and development of WT and OE plants were no significant different. However, following treatment with

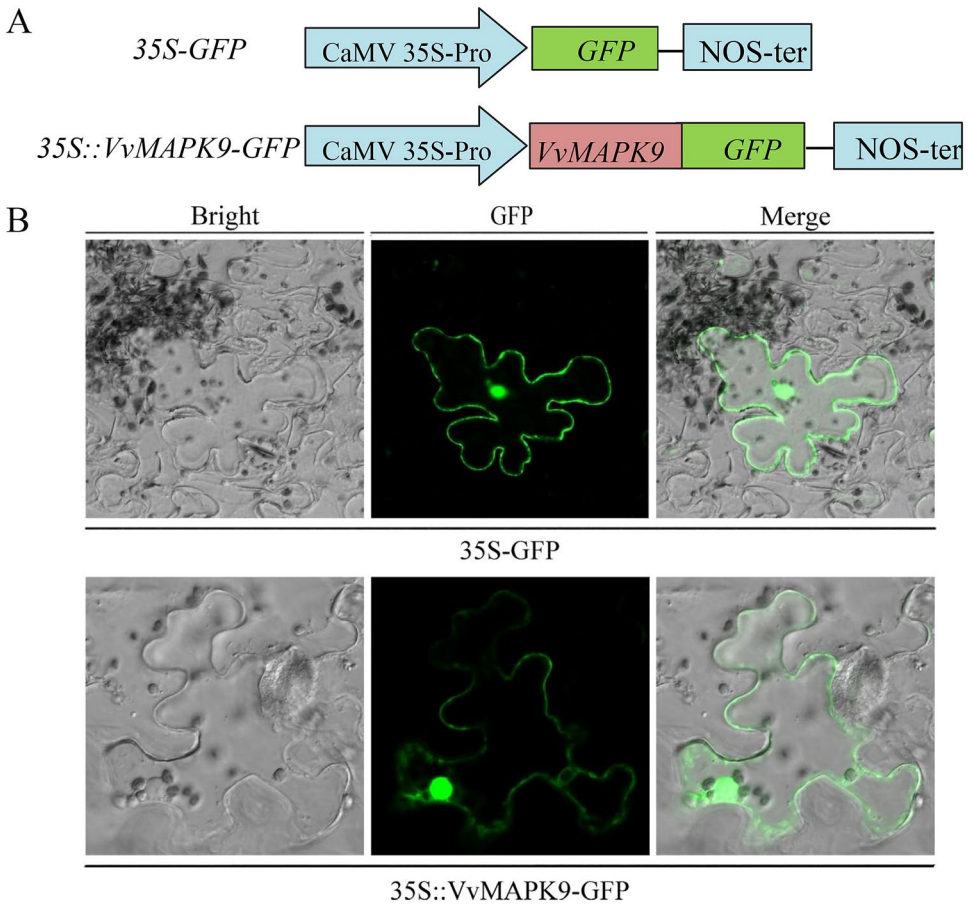


Fig. 2 Subcellular localization of VvMAPK9 in *N. benthamiana* leaves. **A** Schematic diagram of the 35S::VvMAPK9:GFP fusion construct and 35S::GFP construct. **B** Transient expression of the 35S::VvMAPK9:GFP fusion construct and the 35S::GFP construct in *N. benthamiana* leaves. Green fluorescence was observed with an LSM 880 META confocal microscope (Carl Zeiss)

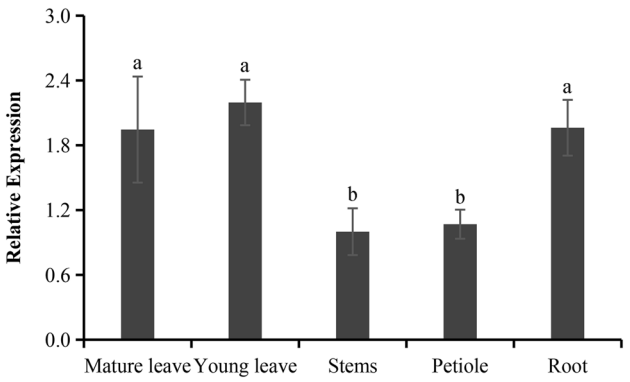


Fig. 3 Expression patterns of *VvMAPK9* in different tissues of grape. Tissue-specific expression of *VvMAPK9* was detected in the roots, stems, petiole, young leaves and mature leaves of 2-month old grape seedlings by qRT-PCR. The β-actin gene was used as the reference gene. The data are the means ± standard error (SE) of three independent experiments (n = 3). Different letters above the bar indicate significant differences ($P \leq 0.01$) based on Duncan's multiple range tests

NaCl, the seeds of the OE lines germinated much earlier, and the germination rate of OE lines were significantly higher than that of WT. At 48 h, the germination rates of OE1, OE2, and OE3 were about 1.3-, 2- and 2.5-fold of WT, respectively (Fig. 5). At the same time, the seeds of WT and OE lines with the same germination status were spread in 1/2 MS medium containing 0, 100 or 200 mM NaCl. After 2 weeks, on MS medium without NaCl, the root length of WT and OE line seedlings was consistent. However, under NaCl treatment, the root length of OE seedlings was significantly longer than that of WT (Fig. 6A, B). To further examine salt tolerance in the transgenic *Arabidopsis* during the vegetative growth stages, 2-week-old WT and OE seedlings were treated with 200 mM NaCl for 14 days. It was found that the leaves of WT were significantly withered and even died, while the OE lines grew significantly better (Fig. 6C). These results showed that overexpression of the *VvMAPK9* gene confers tolerance to salt stress in the early growth of *Arabidopsis*.

Overexpression of *VvMAPK9* improved the salt tolerance of grape callus

To further examine whether *VvMAPK9* participates in salt stress tolerance in grape, *VvMAPK9* was overexpressed in grape callus, three transgenetic lines (OE1, OE2, and OE3) exhibiting different expression levels of *VvMAPK9* were obtained and used for further experiments. WT and

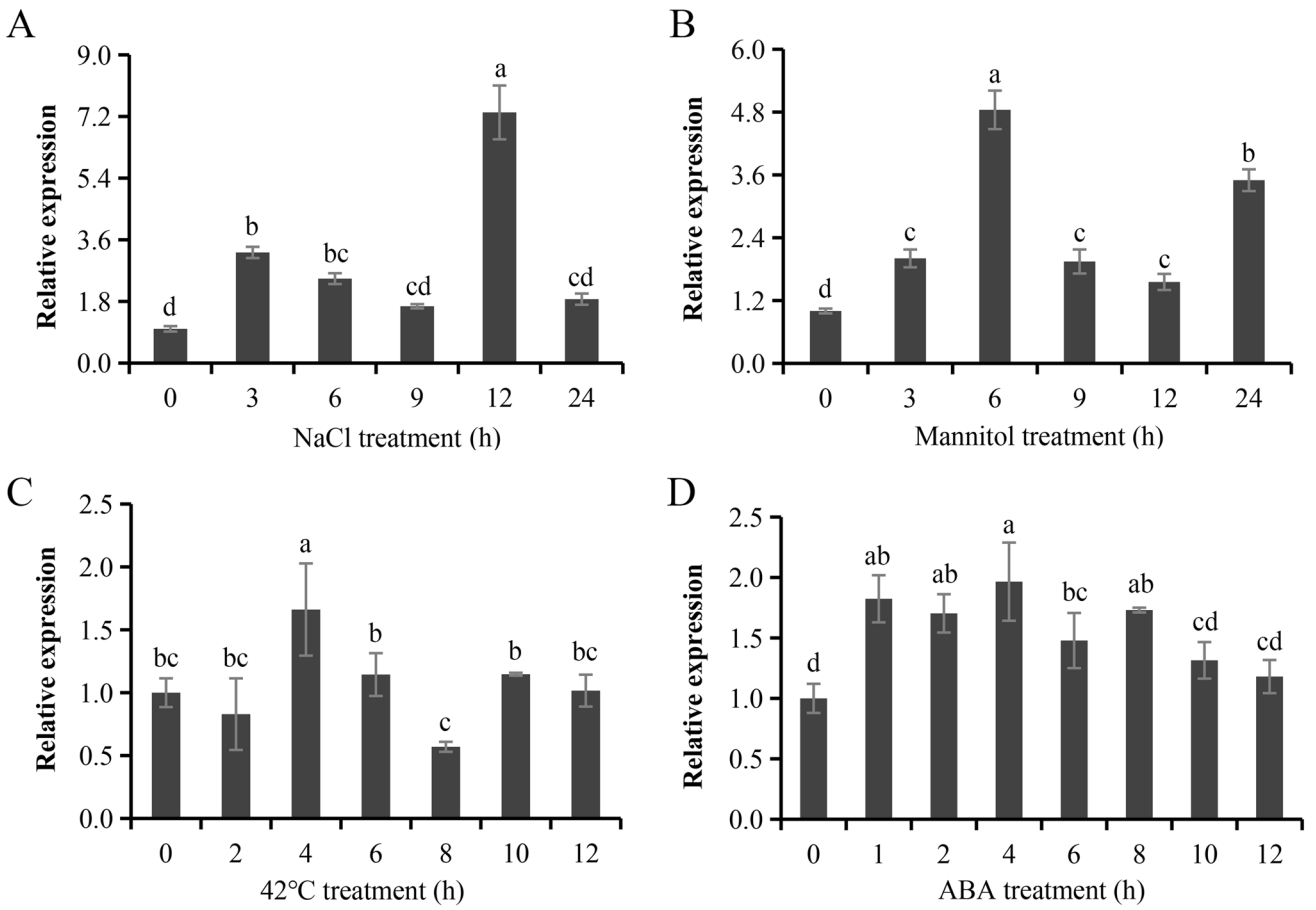


Fig. 4 *VvMAPK9* expression under various abiotic stresses. *VvMAPK9* expression levels were analyzed on 2-month-old grape seedlings treated with 200 mM NaCl (**A**), 200 mM Mannitol treatment (**B**), 42 °C (**C**) and 100 μM ABA (**D**), respectively. The β-actin gene from *Vitis vinifera* was used as an internal control. In addition, the expression levels were normalized to grape without any stress treatment (0 h). The data are means ± SE of three independent experiments (n = 3). Different letters above the bar indicate significant differences ($P \leq 0.01$) based on Duncan's multiple range tests

transgenic grape callus with the same size and growth state were transferred to the medium containing different concentrations of NaCl for 10 days. Under the normal condition, the growth of WT and transgenic callus showed no significant differences. However, under salt stress treatment, the transgenic grape callus grew faster and were significantly larger than WT. Moreover, on the medium with 200 mM NaCl, most of WT callus stopped growing and their color changed to brown, while the transgenic callus grew better with a pale yellow color (Fig. 7A). Additionally, under normal conditions, the relative conductivity had no significant differences between WT and transgenic callus. However, after salt stress, the relative conductivity of transgenic lines was significantly lower than that of WT (Fig. 7B). These results suggested that the overexpression of *VvMAPK9* improved the salt-tolerance ability of grape callus.

Salinity imposes osmotic stress on plants, which will lead to ROS overproduction and cause ROS-associated injury (Krasensky and Jonak 2012). Therefore, the anti-superoxide anion activity of callus was measured in this study. Under normal growth conditions, the anti-superoxide anion activity of transgenic callus was obviously lower than that of WT. However, after salt stress treatment, the anti-superoxide anion activity of transgenic callus was significantly higher than that of WT (Fig. 7C). The results indicated that the *VvMAPK9*-overexpressing callus had a strong ability to remove ROS under salt stress.

VvMAPK9 participates in the metabolism of ROS under salt stress

To explore the possible mechanisms underlying the increased activity of anti-$O_2 \cdot^-$, the antioxidant enzyme activity in grape callus were further examined. As shown in Fig. 8, under normal condition, the POD activity of transgenic callus was not significantly different from that of the WT callus, and SOD activity was significantly lower than that of WT callus. After 200 mM NaCl treatment, the POD

Table 1 Putative *cis*-elements on the promoter of *VvMAPK9*

cis-acting elements	Numbers	Fuction of the *cis*-acting element
Abiotic stress responsive element		
ERE	8	Ethylene responsive element
STRE	2	Osmotic stress response element
ARE	2	*cis*-acting regulatory element essential for the anaerobic induction
MYC	2	ABA, low temperature stress response element
MYB	1	Drought response element
LTR	1	*cis*-acting element involved in low-temperature responsiveness
Light responsive element		
Box 4	4	Part of a conserved DNA module involved in light responsiveness
TCT-motif	2	Part of a light responsive element
3-AF1 binding site	1	Light responsive element
AE-box	1	Part of a module for light response
GATA-motif	1	Part of a light responsive element
GT1-motif	1	Light responsive element
I-box	1	Part of a light responsive element
LAMP-element	1	Part of a light responsive element
TCCC-motif	1	Part of a light responsive element
MRE	1	MYB binding site involved in light responsiveness

and SOD activities of wild-type callus were all decreased. However, the POD and SOD activities of transgenic callus were greatly increased and significantly higher than that of WT callus decreased (Fig. 8A and B). The activities of CAT and APX in transgenic callus were all higher than that in WT under normal condition, but the upregulation degree was not significantly different from the WT after salt treatment (Fig. 8C and D). Synthesizing the above results, *VvMAPK9* mainly improves salt resistance of grape callus by regulating *VvPOD* and *VvSOD*, and enhancing POD and SOD activity.

Discussion

MAPK pathways play an important role in plant response to various adverse environmental stimuli. It is a signal transduction pathway ubiquitous in eukaryotic organisms, in plant cells it transmits various external signals from the cell surface to the nucleus in a cascade signaling pathway, thus regulating plant growth and development and stress response (Nakagami et al. 2005; Zhang and Klessig 2001). Therefore, it is of great significance to study the MAPK pathway in plants. However, most studies on MAPK genes are limited to model plants, and there are few studies on MAPK in other plants, especially in fruit trees. In this study, *VvMAPK9*, a group B MAPK gene, was isolated from grape, which possesses the typical features of group B MAPKs, such as the TEY activation motif and CD domain (Fig. 1) (MAPK group 2002). Overexpression of *VvMAPK9* in *Arabidopsis thaliana* and grape callus enhanced their tolerance to salt stress.

As the most downstream kinase of the MAPK cascade pathway, MAPK, when phosphorylated, can not only continue to stay in the cytoplasm to activate other proteins, but also enter the nucleus to activate transcription factors and regulate the expression of genes (Xu and Zhang 2015). Through the subcellular localization of MAPK, a lot of information can be learned, including its upstream genes and the interaction mechanism between MAPK and protein substrates. In this present study, the analysis of subcellular localization revealed that VvMAPK9 protein was localized in both the cytoplasm and the nucleus (Fig. 4), suggesting that *VvMAPK9* may function in both the cytoplasm and the nucleus.

MAPKs have been confirmed to participate in the regulation of abiotic stress (Ichimura et al. 2000). For example, *AtMPK4* participated in salt stress through a cascade reaction of MEKK1-MKK2-MPK4 (Brader et al. 2007; Furuya et al. 2014). Overexpression of *ZmSIMK* improved the salt and drought tolerance of plants (Gu et al. 2010; Wang et al. 2014a, b). *OsMAPK4* can activate *OsWRKY30* to improve salt tolerance through the MKK1-MPK4 cascade pathway (Wang et al. 2014a, b). Moreover, proteomic analysis showed that *BnMAPK4* activation affects multiple pathways, such as stress and defense responses (Zhang et al. 2019). The present study revealed that *VvMAPK9* has high homology with these MAPK genes, therefore, we speculate that *VvMAPK9* may has the same function with them. The analysis of *VvMAPK9* promoter suggested that some *cis*-acting elements related to abiotic stress, including MYC, MYB, ERE, STRE etc., were identified from the promoter region. Further, the expression

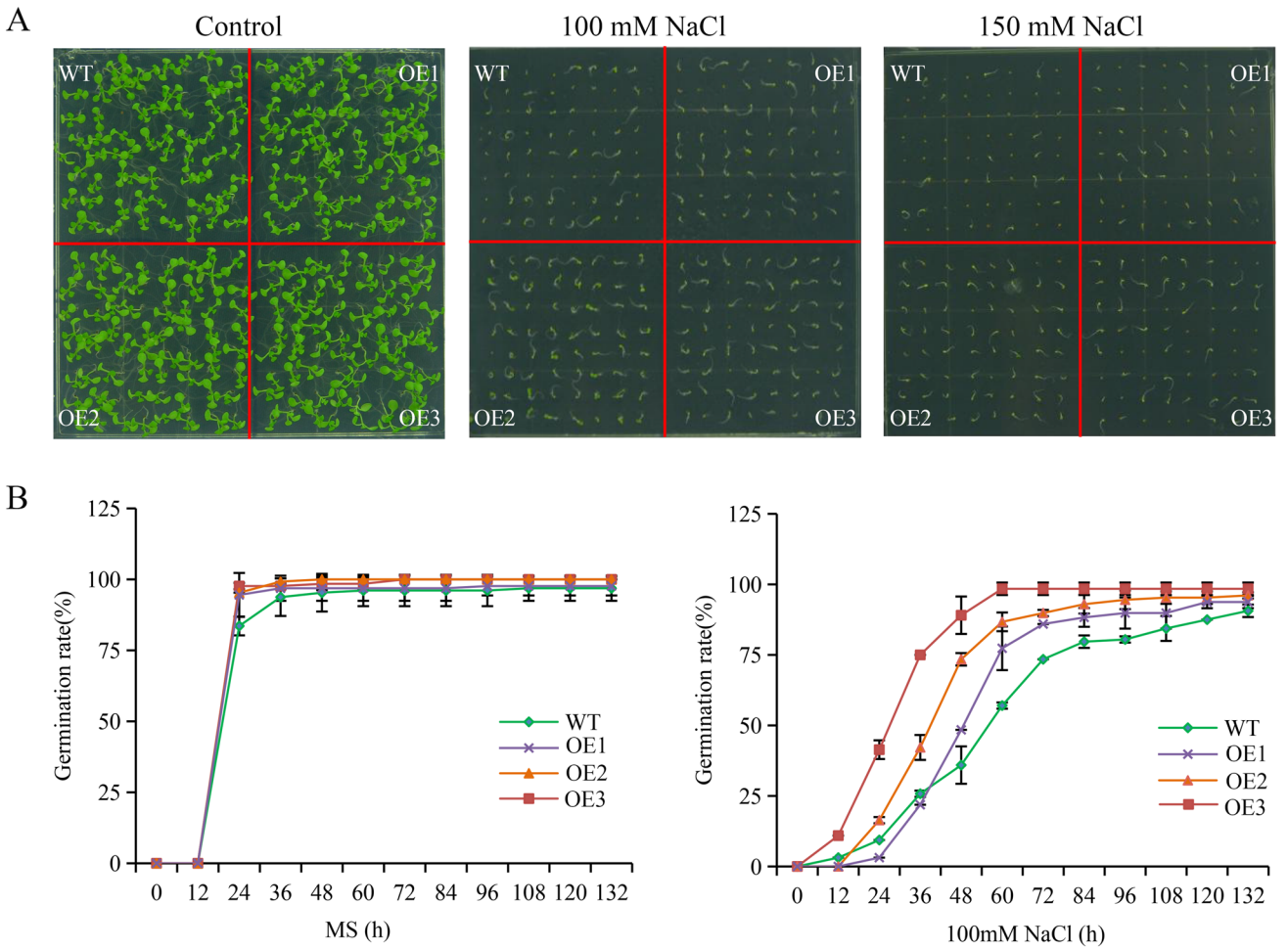


Fig. 5 The germination phenotypes of WT and *VvMAPK9*-overexpressing (OE) lines under salt stress. **A** Seedling phenotype of WT and OE lines in 1/2 MS medium with or without NaCl. **B** The germination rates of WT and OE plants grown on 1/2 MS medium with or without NaCl. Three independent experiments were carried out using 64 seeds in each

of *VvMAPK9* was induced by drought, salt, high temperature and ABA, which suggested that *VvMAPK9* might be involved in regulating responses to various abiotic stresses. In addition, overexpression of *VvMAPK9* in *Arabidopsis* significantly enhanced salt stresses tolerance, with higher germination rates, longer root length, and better growth condition under salt stress. Moreover, the *VvMAPK9* transgenic grape callus displayed better salt tolerance than WT as a result of larger volume and lower relative conductivity under salt stress.

Salt stress can result in the accumulation of excessive ROS, which have been proved to have a negative effect on abiotic stress resistance in plants. Therefore, an increased ROS-scavenging ability might be beneficial to plant tolerance to abiotic stresses (Gill and Tuteja 2010). Previous studies revealed that MAPK pathways play an important role in mediating the antioxidative system under abiotic stresses (Jalmi and Sinha 2015). In the present study, grape callus overexpressing *VvMAPK9* had higher anti-superoxide anion activity than WT under salt stress treatment, indicating that transgenic callus could eliminate excessive ROS in time. Antioxidant enzymes POD and SOD play a key role in ROS clearance, which can reduce or eliminate the damage caused by salt stress (Liang et al. 2003). This study suggested that the activities of SOD and POD were significantly higher in the *VvMAPK9*-overexpressing grape callus than in the WT callus after salt stress, indicating that *VvMAPK9* might improve the salt tolerance of grape callus by positively regulating the ROS pathway.

In conclusion, a grape group B MAPK gene, *VvMAPK9*, was isolated and characterized. The expression of *VvMAPK9* was induced by various abiotic stresses, and it was proved that this gene was involved in the process of salt stress resistance in *Arabidopsis* and grape callus. *VvMAPK9* can positively regulate the antioxidative system to reduce accumulation of ROS under salt stress. These findings not only extend

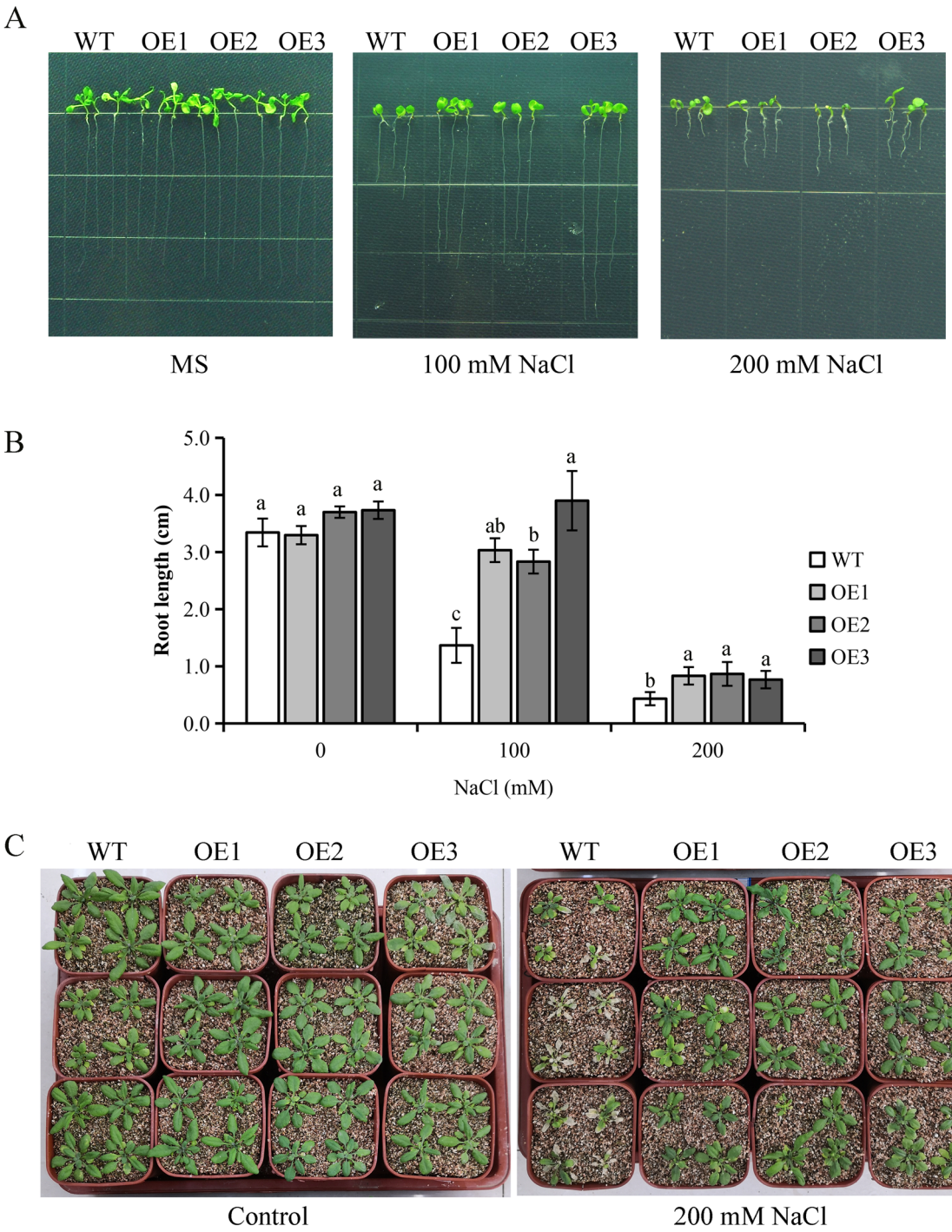


Fig. 6 The phenotype of WT and *VvMAPK9*-overexpressing plants under salt stress. **A** Root phenotypes of WT and OE lines in 1/2 MS medium containing NaCl (0, 100, 200 mM). **B** Root length of WT and OE lines in 1/2 MS medium containing NaCl (0, 100, 200 mM). **C** Phenotypes of WT and OE seedlings were treated with 200 mM NaCl for 14 days

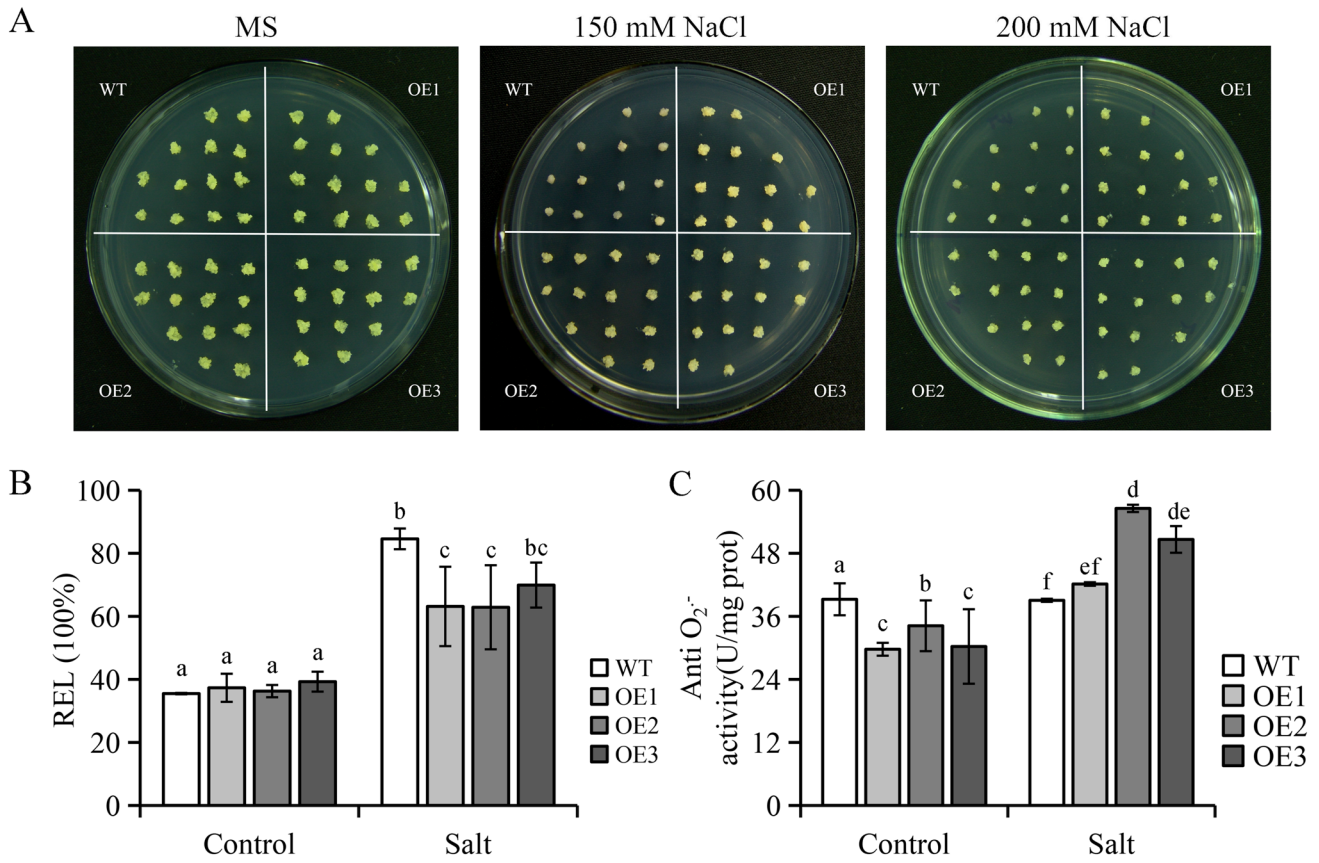


Fig. 7 The phenotypes, relative conductivity and anti-superoxide anion activity of the WT and *VvMAPK9*-overexpressing grape callus after cultured on the medium containing NaCl (0, 150, 200 mM) for 10 days. **A** The phenotypes. **B** The relative conductivity. **C** The anti-superoxide anion activity. The data are means ± SE of three independent experiments (n = 3). Different letters above the bar indicate significant differences ($P \leq 0.01$) based on Duncan's multiple range tests

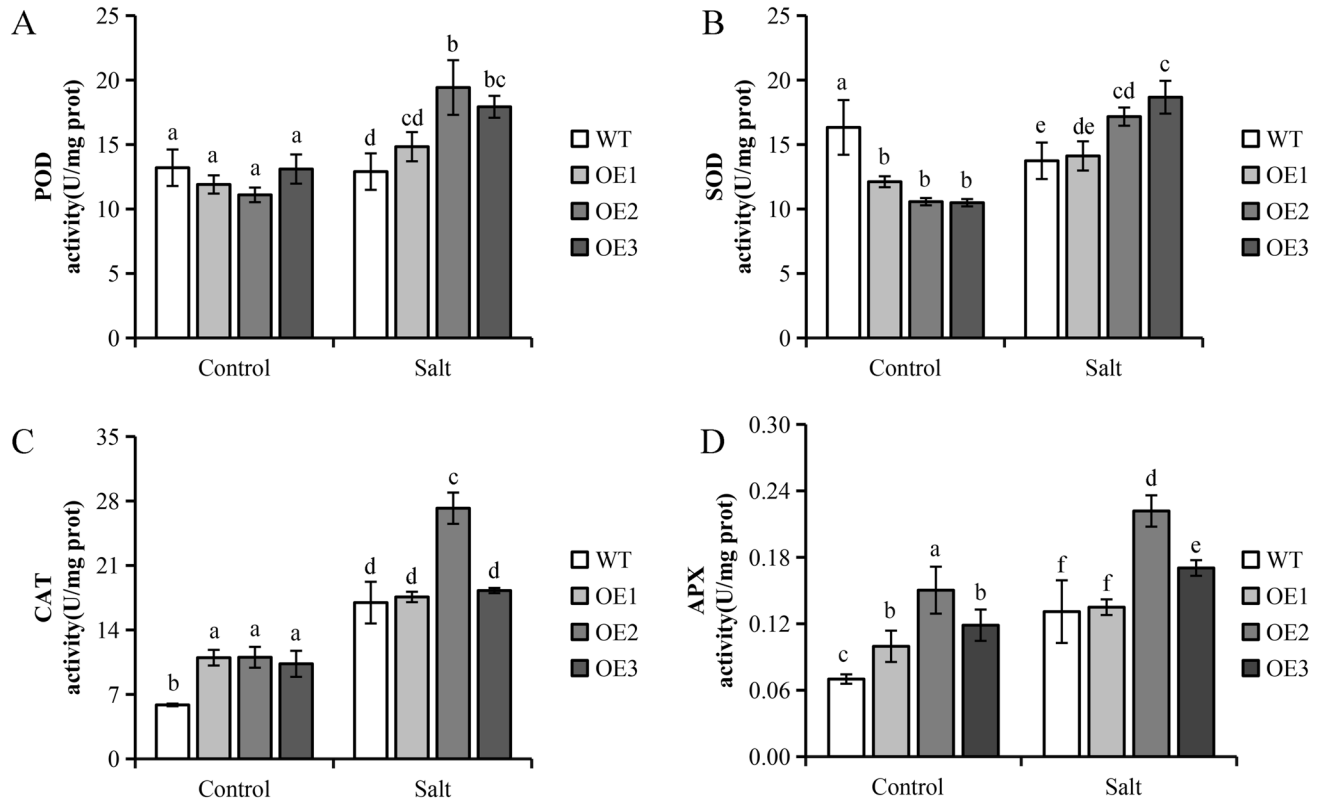


Fig. 8 Antioxidant enzyme activity of WT and *VvMAPK9*-overexpressed grape callus. The grape callus was cultured on the medium containing NaCl (0, 200 mM) for 10 days. The activity of **A** POD, **B** SOD, **C** CAT and **D** APX in WT and transgenic callus were measured, respectively. The data are means ± SE of three independent experiments (n = 3). Different letters above the bar indicate significant differences ($P \leq 0.01$) based on Duncan's multiple range tests

our knowledge of the group B MAPKs but als provide new clues in the regulation of salt tolerance in grape.

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Author contributions QS and BL conceived and designed the experiments. XJ, CS, and YY conducted the experiments. XJ and XL analyzed the data. XJ and QS wrote the manuscript. All authors read and approve the final manuscript.

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Data availability The amino acid sequences of *Arabidopsis* was downloaded from The *Arabidopsis* Information Resource (https://www.arabidopsis.org).

Code availability Amino acid sequences of other plants MAPK genes were retrieved from GenBank (http://www.ncbi.gov/Genbank). Amino acid sequence alignments were done using the DNAMAN program (version 5.2.2). Analysis of the promoter sequence of *VvMAPK9* were performed using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The phylogenetic tree was constructed by the NJ (Neighbor-Joining) method using MEGA 4. Statistical significance was analyzed using Duncan's multiple range tests with analysis of variance (ANOVA), and calculations were performed with SPSS Statistics.

Declarations

Conflict of interest No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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