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ORIGINAL ARTICLE

Expression of a grape bZIP transcription factor, *VqbZIP39*, in transgenic *Arabidopsis thaliana* confers tolerance of multiple abiotic stresses

Mingxing Tu[1,2] · Xianhang Wang[1,2] · Li Huang[1,2] · Rongrong Guo[1] · Hongjing Zhang[2] · Junshe Cai[3] · Xiping Wang[1,2]

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Abstract The basic region/leucine zipper (bZIP) transcription factors are known to play key roles in response to abiotic stress. In this study, a bZIP gene (*VqbZIP39*) was isolated from grape (*Vitis quinquangularis*) and constitutively expressed in Arabidopsis under control of the cauliflower mosaic virus 35S promoter. The transgenic *Arabidopsis thaliana* plants showed enhance salt and drought stress tolerance during seed germination and in the seedling and mature plant stages. Various physiological parameters related to stress responses were analyzed to gain further insight into the role of *VqbZIP39* and it was found that osmotic stress caused less damage to the transgenic seedlings than to the corresponding wild type plants. This correlated with an increase in endogenous ABA content as a consequence of the constitutive overexpression of *VqbZIP39*, and the up-regulated expression of stress-inducible target genes associated with tolerance of drought, high-salt, and oxidative stresses. Our results suggest that the expression of *VqbZIP39* in *A. thaliana* likely enhances the tolerance to multiple abiotic stresses through the ABA signaling pathway, and may therefore have a similar function in the response to abiotic stresses in grape.

Keywords Grape · *VqbZIP39* · Drought · Salt · ABA biosynthesis

Abbreviations

bZIP	Basic region/leucine zipper
WT	Wild type
CaMV	Cauliflower mosaic virus
ORF	Open reading frame
RT-PCR	Reverse transcription-polymerase chain reaction
GFP	Green fluorescent protein
GUS	β-Glucuronidase
MS	Murashige and Skoog
MDA	Malondialdehyde
TCA	Trichloroacetic acid
TBA	Thiobarbituric acid
ELISA	Enzyme-linked immunosorbent assay
NBT	Nitro blue tetrazolium
DAB	Diaminobenzidine
ROS	Reactive oxygen species
MV	Methylviologen
SOD	Superoxide dismutase
CAT	Catalase
POD	Peroxidase
H_2O_2	Hydrogen peroxide
O_2^-	Superoxide anion
ABA	Abscisic acid
cDNA	Complementary deoxyribonucleic acid
FW	Fresh weight
EDTA	Ethylene diamine tetraacetic acid
NCED3	9-*Cis*-epoxycarotenoid dioxygenase
RD	Responsive to dehydration

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✉ Xiping Wang
wangxiping@nwsuaf.edu.cn

[1] State Key Laboratory of Crop Stress Biology in Arid Areas, College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, China

[2] Key Laboratory of Horticultural Plant Biology and Germplasm Innovation in Northwest China, Ministry of Agriculture, Northwest A&F University, Yangling 712100, Shaanxi, China

[3] Research Institute of Grapes and Melon in Xinjiang Uygur Autonomous Region, Shanshan 838200, Xinjiang, China

KIN Protein kinase
ERD Early responsive to dehydration

Introduction

Exposure of plants to various abiotic stresses, such as high salt conditions, cold and drought, can seriously affect their growth and development. Since plants are sessile, they respond and adapt to such stresses by altering their biochemical and physiological status, and by activating the transcription of numerous stress responsive genes (Shinozaki and Yamaguchi-Shinozaki 2000). Exposure to salt and water stress results in a major change in global gene expression (Moretti et al. 2006), which appears to be controlled by multiple cellular pathways, some of which are dependent on the plant hormone abscisic acid (ABA) (Rock 2000). The phytohormone ABA can protect plants from damage induced by drought and salinity, during the growth of seedings and plant maturation (Finkelstein et al. 2002). Since the accumulation of ABA in plant cells are increased in response to osmotic stresses such as drought and salinity, leading to expression of stress responsive genes (Zhu 2002), such as *RD29A*, *RD29B*, *RD22*, *KIN2* and *NCED3*, which have been demonstrated to be induced by drought stress (Yamaguchi-Shinozaki and Shinozaki 2006; Abe et al. 2003; Fujita et al. 2005; Iuchi et al. 2001). Over-expression of *AtNCED3* improved dehydration stress tolerance in transgenic plants (Iuchi et al. 2001). ABA-dependent gene expression plays an important roles in transcriptional regulatory networks under osmotic stress conditions (Yamaguchi-Shinozaki and Shinozaki 2006). Dehydration-inducible gene (*ERD1*) was not responsive to ABA treatment, suggesting the existence of ABA-independent pathway in the dehydration stress response (Yamaguchi-Shinozaki and Shinozaki 2006).

As regulators of gene expression, transcription factors (TFs) influence essentially all aspects of growth, development, and responses to abiotic and biotic stresses. One of the largest TF families in higher plants (Liu et al. 2012a) is the basic region/leucine zipper (bZIP) family, which has been characterized in a range of plant species. For example, the *Arabidopsis thaliana* genome contains 75 bZIP genes (Jakoby et al. 2002), both 89 and 92 have been reported to be present in rice (*Oryza sativa*; Nijhawan et al. 2008; Correa et al. 2008), 47 have been identified in soybean (*Glycine max*; Liao et al. 2008), 92 in sorghum (*Sorghum bicolor*; Wang et al. 2011), 125 in maize (*Zea mays*; Wei et al. 2012), 47 (Gao et al. 2014) and 55 (Liu et al. 2014) in grapevine (*Vitis vinifera*). Plant bZIP TFs are involved in regulating many metabolic processes, such as energy metabolism (Baena-González et al. 2007), cell elongation (Fukazawa et al. 2000), organ and tissue differentiation (Silveira et al. 2007), embryogenesis (Guan et al. 2009), floral induction and development (Abe et al. 2005; Zou et al. 2008), seed maturation (Jakoby et al. 2002; Cheng et al. 2014), plant senescence (Smykowski et al. 2010), hormone signaling (Fujita et al. 2005; Kang et al. 2002; Kim et al. 2004), photomorphogenesis (Huang et al. 2012), and light signaling (Mallappa et al. 2006). In addition, they control responses to a variety of abiotic stimuli, such as high salinity (Hsieh et al. 2010; García et al. 2012), drought (Yoshida et al. 2010), cold stress (Liu et al. 2012a) and heat stress (Liu et al. 2012b). Thus, bZIP genes are important regulators of many stress conditions. To date, only a few grapevine bZIP TF has been characterized (Tak and Mhatre 2013), and its role in stress responses has not been studied in detail.

Grapes are one of the most economically important fruit crops in the world, and are widely cultivated around the world. However, drought can negatively affect the growth, development, and productivity of vines, during the grape-growing season (Xu 2004). China is one of the major centers of origin of *Vitis* species (He et al. 1991). Among them, Chinese wild *V. quinquangularis* have been reported to be resistant to grape powdery mildew (Wang et al. 1995), and drought stress (Yang 2003). Previously, we identified 47 bZIP genes in the grape genome, and of these it was found that the expression profile of *bZIP39* was up-regulated in response to exogenous ABA treatment, salt and drought stress conditions (Gao et al. 2014), suggesting that it may be associated with tolerance to abiotic stress. In support of this idea, expression of the homologous *A. thaliana* gene *AREB1/ABF2* (Yoshida et al. 2010; Fujita et al. 2005; Kim et al. 2004; Uno et al. 2000; Choi et al. 2000), was reported to be highly induced by osmotic stress and ABA treatment in vegetative tissues, while the tomato (*Solanum lycopersicum*) *SlAREB1* gene was shown to confer drought and salt stress tolerance and to regulate biotic and abiotic stress-related genes (Orellana et al. 2010). AREB/ABFs are ABA-responsive transcription factors containing a bZIP domain that binds the ABA-responsive element (ABRE; T/CACGTGGC) in the promoter of downstream genes (Uno et al. 2000).

Genetic transformation of grape has low transformation efficiency and long period. Therefore, we have difficult to verify the function of *bZIP39* in grape. In the current study, we further tested this hypothesis by characterizing the responses to osmotic stress of transgenic *A. thaliana* constitutively over-expressing the grape *VqbZIP39* gene. It was found that overexpression of *VqbZIP39* in the transgenic plants improved drought, salt and oxidative stress tolerance, suggesting that *VqbZIP39* is a good candidate gene for improving stress tolerance in crop species.

Materials and methods

Isolation and bioinformatic analysis of the *VqbZIP39* gene

Total RNA was extracted from leaves of *V. quinquangularis* clone Shang-24, as in Zhang et al. (2003), and was treated with 10 units of RNase-free DNase I (TaKaRa Biotechnology, Dalian, China) to remove genomic DNA contamination. First-strand cDNA was synthesized using PrimerScript^{TM}RTase (TaKaRa Biotechnology), according to the manufacturer's instructions. The full length grape *VqbZIP39* open reading frame (ORF) was amplified by PCR using gene-specific primers F1 (5′-ATG GGG AGT AAT TTG AAC TTC A-3′) and R1 (5′-TCA CCA GGG GCC AGT CAG T-3′). The PCR product was cloned into the pGEM-Teasy vector (Promega, Madison, WI, USA), and the plasmid (pGEM-Teasy-*VqbZIP39*) was sequenced to confirm sequence fidelity.

The *VqbZIP39* protein sequence was used as a query to search the GenBank database, using the BLASTp algorithm at the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi), to identify related bZIP TFs from several plant species. To investigate the relationship between *VqbZIP39* and other bZIP genes, the corresponding amino acid sequences were compared using DNAMAN software (Version 5.2.2.0, Lynnon Biosoft, USA). Phylogenetic analysis were carried out with the MEGA (version 5.05) software using the neighbor-joining (NJ) method, and the bootstrap test was replicated 1000 times.

Generation and selection of transgenic *A. thaliana* plants over-expressing *VqbZIP39*

The *VqbZIP39* open reading frame (ORF) (with *Xba*I and *Kpn*I sites added to the 5′ and 3′ end, respectively) was amplified from the pGEM-Teasy-*VqbZIP39* vector using the gene-specific primers F2 (5′-CGC TCT AGA ATG GGG AGT AAT TTG AAC TTC A-3′ *Xba*I site underline) and R2 (5′-GGC GGT ACC TCA CCA GGG GCC AGT CAG T-3′ *Kpn*I site underlined), and was inserted immediately downstream of the CaMV 35S promoter in the plant overexpression vector, pCambia 2300 (Cambia, Brisbane, QLD, Australia). The construct was introduced into *A. tumefaciens* strain GV3101 via electroporation and *A. thaliana* transformation was carried out using the floral dip method (Clough and Bent 1998). T0 seeds were harvested and sown on Murashige–Skoog (MS) agar medium (Sigma) (Murashige and Skoog 1962) supplemented with 100 mg L^{-1} kanamycin. Three lines (L7, L27 and L42) were selected from 60 independent lines, and T3 homozygous lines were generated and used for all further experiments.

Plant growth

A. thaliana plants were grown in a growth chamber (16 h light/8 h dark at 21 °C). Seeds from each of three selected T3 homozygous lines and from wild-type (WT) plants were vernalized for 3 day at 4 °C, surface sterilized in 70 % ethanol for 1 min, and then treated with 10 % (v/v) NaClO for 10 min, followed by five washes with sterilized distilled water. The seeds were then plated on MS medium solidified with 0.7 % agar and 2 % sucrose. 1-week old wild-type and transgenic seedlings were transferred from plates into the same pot filled with compost soil and used for all further experiments.

Osmotic stress treatments

In preparation for the germination assays, ~100 seeds were surface-sterilized and sown on MS medium or MS agar medium supplemented with 125 mM NaCl and 200 mM mannitol. Seeds were vernalized at 4 °C for 3 days before growing at 21 °C under 16 h light/8 h dark. Germination rates were scored based on radicle protrusion and the greening of expanded cotyledons at day 5 after germination (Saleki et al. 1993).

For the root growth assays, 7-day old transgenic and WT seedlings, grown on MS agar medium plates, were vertically plated on MS agar medium or MS agar medium supplemented with 125 mM NaCl and 200 mM mannitol. The root lengths were measured 7 days after the transfer. All experiments were repeated three times.

Measurements of physiological indices and endogenous ABA content

Seven-day old seedlings were grown on MS agar medium, transferred to new MS medium or MS medium supplemented with 125 mM NaCl and 200 mM mannitol, and grown for 1 week before seedlings were harvested for measurements. All experiments were repeated three times.

For chlorophyll content measurements, ~50 mg fresh leaf tissue was placed in 5 ml of 96 % ethanol and incubated at 4 °C overnight in the dark. The absorbance of the extracted pigments at 665 nm (A_{665}) and 649 nm (A_{649}) was measured using a spectrophotometer (Hitachi Limited, Tokyo, Japan). The chlorophyll content (mg/g FW) = $(18.08 \times A_{649} + 6.63 \times A_{665})$/fresh weight (Zhang et al. 2012a, b).

Relative electrolyte leakage was measured using the protocol of Cao et al. (2007). Seedlings were vacuum-infiltrated with deionized water for 20 min. After 2 h, the conductivities (C1) of the solutions were determined using

a conductivity detector and the seedlings were boiled for 20 min in deionized water before cooling to room temperature. The conductivities (C2) of the solutions were then determined and the C1 to C2 (C1/C2) ratios were calculated and used as a measure of the relative electrolyte leakage.

Malondialdehyde (MDA) content was measured as described by Heath and Packer (1968). Proline concentration was measured according to the method described by Bates (1973). Abscisic acid (ABA) content was measured using an enzyme-linked immunosorbent assay (ELISA), as previously described (Yang et al. 2001).

Stress tolerance assays

Seven-day old transgenic and WT seedlings aseptically grown on MS agar medium were transferred into pots filled with compost soil and watered well for 2 weeks before stress treatments were applied. For the salt tolerance assay, soil-grown plants were irrigated at 2 day intervals with a 200 mM NaCl solution for 1 week. For the drought tolerance assay, irrigation was withheld from soil-grown plants for 1 week, followed by re-watering. Survival rates were scored 2 days after re-watering. Well-watered plants were used as the negative control. For the oxidative stress treatment, five detached leaves from 4-week-old transgenic and WT plants at similar developmental stages were floated on MS medium or MS medium supplemented with 6 μM methylviologen (MV) (Sigma M2254) for 24 h in the light (Moon et al. 2003) before the chlorophyll content was determined (Zhang et al. 2012a, b). All experiments were repeated three times.

Determination of the water loss rate

For the determination of water loss, ten leaves were detached from 4-week old transgenic and WT plants and weighed immediately; the samples were then placed on dry filter paper with a relative humidity of 45–50 %, at room temperature (25–28 °C) and weighed at designated time intervals. The proportion of fresh weight loss was calculated relative to the initial fresh weight (Zhang et al. 2014), and the experiment was repeated three times.

Detection of reactive oxygen species (ROS) and cell death

A histochemical staining procedure was used to detect superoxide and hydrogen peroxide in situ as previously described (Fryer et al. 2002). Rosette leaves from 4-week-old transgenic and WT plants that had either been drought treated or treated with 200 mM NaCl for 1 week, as well as the corresponding non-treated controls, were infiltrated with a diaminobenzidine (DAB) solution or nitro blue tetrazolium (NBT). Cell death was examined by trypan blue staining. All experiments were repeated three times.

For the detection of H_2O_2, leaves were placed in a 1 mg/ml DAB solution for 8 h or until brown spots became visible. The chlorophyll was cleared at 80 °C in 80 % (v/v) ethanol for 2 h and samples place in 10 % (v/v) glycerol for observation (Kotchoni et al. 2006). For the detection of O_2^-, leaves were infiltrated with HEPES buffer (pH 7.5) containing 6 mM NBT for 2 h, as in Kim et al. (2011). To visualize dead cells, leaves were stained with a boiled trypan blue solution (10 mg trypan blue powder dissolved in 10 ml glycerol, 10 ml 85 % (v/v) lactic acid, 10 ml sterile water and 10 mg phenol) for 5 min, and washed with sterilized water. Chloral hydrate (2.5 g/ml) was used as a decolorant (Koch and Slusarenko 1990).

Antioxidant enzyme assays

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of the photochemical reduction of NBT, which produces a blue coloration in the presence of O_2^-, using a spectrophotometer as previously described (Giannopolitis and Ries 1977). Catalase (CAT, EC 1.11.1.6) activity was determined by measuring the consumption of H_2O_2 at 240 nm for 3 min, as previously described (Aebi 1984). CAT activity was reported as the amount of change in absorbance/min/g FW. Peroxidase (POD, EC 1.11.1.7) activity was assayed as previously described (Chance and Maehly 1955) and expressed as the change in absorbance/min/g FW.

Measurement of stomatal closure in response to ABA treatment

Stomatal aperture assays were performed essentially as previously described (Pei et al. 1997). Briefly, detached rosette leaves from 4-week-old transgenic and WT plants were first floated in a buffer containing 10 mM KCl, 10 mM MES-Tris, pH 6.15 and 50 μM $CaCl_2$, before being exposed to light for 2 h. ABA was then added to the solution to a final concentration of either 5 or 10 μM. Stomatal apertures were observed after 2 h under a microscope (BX53, Olympus, Japan) and recorded as the ratio of stomatal width to length. All assessments were carried out in triplicate.

Quantitative real-time RT-PCR

Total RNA extraction was performed from the leaves of WT and transgenic plants using an RNAprep plant kit (Tiangen Biotech, China), following the manufacturer's instructions, and first-strand cDNAs were synthesized as

described above. Subsequent quantitative real-time PCR analyses were conducted using SYBR green (TaKaRa Biotechnology) using an IQ5 real-time PCR instrument (Bio-Rad, Hercules, CA, USA) with the following thermal profile: 95 °C for 30 s, 45 cycles of 95 °C for 5 s, and 60 °C for 30 s. To perform the melt-curve analysis, the following program was added after 45 PCR cycles: 95 °C for 15 s, followed by a constant increase from 60 to 95 °C. The expression level of the *A. thaliana ACTIN2* gene (AT3G18780) was used as a reference. Primers used for qRT-PCR are listed in Supplementary table S1.

Statistical analysis

All experiments were repeated three times as independent analyses. Data analysis of was performed using Microsoft Excel and generation of graphs was done using Sigmaplot software (v. 10.0, Systat Inc., CA USA). Paired t tests were performed to assess significant differences using the SPSS Statistics 17.0 software (IBM China Company Ltd., Beijing, China).

Results

Isolation and bioinformatic analysis of the *VqbZIP39* gene

The cDNA of *VqbZIP39* was predicted to be 1344 bp in length (Gene Bank accession number: NM_001281221.1. GeneID: 100232889) with a complete open reading frame corresponding to 447 amino acids. Analysis of the deduced amino acid sequence using the SMART program (http://smart.embl-heidelberg.de) revealed that the protein contained a characteristic bZIP domain at its N terminal (Supplemental Figure S1a), and had 53, 65, 61, and 67 % sequence identity, respectively, with the corresponding proteins from *A. thaliana*, *S. lycopersicum*, *G. max*, *Nicotiana tabacum* (Supplemental Figure S1b). Furthermore, phylogenetic analysis indicated that *VqbZIP39* was most closely to *S. lycopersicum* and *Nicotiana tabacum*, while it is most distant to *O. sativa* and *Triticum aestivum*, which are monocotyledons (Fig. 1).

Identification of transgenic *A. thaliana* lines

Transgenic plants were screened using kanamycin-selective medium. A total of 60 independent transgenic lines were obtained and confirmed to be transformants by PCR analysis. Three transgenic lines (L7, L27, and L42) with the best performance under salt and drought stress were selected for generating homozygous T3 lines, which were used for all further analyses, and the expression level of

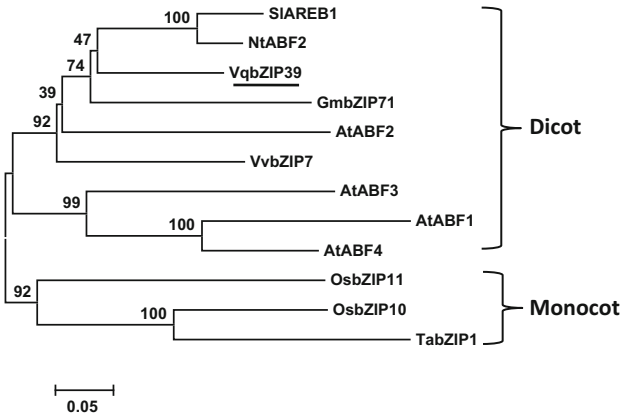

Fig. 1 Phylogenetic analysis of *VqbZIP39*. The phylogenetic tree represents *VqbZIP39* (*underlined*) and its orthologs from several plant species, including *SlAREB1* (NP_001234596.1), *NtABF2* (AHD24943.1), *GmbZIP71* (XP_003528292.1), *AtABF2* (NP_001185157.1), *VvbZIP7* (XP_002285116.1), *AtABF3* (NP_849490.2), *AtABF1* (AEE32464.1), *AtABF4* (NP_566629.1), *OsbZIP11* (NP_001048225.1), *OsbZIP10* (NP_001062018.1) and *TabZIP1* (BAG16727.1). The phylogenetic tree was constructed with the MEGA (5.05) software by neighbor-joining method. Reliability values at each branch represent bootstrap samples (1000 replicates)

VqbZIP39 in the three lines was verified by quantitative real-time RT-PCR (qRT-PCR) (Fig. 2a).

The response of *VqbZIP39* to abiotic stress in the three transgenic lines was verified using qRT-PCR following salt and drought stress treatments, and higher transcript levels were 1.5–2.5 times higher in the treated transgenic plants than in untreated transgenic lines (Fig. 2b).

Effect of osmotic stress on seed germination and post-germination growth in transgenic *A. thaliana* lines over-expressing *VqbZIP39*

To determine whether *VqbZIP39* is involved in osmotic stress responses, we compared the osmotic stress tolerance of the transgenic lines and WT plants at the time of seed germination and in post-germination growth. The germination rates of seeds from the transgenic lines and WT plants were evaluated following treatments with 125 mM NaCl and 200 mM mannitol. When sown on regular MS medium, nearly 100 % of the seeds from both the transgenic lines and WT germinated successfully; however, seeds from the transgenic lines exhibited significantly higher germination rates than those of WT in response to osmotic stress treatments (Fig. 3a). Indeed, transgenic seeds sown on MS medium containing NaCl or mannitol exhibited 40–54 % higher germination rates than WT seeds sown on the same media (Fig. 3b). The root of transgenic and WT seedlings displayed similar growth characteristics on MS basal medium; however, while the post-germination growth of both transgenic and WT seedlings was affected

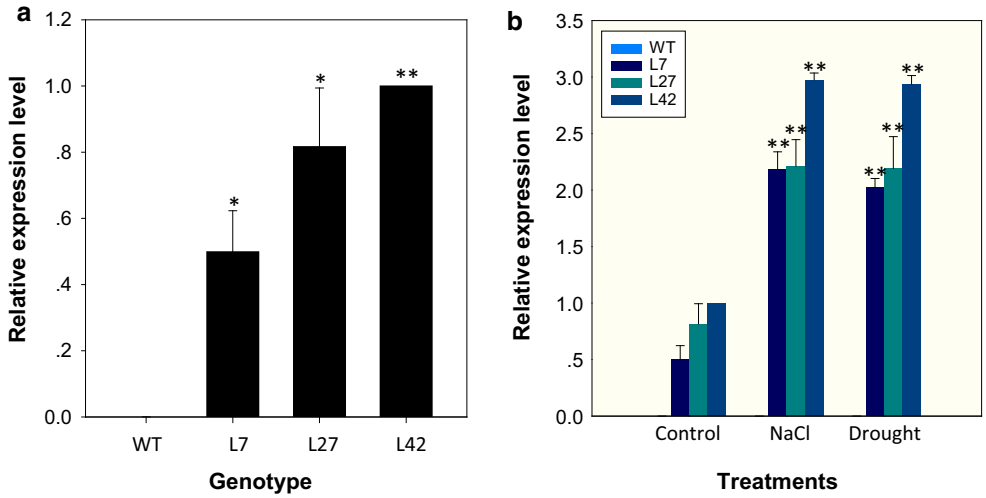

Fig. 2 Validation of *VqbZIP39* expression and stress-responsiveness in transgenic *A. thaliana.* **a** The transcript levels of *VqbZIP39* were analyzed by qRT-PCR. Total RNA from 4-week old untreated WT and three independent transgenic lines (L7, L27 and L42) over-expression *VqbZIP39*. **b** Induction of *VqbZIP39* expression by NaCl or drought stress. Total RNA from WT and transgenic lines treated with 200 mM NaCl and drought for 1 week. The *ACTIN2* gene was used as an internal control. The highest expression level in the transgenic plants under non-stressed condition was defined as 1.0. Data represent the mean values and standard deviation (SD) from three independent experiments. *Asterisks* indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's t test) between the transgenic and WT plants

by both NaCl and mannitol treatments, the roots of the transgenic plants were longer than those of WT plants upon exposure to NaCl or mannitol (Fig. 3c, d).

VqbZIP39 transgenic lines have improved physiological traits associated with osmotic stress tolerance compared with WT

In order to further investigate the *VqbZIP39*-mediated enhanced tolerance of osmotic stress, several physiological parameters and the endogenous ABA content of transgenic *VqbZIP39 A. thaliana* seedlings grown on MS medium containing 125 mM NaCl and 200 mM mannitol were measured. While the leaves of WT and transgenic plants both became bleached and wilted after 1 week of treatment with 125 mM NaCl and 200 mM mannitol, WT leaves showed more serious damage (data not shown). To verify this phenomenon, the chlorophyll content of the leaves was measured and it was found that chlorophyll levels in the transgenic leaves (0.28–0.39 mg g fresh weight^{-1}) was significantly higher than in those of the WT (0.10–0.15 mg g fresh weight^{-1}) under osmotic stress (Fig. 4a).

We also measured relative electrolyte leakage, as well as MDA, proline, and endogenous ABA levels, all of which are important markers of osmotic stress tolerance, under normal growth and osmotic stress conditions. Under normal growth conditions, the MDA contents and relative electrolyte leakage of the transgenic lines was similar to those of the WT plants, but following osmotic stress treatments they became significantly lower in the transgenic lines than in WT (Fig. 4b, e). The proline content and endogenous ABA levels were similar in transgenic and WT seedlings under normal conditions; however, after osmotic stress treatment for 7 days, the transgenic lines exhibited significantly higher proline and ABA levels compared to WT plants (Fig. 4c, d).

VqbZIP39 overexpression increases the tolerance of *A. thaliana* to abiotic stresses

The performance of 3-week old transgenic and WT plants subjected to abiotic stress was also investigated. Before stress treatments, the plants were watered for 2 weeks, and no obvious morphological differences between the transgenic and WT plants (Fig. 5a-A) were observed. However, after 1 week of treatment with 200 mM NaCl, most WT leaves became etiolated, while the transgenic plants showed only mild etiolation under the same conditions (Fig. 5a-B). After a 7-day water-withholding period, which represented a drought treatment, all of the WT plants exhibited severe wilting, while only slight wilting was observed in most of the transgenic plants (Fig. 5a-C). After 2 days of re-watering, almost all of the WT plants were dead, whereas 62–91 % of the transgenic seedlings survived (Fig. 5a-D, b).

To assess the water retention ability of the transgenic *A. thaliana*, detached rosette leaves from 4-week-old transgenic and WT plants were subjected to a water loss rate

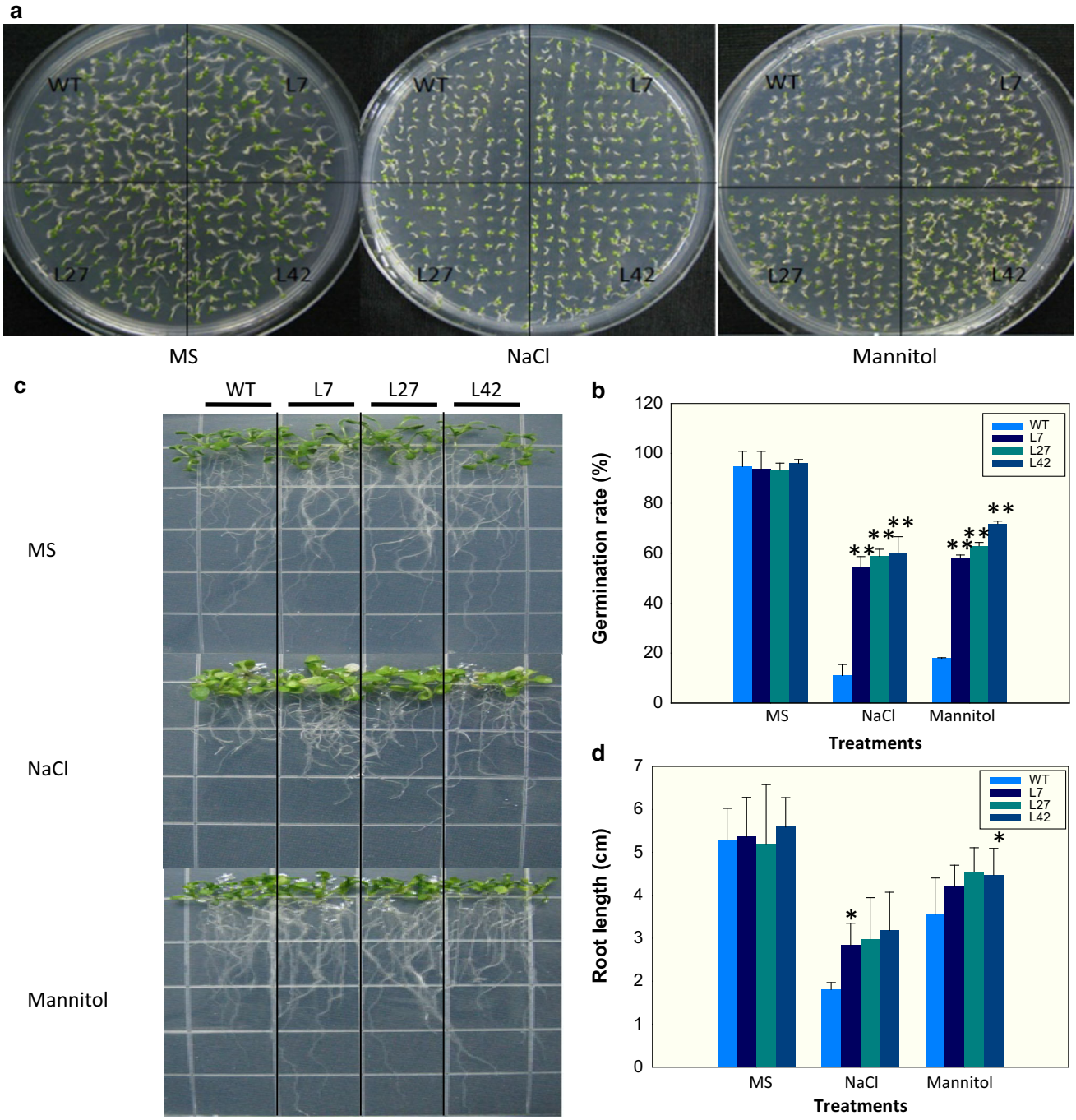

Fig. 3 Effect of osmotic stress on seed germination and post-germination growth of WT and *VqbZIP39*-expressing transgenic *A. thaliana* lines. **a** Photographs of WT and transgenic seedlings 5 days after seeds were cultivated on MS basal medium or MS basal medium supplemented with 125 mM NaCl and 200 mM mannitol; treatments that induce salt and drought stress, respectively. **b** Seed germination rates of WT and transgenic lines cultivated on MS basal medium or MS basal medium containing 125 mM NaCl and 200 mM mannitol. Three independent experiments were performed with ~100 seeds per experiment. *Error bars* indicate SD. *Asterisks* indicate statistical significance ($*0.01 < P < 0.05$, $**P < 0.01$, Student's t test) between the transgenic and WT plants. **c** Photographs of seedlings at 7 days after transfer to MS basal medium or MS basal medium supplemented with 125 mM NaCl and 200 mM mannitol. Seedlings were 7 days old at the time of transfer. **d** Root length of WT and transgenic lines after 7 days with or without 125 mM NaCl and 200 mM mannitol. Three independent experiments were performed using 20 plants per experiment. Error bars indicate SD. *Asterisks* indicate statistical significance ($*0.01 < P < 0.05$, $**P < 0.01$, Student's t test) between the transgenic and WT plants

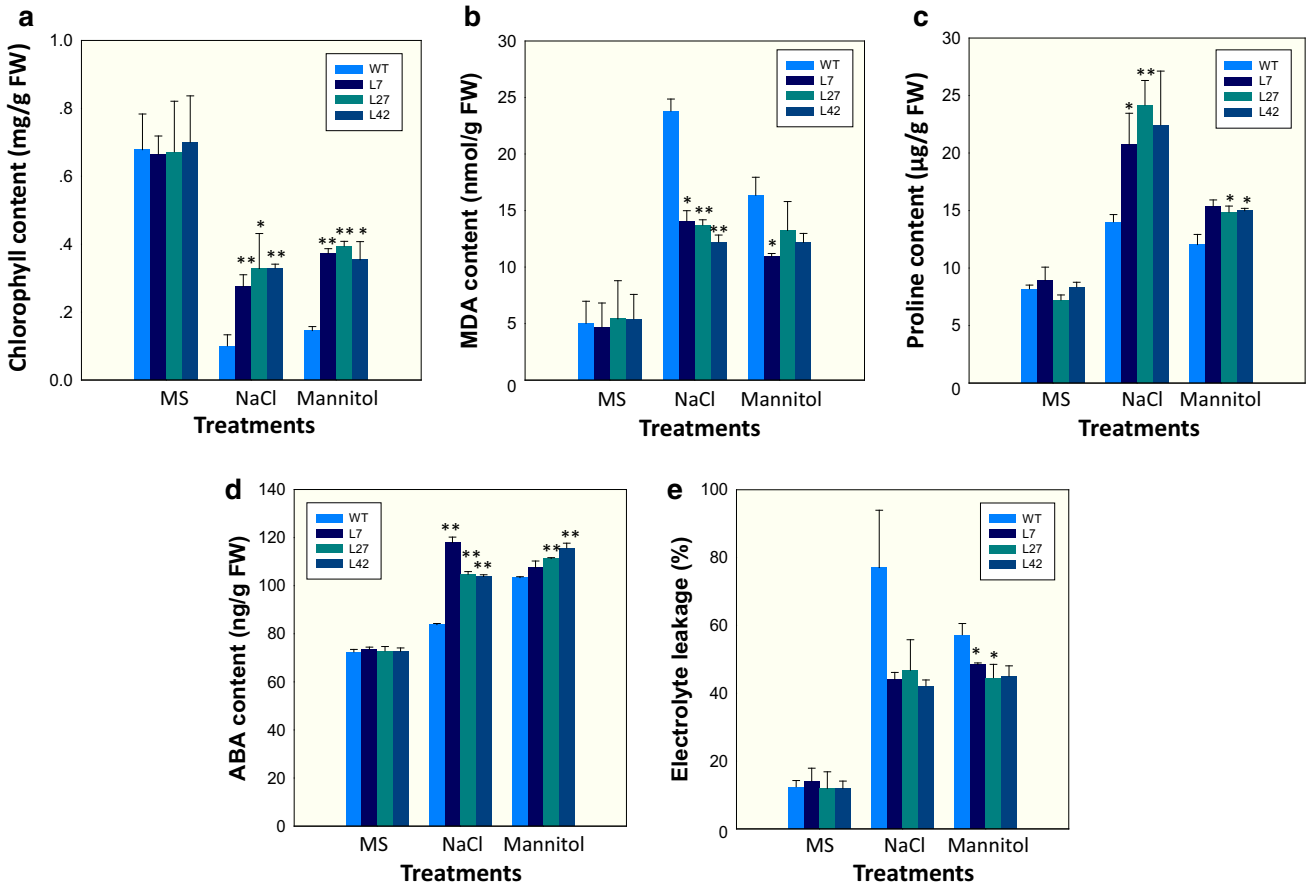

Fig. 4 Physiological changes associated with osmotic stress response in WT and *VqbZIP39*-expressing transgenic *A. thaliana* seedlings. **a**, **b**, **c**, **d** Chlorophyll content (**a**), MDA content (**b**), Proline content (**c**) and endogenous ABA content (**d**) in the leaves of WT and transgenic seedlings under osmotic stress or under non-stress conditions. **e** Relative electrolyte leakage from detached leaves of WT and transgenic seedlings grown under osmotic stress or non-stress conditions. In all cases, data represent mean values ± SD from three independent experiments. *Asterisks* indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the transgenic and WT plants

assay. Fresh weights were recorded ten times over a 10 h period and the three transgenic lines showed lower rates of water loss than WT plants at each time point (Fig. 5c). Leaf cell death in the WT and transgenic lines was evaluated by trypan blue staining following 1 week of abiotic stress and, as shown in Fig. 5d, a deeper staining of the WT leaves was observed, suggesting a higher rate of cell death after abiotic stress treatments.

Since ABA mediates adaptive responses not only to drought but also to high salinity and oxidative stress, we investigated whether the *VqbZIP39* gene contributed to an oxidative stress response. The cellular levels of H_2O_2 and O_2^-, two reactive oxygen species (ROS), in transgenic and WT plants were evaluated by DAB and NBT staining, respectively. As shown in Fig. 6c and d, the leaves of the WT plants displayed a deeper brown and dark blue staining than those of the transgenic lines under salt and drought stress conditions, respectively. Subsequently, the oxidative stress tolerance of the transgenic and WT plants was evaluated by floating leaves on MS medium supplemented with 6 μM methylviologen (MV). MV is a herbicide that generates ROS and causes membrane damage and chlorophyll degradation (Slade 1966). As shown in Fig. 6a, b, the WT leaves became bleached after 24 h of treatment and quantitative determination of their chlorophyll contents indicated that they lost 72 % of their chlorophyll compared with the control leaves, which had not been treated with MV. In contrast, the leaves from the transgenic plants remained green under the same conditions and lost only 56–59 % of the chlorophyll.

In order to further understand the relationship between the observed reduction in levels of ROS and the activity of anti-oxidases in the *VqbZIP39* transgenic plants, the activities of known antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) were assessed in plants grown under the same abiotic stress conditions, as described above. We observed that the activities of all three antioxidant enzymes were

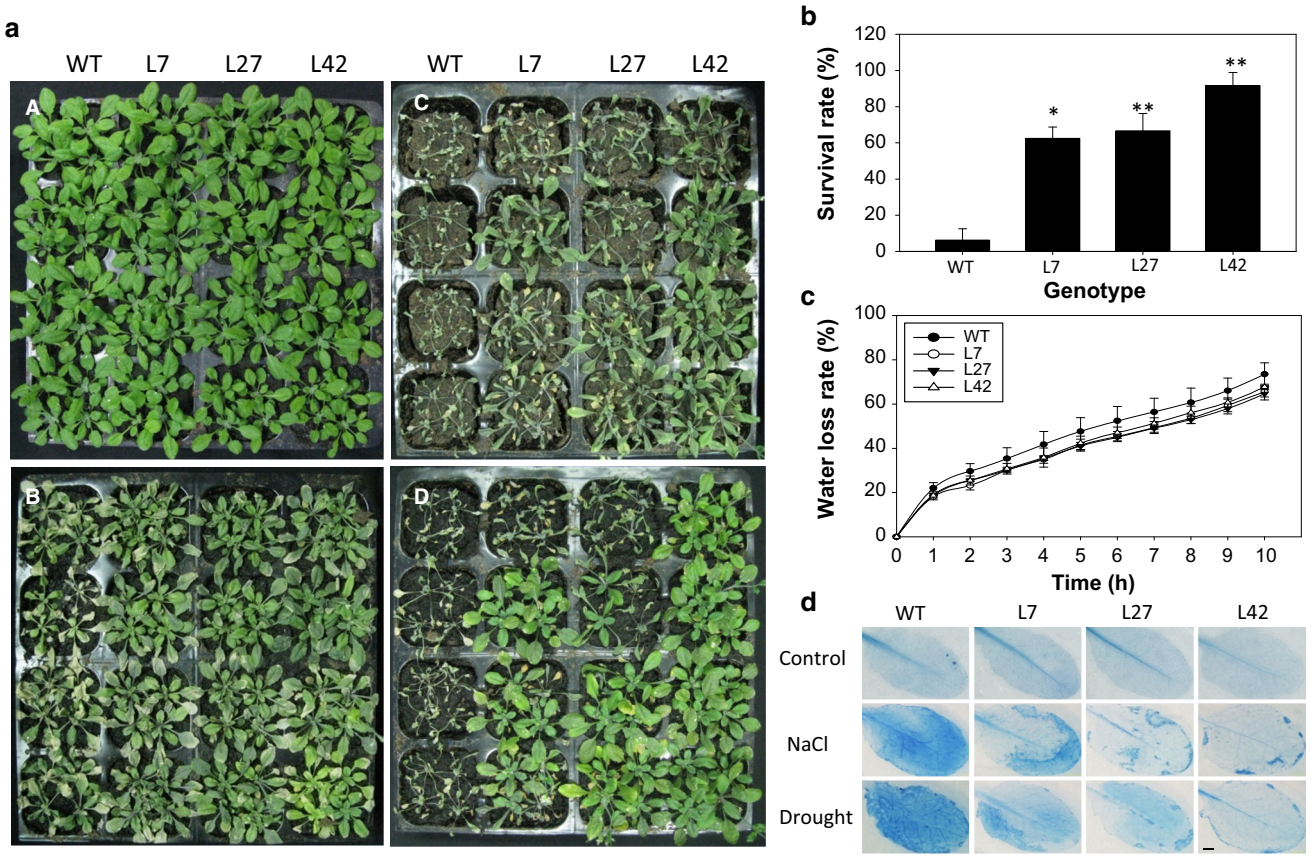

Fig. 5 Performance of WT and *VqbZIP39*-expressing transgenic *A. thaliana* plants under non-stress or abiotic stress conditions. **a** Representative images of 4-week old WT and transgenic lines grown under normal and abiotic stress conditions. **A** WT and transgenic plants grown for 4 weeks under normal conditions. **B** 4-week old WT and transgenic plants treated with 200 mM NaCl for 7 days. **C** 4-week old WT and transgenic plants deprived of water for 7 days. **D** WT and transgenic plants deprived of water for 7 days and then re-watered. **b** Survival rates of WT and transgenic lines 2 days after re-watering. Each data point is the mean of three replicates of 32 plants. Error bars indicate SD. Asterisks indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the transgenic and WT plants. **c** Water loss rates of detached leaves of WT and transgenic lines. Each data point is the mean of three replicates of ten detached leaves. *Error bars* indicate SD. **d** Staining with trypan blue of detached leaves from WT and transgenic lines subjected to abiotic stress for 7 days or non-stressed control leaves. The experiment was repeated three times with 5 leaves. *Scale bar* 2 mm

significantly higher in the transgenic lines than in the WT plants following NaCl and drought stress treatments (Fig. 6e–g). Furthermore, the level of SOD and CAT activity in the transgenic lines was slightly higher than in the WT even without a stress treatment (Fig. 6e, f), which is consistent with the results obtained with the DAB and NBT staining.

Stomata from *VqbZIP39* over-expressing *A. thaliana* lines exhibit increased sensitivity to ABA treatment

Water loss from plants largely depends on stomatal regulation, which is in turn affected by ABA (Yan et al. 2014; Guo et al. 2014). Since the water loss rate was lower in the leaves from the transgenic seedlings than in the leaves from the WT plants at each time point (Fig. 5c), the responses of the stomata to different concentrations of exogenous ABA were compared. As shown in Fig. 7, without ABA treatment, no significant difference was detected in the size of stomatal aperture between transgenic and WT plants, whereas the transgenic plants displayed a lower stomatal width to length ratio when ABA was applied, indicating that they are more sensitive to ABA than those of WT.

VqbZIP39 expression alters the expression of abiotic stress-response genes in transgenic *A. thaliana*

When the expression profiles of 6 known abiotic stress-response genes were analyzed in 4-week old *VqbZIP39* over-expressing *A. thaliana* plants after 7 days of salt and drought stress (Fig. 8), the expression levels of *RD29A*, *RD29B*, *RD22* and *NCED3* were found to be similar in WT and transgenic plants under control conditions. In contrast, the expression of *RD29A*, *RD29B*, *RD22* and *NCED3* was

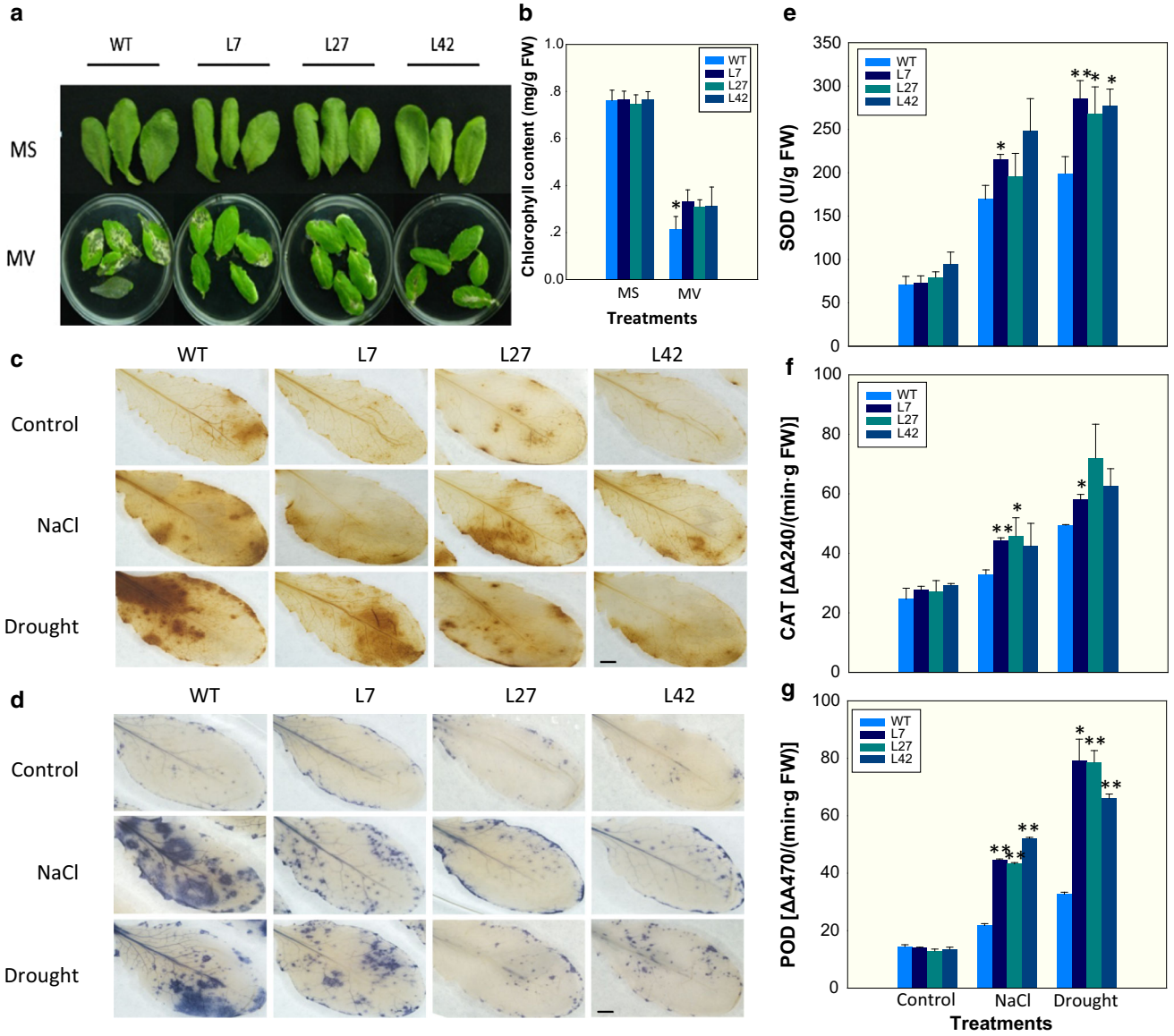

Fig. 6 ROS levels and oxidative enzyme activity assays of WT and *VqbZIP39*-expressing transgenic *A. thaliana* plants. **a** Representative images of 4-week old WT and transgenic plants after leaves were floated on MS medium or MS medium supplemented with 6 μM methyl viologen (MV) for 24 h. The experiment was repeated three times with 5 leaves. **b** The chlorophyll contents of WT and transgenic leaves under oxidative stress or under non-stress conditions. **c**, **d** Histochemical staining assay detection of H_2O_2 and O_2^- accumulation with diaminobenzidine (DAB) (**c**) and nitro blue tetrazolium (NBT), respectively (**d**), in WT and transgenic leaves following growth under normal or osmotic stress conditoins. The experiment was repeated three times with 5 leaves. *Scale bar* 2 mm. **e–g** Activities of superoxide dismutase (SOD) (**e**), catalase (CAT) (**f**) and peroxidase (POD) (**g**) in the leaves of WT and transgenic plants grown under non-stress and osmotic stress conditions. Data represent mean values ± SD from three independent experiments. *Asterisks* indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the transgenic and WT plants

significantly greater in the transgenic lines compared with WT plants under both salt and drought stress. However, the expression level of *ERD1* was not significantly different in WT and transgenic plants, with or without abiotic stress treatment. Interestingly, the expression levels of *KIN2*, a multiple stress marker gene (Fujita et al. 2005), was significantly higher in the transgenic lines than in WT plants, whether they were subjected to a stress treatment or not.

Discussion

The bZIP gene family is one of the largest TF families in plants and is known to regulate a wide range of biological processes, including responses to abiotic stresses. Transgenic *A. thaliana* plants over-expressing a soybean bZIP gene exhibit a higher tolerance of salt and cold stress (Liao et al. 2008), and *A. thaliana* constitutively expressing

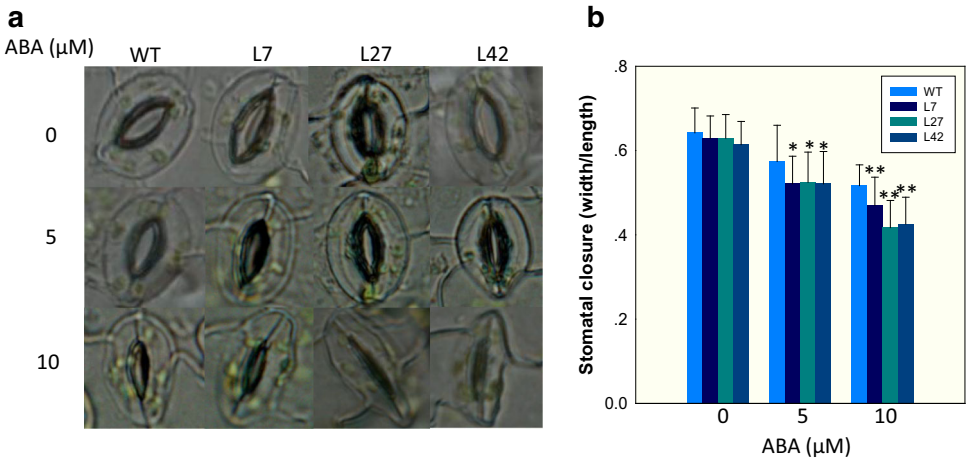

Fig. 7 Stomatal closure in response to ABA treatment in WT and *VqbZIP39*-expressing transgenic *A. thaliana* plants. **a** Comparison of stomatal apertures in response to different concentrations of exogenous abscisic acid (ABA) in WT and transgenic plants. **b** Stomatal aperture width to length ratios following treatment with different concentrations of exogenous ABA. Data represent mean values ± SD from three independent experiments. *Asterisks* indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the transgenic and WT plants

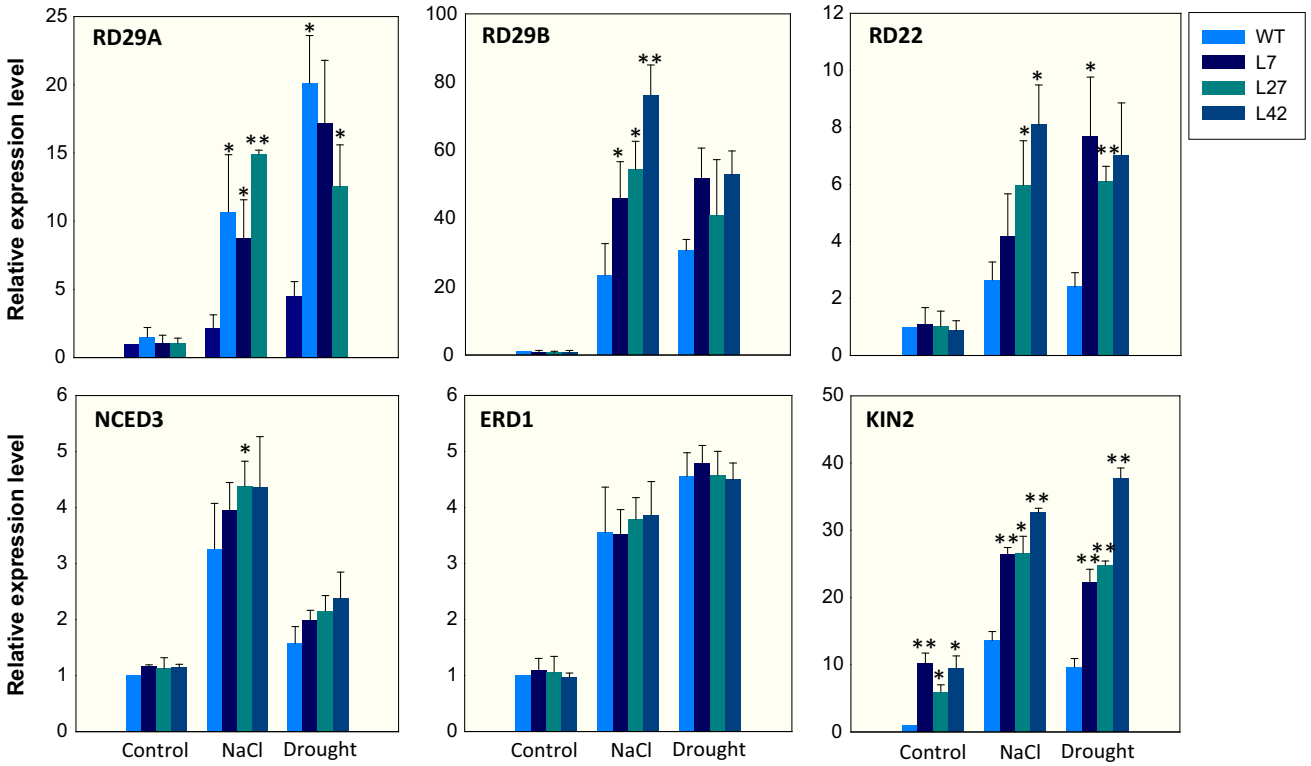

Fig. 8 Gene expression profiles of stress-marker genes in WT and *VqbZIP39*-expressing transgenic *A. thaliana* plants analyzed using quantitative real-time RT-PCR. Total RNA from transgenic and WT plants that had been treated with 200 mM NaCl or deprived of water for 7 days. The *ACTIN2* gene was used as an internal control. For each gene, the expression level in the WT plants under non-stressed conditions was defined as 1.0. Data represent mean values ± SD from three independent experiments. *Asterisks* indicate statistical significance (*$0.01 < P < 0.05$, **$P < 0.01$, Student's *t* test) between the transgenic and WT plants

ABP9, another bZIP gene, is more tolerant of heat, water, and drought stress (Zhang et al. 2008). Additionally, it has been shown that *A. thaliana* over-expressing wheat *bZIP60* exhibits increased drought, salt and freezing tolerance (Zhang et al. 2014). However, to date there is only one report that has addressed the function of a bZIP gene from

grapevine (Tak and Mhatre 2013), one of the most economically important perennial fruit crops globally, due to its diverse uses, including the production of wine, jam, juice and jelly, grape seed extracts, raisins, vinegar, and grape seed oil. Since the production of grape is limited by a range of biotic and abiotic stresses, which cause significant losses in yield every year, as well as a reduction in berry quality (Ferreira et al. 2004), the expression and function of grape regulatory gene families is of considerable interest.

In the present study, of the 47 bZIP genes in the grape genome that were previously identified by our group (Gao et al. 2014), *bZIP39* was selected for further study as its expression is up-regulated in response to exogenous ABA treatment, salt and drought stress conditions (Gao et al. 2014). Multiple sequence alignment (Fig. S1b) and phylogenetic analysis (Fig. 1) showed that VqbZIP39 is highly homologous to AREB1, SLAREB1, GmbZIP71, and NtABF2, of which AREB1/ABF2 is known to be a key regulator of ABA signaling under drought stress (Fujita et al. 2005). SLAREB1 has been shown to enhance tolerance to drought and salt stress in transgenic tomato (Orellana et al. 2010). This homology thus further suggested a potential association between *VqbZIP39* and abiotic stress tolerance. To better understand the function of *VqbZIP39*, the gene was constitutively expressed in the transgenic plants exposed to drought and salt treatments (Fig. 2a, b). In terms of general drought and salt stress tolerance, the transgenic seeds exhibited significantly higher levels of germination under osmotic stress compared to WT seeds (Fig. 3a, b), and the roots of the transgenic seedlings were significantly longer than those of WT under the same stress conditions (Fig. 3c, d). Furthermore, the transgenic plants showed greater tolerance of salt and dehydration than WT plants (Fig. 5a, b). Taken together these results suggest that germination and post-germination growth was improved under osmotic stress conditions due to the presence of the transgene.

To gain insight into the mechanism associated with *VqbZIP39*-induced abiotic stress tolerance, we monitored changes in several physiological and biochemical characteristics that are known to be associated with responses to abiotic stress. Assessment of the chlorophyll fluorescence of intact leaves, especially fluorescence induction patterns, is a reliable, non-invasive method to evaluate the physiological status of plants (Strasser et al. 2002). We observed that, compared with WT, the chlorophyll content was higher in *VqbZIP39* transgenic seedlings after the application of osmotic stress (Fig. 4a). Salt and drought stresses causes membrane lipid peroxidation, which results in the accumulation of MDA and changes the levels of electrolyte leakage in plants (Levine et al. 1994). MDA levels and the degree of electrolyte leakage have therefore been used as efficient indicators to evaluate the degree of abiotic stress resistance (Tang et al. 2013; Neill et al. 2002; Pompelli et al. 2010). Here, MDA content and the levels of electrolyte leakage in WT plants were significantly higher than in any of the transgenic lines under salt and mannitol imposed stress conditions (Fig. 4b, e), indicating that the degree of membrane damage in transgenic lines was less than that in WT seedlings under osmotic stress. Free proline is a common multifunctional osmolyte in many organisms and plays important roles in enhancing osmotic stress tolerance (Bartels and Sunkar 2005). We determined that the transgenic plants accumulated higher levels of proline than the WT plants in response to osmotic stresses (Fig. 4c), suggesting that proline contributed to the enhanced osmotic stress tolerance of the *VqbZIP39* transgenic plants. In summary, the transgenic plants exhibited higher chlorophyll content and free proline levels, but lower levels of MDA and relative electrolyte leakage, indicating that the over-expression of the *VqbZIP39* gene in *A. thaliana* improved tolerance of multiple abiotic stresses. In support of this conclusion, trypan blue staining revealed a considerably greater number of dead cells in WT leaves than those of the leaves of the transgenic lines (Fig. 5d).

Exposure to abiotic stress leads to increased production of ROS, causing oxidative damage to cellular components, which is considered to be a primary factor in cellular injury in plants (Kasukabe et al. 2004). Here we found that H_2O_2 and O_2^- accumulation in WT plants was higher than in the transgenic lines after 7 days of salt and drought stress treatments (Fig. 6c, d), suggesting that *VqbZIP39* expression is associated with oxidative stress responses. MV is a herbicide that generates ROS and causes membrane damage and chlorophyll degradation (Slade 1966). The damage induced by this free radical-generating chemical was less severe in the transgenic plants than in WT plants (Fig. 6a, b), indicating that expression of *VqbZIP39* enhanced the oxidative stress tolerance of the transgenic *A. thaliana* plants. To minimize damage caused by ROS, plants produce antioxidants and ROS-scavenging enzymes (Miller et al. 2010), such as SOD, CAT and POD (Mittler 2002) and so we measured the activities of these enzymes in plants frown under stress conditions. As shown in Fig. 6e–g, the transgenic plants had higher activities of all three enzymes, suggesting that they possess a more efficient enzymatic antioxidant system compared with the WT plants. Consistent with this idea, we observed lower levels of oxidative damage and reduced levels of H_2O_2 and O_2^- in the transgenic lines under abiotic stress (Fig. 6c, d). We propose that the increased activities of ROS-scavenging enzymes constitute an important physiological mechanism underlying the promotion of abiotic stress tolerance by *VqbZIP39*.

The rate of water loss from detached leaves has been proposed as a useful indicator of water status (Dhanda and

Sethi 1998). We found the rate to be lower in the leaves of the transgenic seedlings than in those of the WT plants at all the investigated time points (Fig. 5c), indicating that the transgenic seedlings have a stronger water retention capability. The production of ABA has been shown to promote stomatal closure, thereby reducing transpirational water loss (Kim et al. 2010), and we hypothesized that the transgenic plants had a stronger water retention capacity due to increased ABA production. The endogenous ABA levels of the transgenic plants were indeed significantly higher than those of WT after exposure to abiotic stress (Fig. 4d), as was the transcript abundance of *NCED3*, whose expression is often used as an indicator of ABA biosynthesis (Iuchi et al. 2001), under salt and drought stress (Fig. 8). A key ABA-controlled process involved in adaptation to water deficit conditions is stomatal closure (Kang et al. 2002). In this study, we compared the responses of stomata to various concentrations of exogenous ABA and found a lower width to length ratios in the transgenic plants than in WT plants following ABA treatment (Fig. 7a, b), indicating that transgenic lines exhibited increased stomatal closure. Thus, we propose that stomatal movement and ABA signaling are factors in the enhanced stress tolerance shown by the transgenic *VqbZIP39* plants.

We also observed that the expression levels of members of the LEA class of genes, including *RD29A*, *RD29B*, *RD22* and *KIN2*, which are involved in ABA-signaling pathways (Yamaguchi-Shinozaki and Shinozaki 2006; Abe et al. 2003; Fujita et al. 2005), were significantly higher in the transgenic plants after salt or drought treatments. The expression of *KIN2* was up-regulated in the transgenic plants under normal growth conditions, while the other genes showed no significant difference between WT and transgenic plants under such conditions (Fig. 8). The increased expression of these stress-responsive genes correlates with the increased salt and drought stress tolerance of the transgenic *VqbZIP39* plants. The expression level of *ERD1*, an ABA-independent gene (Yamaguchi-Shinozaki and Shinozaki 2006), was similar in the WT and transgenic plants regardless of growth conditions (Fig. 8), indicating that the over-expression of *VqbZIP39* may does not influence the ABA-independent signaling pathway. This further supports our contention that *VqbZIP39* mediates responses to abiotic stress through an ABA-dependent pathway. In support of this idea, we analysed the promoter of the stress-related genes, which tested in Fig. 8. We found that all of the ABA responsive genes, including *AtRD29A*, *AtRD29B* (Yamaguchi-Shinozaki and Shinozaki 1993), *AtRD22* and *AtNCED3*, have the ABRE in their promoters. However, no ABRE was found in the promoter of the ABA-independent gene *AtERD1* (Nakashima et al. 1997) (Supplemental table S2). Meanwhile, *VqbZIP39* is an AREB/ABF-like transcription factor (Davies and Robinson 2000), containing a bZIP family-type DNA-binding domain that binds the ABRE (Uno et al. 2000). The result was consistent with the qRT-PCR. In order to realize whether observations made in *A. thaliana* may hold in grapevine, we analysed the promoter of grape stress-related genes, which homologous to the stress-related genes from *A. thaliana.* We observed that all of the ABA responsive genes, except *VvKIN2*, have the ABRE in their promoters, and no ABRE was found in the promoter of the *VvERD1* (Supplemental table S2). It means that the *VqbZIP39* may be a good candidate gene for improving stress tolerance in grape.

In conclusion, in this study we identified a grape bZIP gene, *VqbZIP39*, which was predicted to encode a nuclear-localized protein. Over-expression *VqbZIP39* in transgenic *A. thaliana* lead to enhanced tolerance of multiple abiotic stresses through changes in several physiological parameters and the regulation of stress-responsive genes. Our results further suggest that *VqbZIP39* plays a role in abiotic stress responses via the ABA dependent signaling pathway.

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