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# Expression of a grape (*Vitis vinifera*) bZIP transcription factor, VlbZIP36, in *Arabidopsis thaliana* confers tolerance of drought stress during seed germination and seedling establishment

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ABSTRACT

Drought is one of the most serious factors that limit agricultural productivity and there is considerable interest in understanding the molecular bases of drought responses and their regulation. While numbers of basic leucine zipper (bZIP) transcription factors (TFs) are known to play key roles in response of plants to various abiotic stresses, only a few group K bZIP TFs have been functionally characterized in the context of stress signaling. In this study, we characterized the expression of the grape (*Vitis vinifera*) group K bZIP gene, *VlbZIP36*, and found evidence for its involvement in response to drought and the stress-associated phytohormone abscisic acid (ABA). Transgenic *Arabidopsis thaliana* lines over-expressing *VlbZIP36* under the control of a constitutive promoter showed enhanced dehydration tolerance during the seed germination stage, as well as in the seedling and mature plant stages. The results indicated that *VlbZIP36* plays a role in drought tolerance by improving the water status, through limiting water loss, and mitigating cellular damage. The latter was evidenced by reduced cell death, lower electrolyte leakage in the transgenic plants, as well as by increased activities of antioxidant enzymes. We concluded that *VlbZIP36* enhances drought tolerance through the transcriptional regulation of ABA-/stress-related genes.
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## 1. Introduction

Drought is one of the most serious factors limiting plant growth and development worldwide [1], and since they are sessile, plants must rapidly recognize and adapt to drought stress conditions in order to maintain growth and productivity. This involves the activation of a broad range of physiological and biochemical responses [2], including limiting water loss, maintaining intracellular ion homeostasis and scavenging reactive oxygen species (ROS) that are generated as a secondary effect of drought [3]. An improved understanding of the mechanisms of these adaptations has value in informing plant breeding strategies [4]. Moreover, drought stress leads to changes in the expression of numerous drought stress-related genes [5], and the identification and functional analysis of such drought stress-related genes is an important step in the development of drought tolerant plants [6].

The phytohormone abscisic acid (ABA) is known to regulate processes that protect plants from damage induced by drought, during both seedling growth and subsequent maturation [7]. Accordingly, ABA accumulates in plant cells in response to drought, leading to expression of stress responsive genes [1,8]. Additionally, ABA-dependent gene expression plays an important role in transcriptional regulatory networks under osmotic stress conditions [8]. However, in addition to the ABA-dependent regulatory gene expression networks, it has also been reported that plants have stress responsive regulatory systems that are ABA-independent [9].

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 [10] is the basic leucine zipper (bZIP) family. Previous studies have shown that bZIP TFs (groups A, S and G) play an important role in the ability of plants to resist various stresses. For example, the group A bZIP TF, AtAREB1, enhances drought tolerance in vegetative tissues through ABRE-dependent ABA sig-

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naling [6], and the *areb1*, *areb2* and *abf3 Arabidopsis thaliana* triple mutant has reduced drought stress tolerance compared to the single and double AREB/ABF TF knockout mutants [11]. 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 [12]. In addition, rice (Zhonghua 11) *OsbZIP16* has been shown to be a positive regulator of ABA responses [13]. The member of group S bZIP TF, *OsLIP19*/*OsbZIP86*, has been characterized and is thought to be involved in the cold signaling [14]. Group G bZIP TFs, including *OsbZIP53* and *OsbZIP54*, also participate in abiotic stress signaling [15]. Thus, bZIP genes have been shown to function as important regulators of many stress conditions; however, to date, only a few group K bZIP TFs have been functionally characterized in the context of stress signaling [16–18]. For example, *AtbZIP60* [16] and *OsbZIP74* [17] have previously been shown to be involved in the endoplasmic reticulum (ER) stress response, and overexpression of *AtbZIP60* in rice and white pine cell lines improve drought stress tolerance by regulating the expression of several $Ca^{2+}$-dependent protein kinase genes [18].

In this current study, we found that the expression patterns of grapevine *VlbZIP36* (GenBank accession number: XM_003634288) from group K [19,20] was up-regulated in response to exogenous ABA treatment and drought stress conditions, suggesting that it may be associated with tolerance to abiotic stress. Therefore, *VlbZIP36* was cloned and its function on abiotic stress responses was investigated by ectopic expression in *A. thaliana*, and the drought stress tolerance of the transgenic lines was compared to that of wild type (WT) plants. A number of physiological parameters of the transgenic and WT plants were measured, including the water loss rate, electrolyte leakage, malondialdehyde (MDA) levels and survival rates. In addition, antioxidant enzyme activities were assayed and the levels of ROS were quantified in the different genotypes. The results of these analyses are presented and discussed in the context of the importance of *VlbZIP36* in regulating stress responses and its potential value in crop breeding.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Two-year-old 'Kyoho' grape (*Vitis labrusca* × *V. vinifera*) plants used in this study were grown in the grape repository of Northwest A&F University, Yangling, Shaanxi, China. The drought and ABA treatment assays using grape seedlings were performed as previously described [21].

*A. thaliana* Columbia-0 (WT) plants, were grown in soil at 21 °C under long-day (LD) conditions (16 h light/8 h dark) for 3 weeks and used for experiments prior to bolting. Seeds from each of three selected T3 homozygous lines and from WT plants were vernalized for 3 days 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 placed on Murashige-Skoog (MS) medium (Sigma) [22] solidified with 0.7% agar containing 2% sucrose.

### 2.2. Vector construction

Total RNA was extracted from the leaves of 2-year-old 'Kyoho' seedlings as previously described [23], and 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™Reverse Transcriptase (TaKaRa Biotechnology), according to the manufacturer's instructions. The full length grape *VlbZIP36* open reading frame was amplified by PCR using the gene-specific primers F1 (5′-ATG GAC GAT TTG GAA ATT GGG G-3′) and R1 (5′-TCA CAC CAA AAC TCC ATG AG-3′). The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and the resulting plasmid (pGEM-Teasy-*VlbZIP36*) was sequenced to confirm sequence fidelity.

To obtain a 35S:*VlbZIP36* vector, the *VlbZIP36* open reading frame (with *Xba*I and *Kpn*I sites added to the 5′ and 3′ end, respectively) was amplified from the pGEM-Teasy-*VlbZIP36* vector using the gene-specific primers F2 (5′-CGC TCT AGA ATG GAC GAT TTG GAA ATT GGG G-3′ *Xba*I site underlined) and R2 (5′-GGC GGT ACC TCA CAC CAA AAC TCC ATG AG-3′ *Kpn*I site underlined), and inserted immediately downstream of the CaMV 35S promoter in the plant overexpression vector, pCambia2300 (Cambia, Brisbane, QLD, Australia).

Grapevine DNA was extracted from the leaves of 2-year-old 'Kyoho' seedlings as previously described [24]. To obtain a $Pro_{VlbZIP36}$:*GUS* (β-glucuronidase) vector, a 1973 bp fragment upstream of the *VlbZIP36* transcription start site was amplified by PCR from the genomic DNA using the gene-specific primers F3 (5′-CCC AAG CTT GGT AAG AAA TGG TAG AAG GG-3′ *HindIII* site underline) and R3 (5′-TCC CCC GGG GAC TAG AGG AAC AAT TCC TC-3′ *Sma*I site underlined), re-sequenced and inserted into the binary vector pBI121 (Clontech Lab. Inc., Palo Alto, CA, USA) containing the *GUS* reporter gene. The integrity of the final constructs was confirmed by sequencing.

### 2.3. Bioinformatic analysis

The protein sequences of group A, G, S and K bZIP TFs from plants have been previously reported [15,25,26], and are available through the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the J. Craig Venter Institute (TIGR) (http://www.tigr.org/). To investigate the relationship between VlbZIP36 and other bZIP TFs from *A. thaliana* and rice, their amino acid sequences were compared using DNAMAN software (Version 5.2.2.0, Lynnon Biosoft, USA) and a systematic phylogenetic analysis was carried out using the neighbor-joining (NJ) method in the MEGA (version 5.05) software, with 1000 replicated bootstrap tests. Highly conserved amino acid residues were analyzed using the SMART program (http://smart.embl-heidelberg.de/).

### 2.4. Plant transformation

All of the above constructs were introduced into *Agrobacterium tumefaciens* (strain GV3101) via electroporation and *A. thaliana* transformation was carried out using the floral dip method [27]. For each construct, T0 seeds were harvested and sown on MS agar medium supplemented with 100 mg/L kanamycin. For the phenotypic investigation, three T3 homozygous lines (13#, 31# and 32#) with high-levels of expression of *VlbZIP36*, were generated and used for all subsequent experiments. To assess the expression of *GUS* driven by the presence of $Pro_{VlbZIP36}$:*GUS* in the transgenic plants, T3 homozygous lines from 3 independent transgenic lines were analyzed.

### 2.5. Histochemical GUS assay

An in situ GUS activity assay was performed as previously described [28]. Briefly, plant materials were immersed in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; Biosynth AG), 100 mM sodium phosphate (pH 7.0), 0.1% Triton X-100, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 0.1 mM EDTA. After applying vacuum for 30 min, the samples were incubated at 37 °C for 24–48 h. Finally, chlorophyll was cleared from

the samples by immersing them in 70% ethanol and the samples were viewed under a microscope (BX53, Olympus, Japan).

### 2.6. Osmotic stress treatments and the response of the seeds to ABA

WT and transgenic seeds were harvested at the same time and stored for 3 months. For seed germination and cotyledon greening analyses, approximately 50 seeds were surface-sterilized and sown on MS agar medium, MS agar medium containing 300, 325 and 350 mM mannitol or 0.5, 0.75 and 1 μM ABA. Seeds were vernalized at 4 °C for 3 days before growth at 21 °C under 16 h light/8 h dark conditions. Seedlings with fully emerged radicle tips and green cotyledons were scored for seed germination and cotyledon greening rates, respectively [29].

For the osmotic stress treatment, 1-week-old WT and transgenic seedlings were transferred from MS medium plates into MS medium or MS medium supplemented with mannitol at the indicated concentrations. The root lengths were measured 7 days after the transfer, and the leaves were sampled following osmotic stress treatment to measure electrolyte leakage and MDA levels. For the drought treatment, plants were initially grown in pots filled with compost soil under a normal watering regime for 3 weeks. Irrigation was then withheld from the soil-grown plants for 8 days, followed by re-watering. Survival rates were scored 3 days after re-watering. Well-watered plants were used as the negative control.

### 2.7. Water loss assay

For water loss measurements, rosette leaves of 3-week-old transgenic and WT plants were detached and immediately weighed on dry filter paper. The samples, together with the paper, were then placed at 21–25 °C, with a relative humidity of 45–50%, and weighed at the indicated times. The proportion of fresh weight loss was calculated based on the initial weight of the plants [30]. The leaves were sampled after dehydration to examine cell death, electrolyte leakage, MDA, antioxidant enzyme activity, and levels of ROS. Leaves collected before dehydration were used as the negative control.

### 2.8. Analysis of electrolyte leakage, MDA and ROS levels and cell death

Relative electrolyte leakage was measured as previously described [31], as was MDA content [32]. A histochemical staining procedure was used to detect in situ superoxide and hydrogen peroxide, as previously described [33]. For the detection of $H_2O_2$, leaves were placed in a 1 mg/ml DAB (3, 3′-diaminobenzidine) solution for 8 h, or until brown spots became visible. Chlorophyll was cleared at 80 °C in 80% (v/v) ethanol for 2 h, and samples were placed in 10% (v/v) glycerol for observation [34]. For the detection of $O_2^-$, leaves were infiltrated with HEPES buffer (pH 7.5) containing 6 mM NBT (nitroblue tetrazolium) for 2 h, as previously described [35]. 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 [36].

### 2.9. Antioxidant enzyme activity

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 $O_2^-$, as previously described [37]. 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 [38]. CAT activity was reported as the amount of change in absorbance/min/g FW (fresh weight). Peroxidase (POD, EC 1.11.1.7) activity was assayed as previously described [39] and expressed as the change in absorbance/min/g FW.

### 2.10. Stomatal aperture analysis

Stomatal aperture assays were performed essentially as previously described [40]. Briefly, detached rosette leaves from 3-week-old transgenic and WT plants were first incubated in a buffer containing 10 mM KCl, 10 mM MES-Tris, pH 6.2 and 50 μM $CaCl_2$, before being exposed to light for 3 h. ABA was then added to the solution to a final concentration of 10 μM. Stomatal apertures were observed after 1 h under a microscope (BX53, Olympus, Japan) and recorded as the ratio of stomatal width to length.

### 2.11. Quantitative real-time PCR

Total grapevine RNA was extracted using the E.Z.N.A._Plant RNA Kit (Omega Bio-tek, USA, R6827-01), and total *A. thaliana* RNA was extracted from the leaves of 3-week-old transgenic and WT plants collected before and after dehydration [41] using the RNAprep plant kit (Tiangen Biotech, China), following the manufacturer's instructions. First-strand cDNA was synthesized as described above. Subsequent quantitative real-time PCR (qRT-PCR) analyses were conducted using SYBR Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa Biotechnology) and an IQ5 RT-PCR instrument (Bio-Rad, Hercules, CA, USA) with the following thermal profile: 95 °C for 3 min, 45 cycles of 95 °C for 10 s, and 60 °C for 30 s. The expression levels of the grape *Actin1* (GenBank accession number: AY680701) or *A. thaliana Actin2* (TAIR: AT3G18780) (TAIR: The Arabidopsis Information Resource; http://www.arabidopsis.org/index.jsp) gene were used as a reference. The primers used for qRT-PCR are listed in Supplementary Table 1. Relative expression levels were analyzed with IQ5 software using the Normalized Expression Method.

### 2.12. Statistical analysis

Data analysis was performed using Microsoft Excel (Microsoft Corporation, USA) and data were plotted using Sigmaplot (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). All experiments were repeated three times as independent analyses.

### 2.13. Accession numbers

The sequences of the *A. thaliana* genes analyzed by qRT-PCR can be found in TAIR under the following accession numbers: *AtRD29A* (AT5G52310), *AtRD29B* (AT5G52300), *AtRAB18* (AT1G43890), *AtNCED3* (AT3G14440), *AtABF3* (AT4G34000), *AtABI1* (AT4G26080), *AtABI5* (AT2G36270), *AtERA1* (AT5G40280), *AtKAT2* (AT4G18290), *ANAC103* (AT5G64060), *AtActin2* (AT3G18780).

## 3. Results

### 3.1. Isolation and bioinformatic analysis of the VlbZIP36 gene

The *VlbZIP36* cDNA was predicted to be 969 bp in length, with an open reading frame of 322 amino acids. A multiple sequence alignment and phylogenetic analysis indicated that VlbZIP36 was most closely related to AtbZIP60 and OsbZIP74, which are members of the *A. thaliana* and rice (*Oryza sativa*) group K bZIP family, respectively (Fig. 1A). Analysis of the deduced amino acid residues using the SMART program (http://smart.embl-heidelberg.de/) revealed that these amino acid sequences of the bZIP proteins belonging

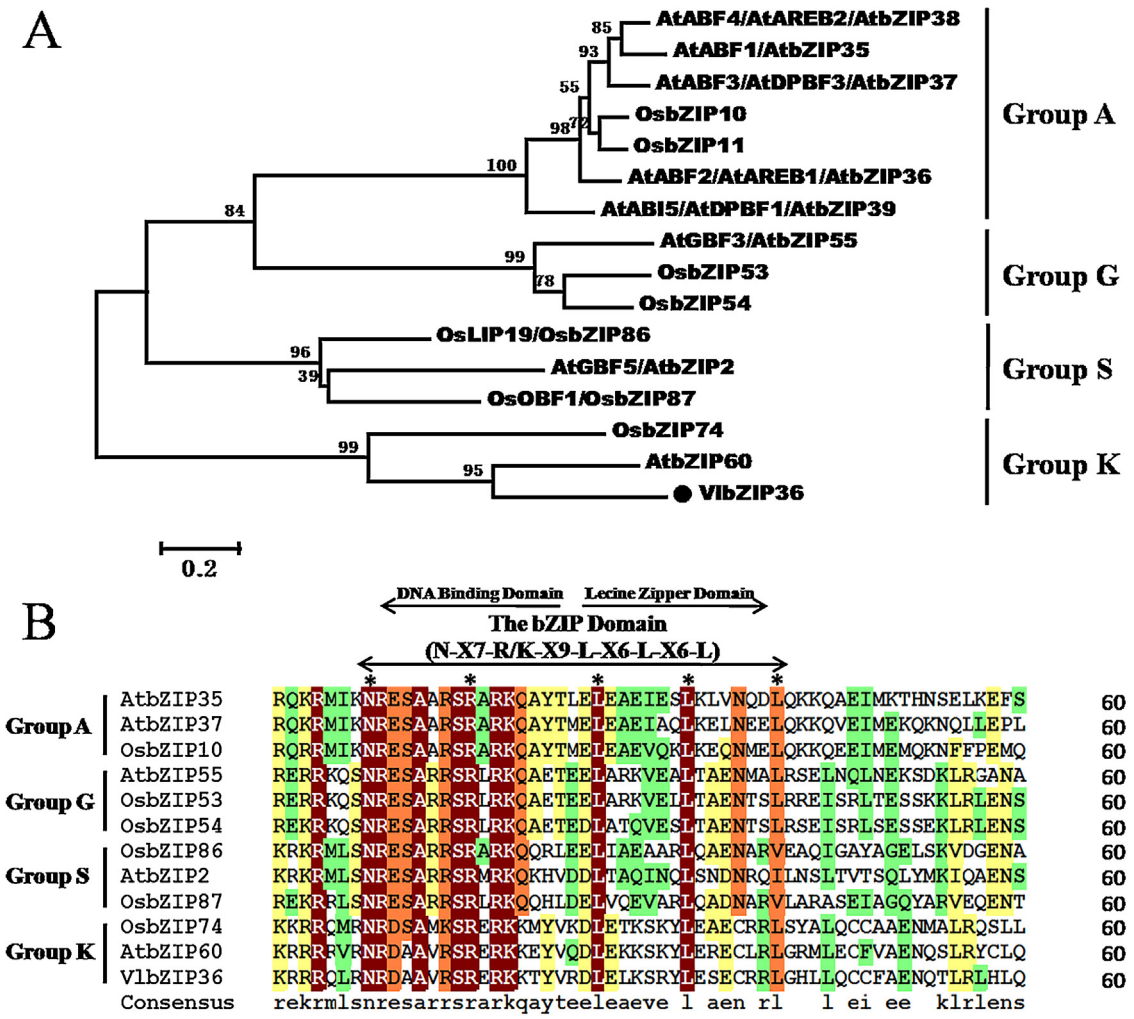


**Fig. 1.** Phylogenetic analysis and multiple sequence alignment. (A) Phylogenetic tree showing the relationship between VlbZIP36 (black circle) and other bZIP amino acid sequences from *Arabidopsis thaliana* and *Oryza sativa*. The bZIP amino acid sequences were divided into groups A, G, S and K, with VlbZIP36 (XM_003634288), AtbZIP60 (NP_174998) and OsbZIP74 (LOC_Os06g41770) belonging to group K. The clustering of amino acid sequences of the group K bZIP proteins and the other group bZIP proteins (AtABF1: NP_564551; AtABF2: NP_001185157; AtABF3: NP_849490; AtABF4: NP_566629; AtABI5: NP_565840; AtbZIP55: NP_182150; AtbZIP2: NP_179408; OsbZIP10: LOC_Os08g36790; OsbZIP11: LOC_Os02g52780; OsbZIP53: LOC_Os01g46970; OsbZIP54: LOC_Os05g49420; OsbZIP86: LOC_Os05g03860; OsbZIP87: LOC_Os12g37410) is in accordance with Jakoby et al. [25], Nijhawan et al. [15] and Correa et al. [26]. (B) Multiple sequence alignments of the amino acid sequences of group A, G, S and K bZIP proteins. Asterisks indicate the well-conserved bZIP domain (N-X7-*R*/K-X9-L-X6-L-X6-L). Partially conserved residues with 100% similarity are highlighted in *brown*; conserved amino acid residues with a similarity >75% are highlighted in *orange*; conserved amino acid residues with 50–75% and 33–50% similarity are highlighted in *yellow* and *green*, respectively.

to the same groups are highly conserved. All these proteins contained a characteristic bZIP domain (N-X7-*R*/K-X9-L-X6-L-X6-L), which included a basic DNA binding domain and a leucine zipper domain, except for the group S bZIP proteins lacked a X6-L motif (N-X7-*R*/K-X9-L-X6-L) (Fig. 1B).

### 3.2. *VlbZIP36 expression is induced in grape by drought and ABA treatments*

The expression patterns of *VlbZIP36* as a result of water deficiency or ABA treatment were evaluated using qRT-PCR. As shown in Fig. 2A, *VlbZIP36* expression was significantly and rapidly induced within 48 h of the drought treatment, and peaked at 72 h, after which it decreased. When drought treated plants were re-watered, *VlbZIP36* expression reverted to original level after 48 h. The expression of *VlbZIP36* was also rapidly induced at 6 h after ABA treatment, but then decreased for the next 48 h, although *VlbZIP36* transcript levels were still 2-fold higher than those in the control (Fig. 2B). These results indicated that *VlbZIP36* may be involved in the response to drought and ABA in 'Kyoho' grape.

### 3.3. *VlbZIP36 expression profile*

To characterize the temporal and spatial expression patterns of *VlbZIP36*, we analyzed transgenic *A. thaliana* plants harboring a *VlbZIP36* promoter-*GUS* reporter construct using histochemical GUS staining.

GUS activity was undetectable in the emerging radicles at the germination stage (Fig. 3A), but it was observed in the cotyledon tips and roots (Fig. 3C) and in most of the vegetative tissues at later developmental stages (Fig. 3D). However, after mannitol treatment, GUS staining was also observed in the tip of emerging radicles at the germination stage (Fig. 3B) and its activity was significantly enhanced in all of the vegetative tissues of 2-week-old seedlings (Fig. 3E). In mature plants, strong GUS staining was detected in petioles, anthers, stigmas and siliques (Fig. 3F, L–O), while only slight staining was observed in guard cells (Fig. 3I) and sepals (Fig. 3N), and none in petals. GUS activity was then assessed in 3-week-old plants that had been subjected to dehydration or ABA stress conditions. As expected, the petioles and leaves showed an increase in GUS staining when dehydrated (Fig. 3G) and treated with exoge-

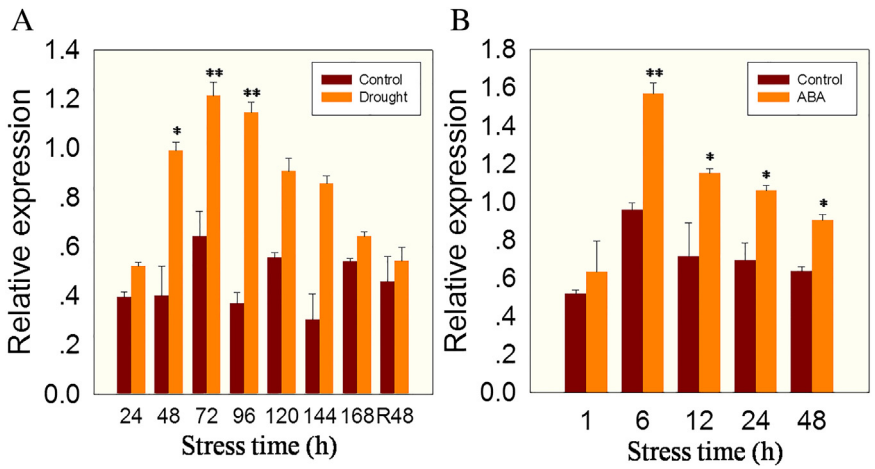


**Fig. 2.** Expression analysis of *VlbZIP36* in 'Kyoho' grapevine plants exposed to drought and ABA treatments by quantitative real-time PCR. Expression profiles of *VlbZIP36* in response to drought (A) and ABA (B) treatments. R48 represents 48 h after re-watering. The grape *Actin1* gene was used as an internal control. 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 treated and untreated control plants.

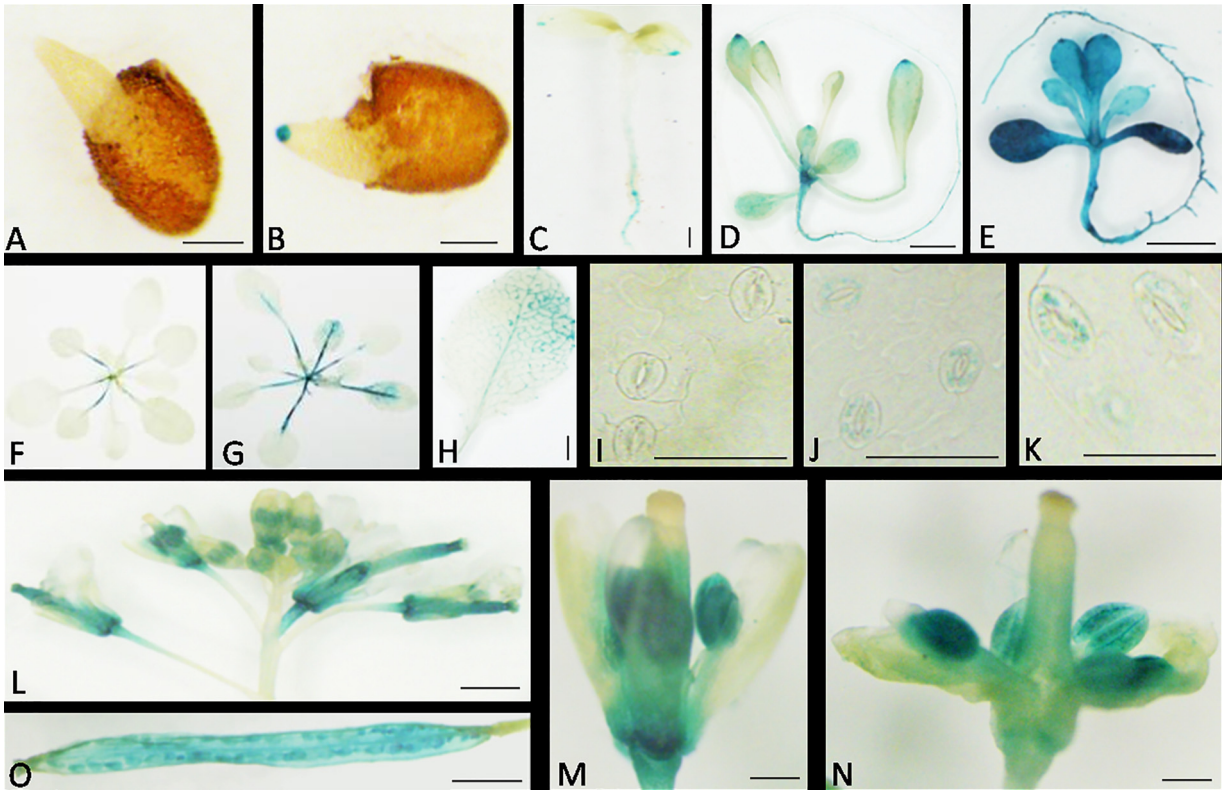


**Fig. 3.** Expression of *VlbZIP36* in transgenic *Arabidopsis thaliana*. T3 homozygous $Pro_{VlbZIP36}$:*GUS* plants were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid at different growth stages. (A) Mature embryo cultivated on Murashige-Skoog (MS) basal medium for 24 h. Scale bar = 200 μm. (B) Mature embryo cultivated on MS basal medium supplemented with 300 mM mannitol for 48 h. Scale bar = 200 μm. (C) 5-day-old seedling. Scale bar = 500 μm. (D) 2-week-old plant. Scale bar = 2 mm. (E) 2-week-old plant treated with 300 mM mannitol for 7 d. Scale bar = 2 mm. (F) 3-week-old plant. (G) 3-week-old plant after dehydration for 2 h. (H) 3-week-old plant treated with 10 μM ABA for 1 h. Scale bar = 2 mm. (I) Guard cells of 3-week-old plant. Scale bar = 50 μm. (J) Guard cells of 3-week-old plant after dehydration for 2 h. Scale bar = 50 μm. (K) Guard cells of 3-week-old plant treated with 10 μM ABA for 1 h. Scale bar = 50 μm. (L) Inflorescence. Scale bar = 2 mm. (M) Flower. Scale bar = 200 μm. (N) Stigma and anthers of flower. Scale bar = 200 μm. (O) Silique. Scale bar = 1 mm.

nous ABA (Fig. 3H). In addition, strong GUS activity was observed in guard cells after dehydration (Fig. 3J) or ABA (Fig. 3K) treatment. The transgenic plants harboring $Pro_{VlbZIP36}$:*GUS* showed a marked increase in GUS staining when treated with exogenous ABA or dehydration stress, confirming that *VlbZIP36* expression is regulated by these factors, and in accordance with the qRT-PCR results (Fig. 2).

### 3.4. Analyses of seed germination and post-germination growth with exogenous ABA and osmotic treatments

We examined the germination rates of seeds from the *VlbZIP36* over-expressing transgenic lines and WT plants following treatments with 300, 325 and 350 mM mannitol or 0.5, 0.75 and 1 μM ABA. When grown on MS agar medium, nearly 100% of the seeds

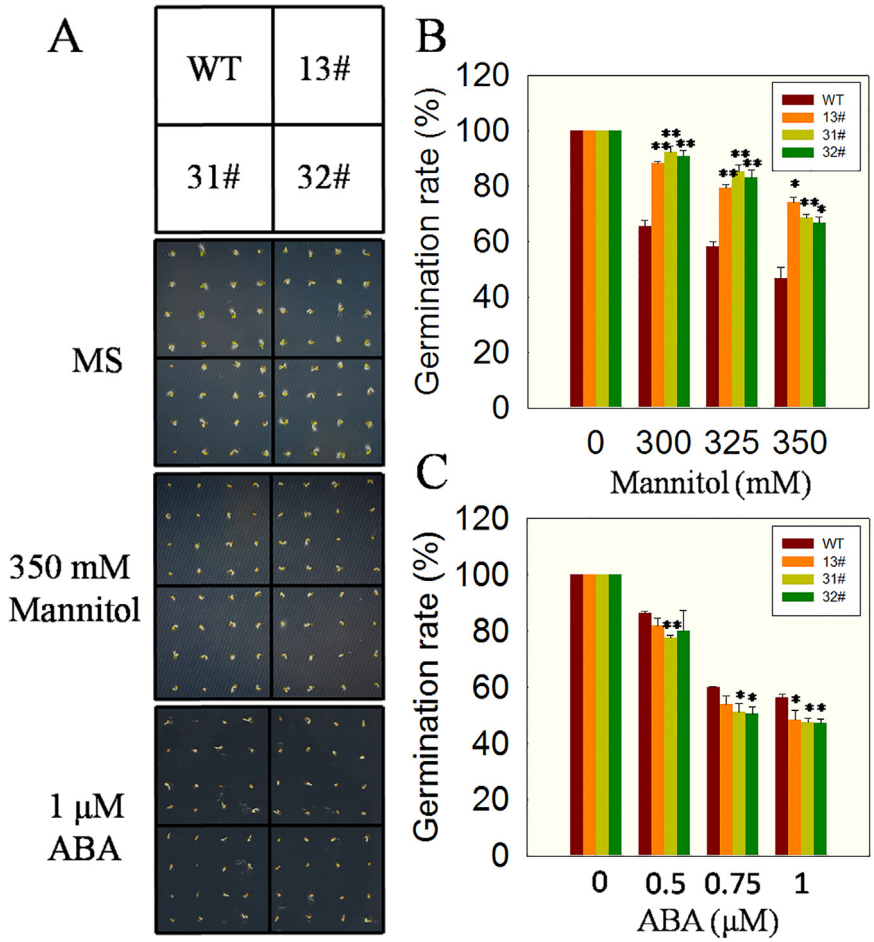


**Fig. 4.** Phenotypes of wild type (WT) and *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* lines at the seed germination stage under osmotic stress and exogenous ABA treatments. (A) Photographs of seed germination in WT and transgenic lines (13#, 31# and 32#) 3 d after seeds were cultivated on Murashige-Skoog (MS) basal medium, MS basal medium supplemented with 350 mM mannitol or 1 μM ABA. (B) Seed germination rates of WT and transgenic lines 3 d after cultivated on MS basal medium, or MS basal medium containing 300, 325 and 350 mM mannitol, respectively. (C) Seed germination rates of WT and transgenic lines 3 d after cultivated on MS basal medium, or MS basal medium containing 0.5, 0.75 and 1 μM ABA, respectively. Three independent experiments were performed with ~50 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.

from both the transgenic lines and WT germinated successfully after 3 d; but seeds from the transgenic lines exhibited significantly higher germination rates than those of WT after osmotic stress treatments, however, the germination rates for the transgenic lines were slightly lower than those for WT plants in the presence of exogenous ABA (Fig. 4A). Although the germination rates of both transgenic lines and WT decreased substantially under osmotic stress and exogenous ABA treatments, the transgenic seeds exhibited 21–28% higher germination rates than those of WT sown on the mannitol medium but showed 4.57–9.2% lower than those of WT sown on the ABA medium (Fig. 4B,C). In the presence of various concentrations of mannitol, the cotyledon greening rates were significantly higher in the transgenic lines compared with the WT (Fig. 5A,B). For example, in the presence of 300 mM mannitol, ~83% of seedlings from the transgenic lines had greening cotyledons after 6 d, while this was the case for only 66% of the WT seedlings. In the presence of 350 mM mannitol, the transgenic lines showed ~27% higher cotyledon greening rates than WT plants. As shown in Fig. 5C, neither the transgenic lines nor WT plants had greening cotyledons 3 d after treatment with 350 mM mannitol, while the transgenic lines exhibited at least 32% higher cotyledon greening rates than WT plants 7 d after treatment. In addition, in the presence of various concentrations of ABA, the cotyledon greening rates were significantly lower in the transgenic lines compared with the WT (Fig. 6). When grown on MS agar medium supplied with 0.5 μM ABA, the cotyledon greening rates of seeds from transgenic lines and WT showed no significant difference; while the concentration of ABA increased into 0.75 and 1 μM supplied in MS agar medium, cotyledon greening rates of transgenic seeds exhibited 13.39–24.57% and 12–15.12% lower than those of WT 11 d after treatments, respectively (Fig. 6A,B). As shown in Fig. 6C, neither the transgenic lines nor WT plants had greening cotyledons 7 d after treatment with 1 μM ABA, while the transgenic lines exhibited 14.44–19.48% lower cotyledon greening rates than WT plants 12 d after treatment, suggesting that over-expressing *VlbZIP36* increased sensitivity of *A. thaliana* seeds to exogenous ABA.

To gain more insights into the possible roles of *VlbZIP36* in osmotic stress responses, we compared the osmotic stress tolerance of the transgenic lines and WT plants at the time of post-germination growth. No difference was observed in root length between the WT plants and the transgenic plants when they were grown under normal condition (Fig. 7A,B); however, under osmotic stress conditions conferred by 300 or 350 mM mannitol, while the root growth of both transgenic and WT seedlings was reduced compared with non-stress conditions, the roots of the transgenic lines were slightly longer than those of WT plants. Another result of osmotic stress is membrane lipid peroxidation, which results in the accumulation of MDA and changes in the level of electrolyte leakage [42]. MDA levels and the degree of electrolyte leakage have therefore been used to indicate the degree of abi-

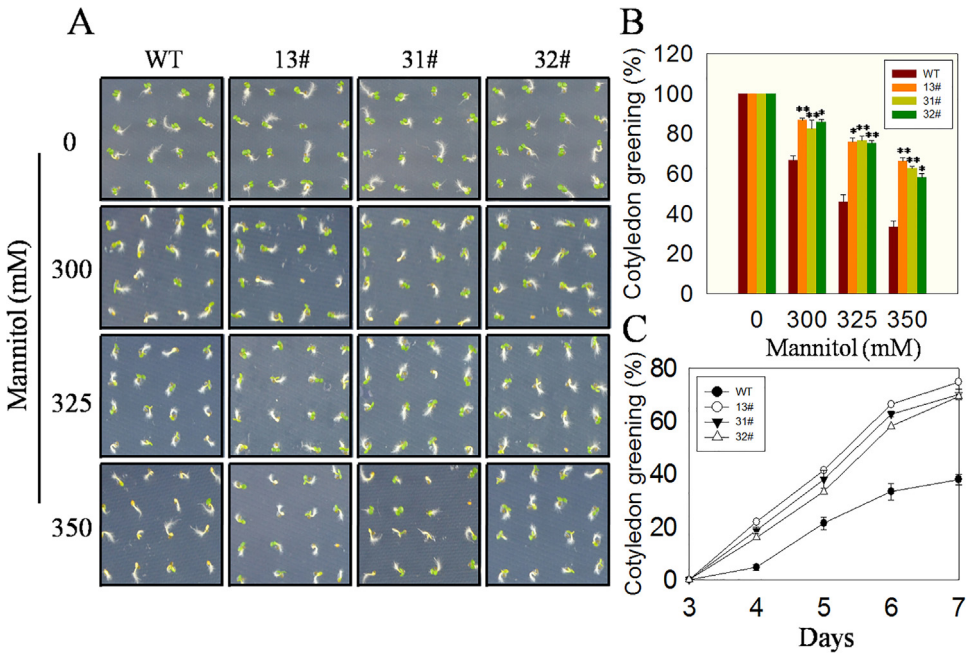


**Fig. 5.** Phenotypes of wild type (WT) and *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* lines at the greening cotyledon stage under osmotic stress conditions. (A) Photographs of greening cotyledons from WT and transgenic lines 6 d after seeds were cultivated on Murashige-Skoog (MS) basal medium, or MS basal medium supplemented with 300, 325 and 350 mM mannitol, respectively. (B) Cotyledon greening rates of WT and transgenic lines cultivated on MS basal medium, or MS basal medium containing 300, 325 and 350 mM mannitol, respectively. (C) Cotyledon greening rates of WT and transgenic lines grown on MS basal medium containing 350 mM mannitol. Three independent experiments were performed with ~50 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.

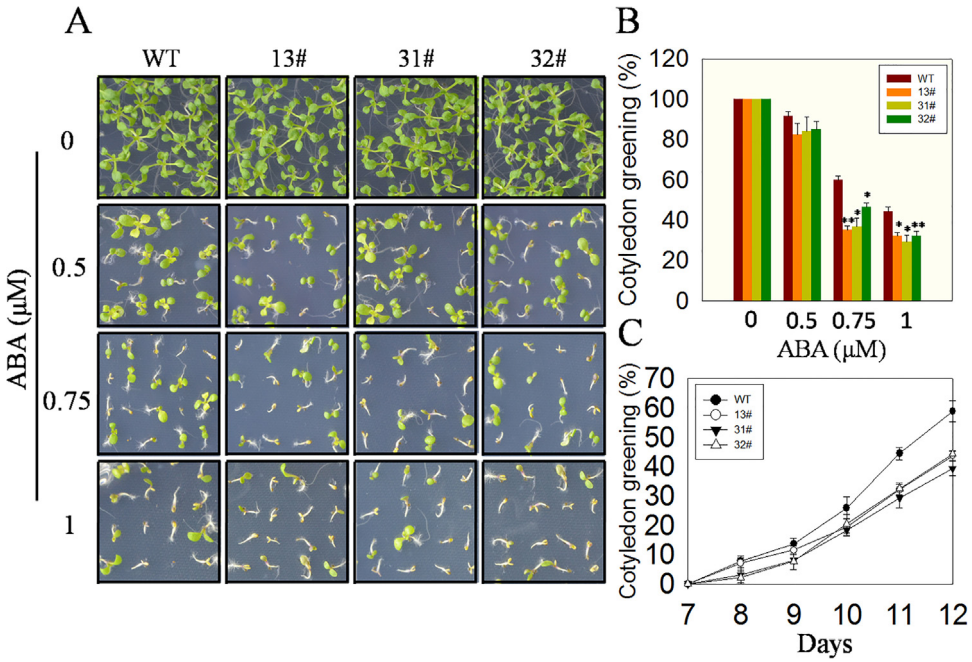


**Fig. 6.** Phenotypes of wild type (WT) and *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* lines at the greening cotyledon stage under exogenous ABA treatments. (A) Photographs of greening cotyledons from WT and transgenic lines 11 d after seeds were cultivated on Murashige-Skoog (MS) basal medium, or MS basal medium supplemented with 0.5, 0.75 and 1 μM ABA, respectively. (B) Cotyledon greening rates of WT and transgenic lines 11 d after cultivated on MS basal medium, or MS basal medium containing 0.5, 0.75 and 1 μM ABA, respectively. (C) Cotyledon greening rates of WT and transgenic lines grown on MS basal medium containing 1 μM ABA. Three independent experiments were performed with ~50 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.

otic stress resistance [43–45]. We measured the relative electrolyte leakage and MDA contents of transgenic and WT seedlings grown on MS medium containing 300 or 350 mM mannitol. Under normal growth conditions, there was little difference between the transgenic lines and WT plants, but following osmotic stress treatments, the values were significantly lower in the transgenic lines than in the WT plants, indicating that the degree of membrane damage was less as a result of over-expressing *VlbZIP36* (Fig. 7C,D).

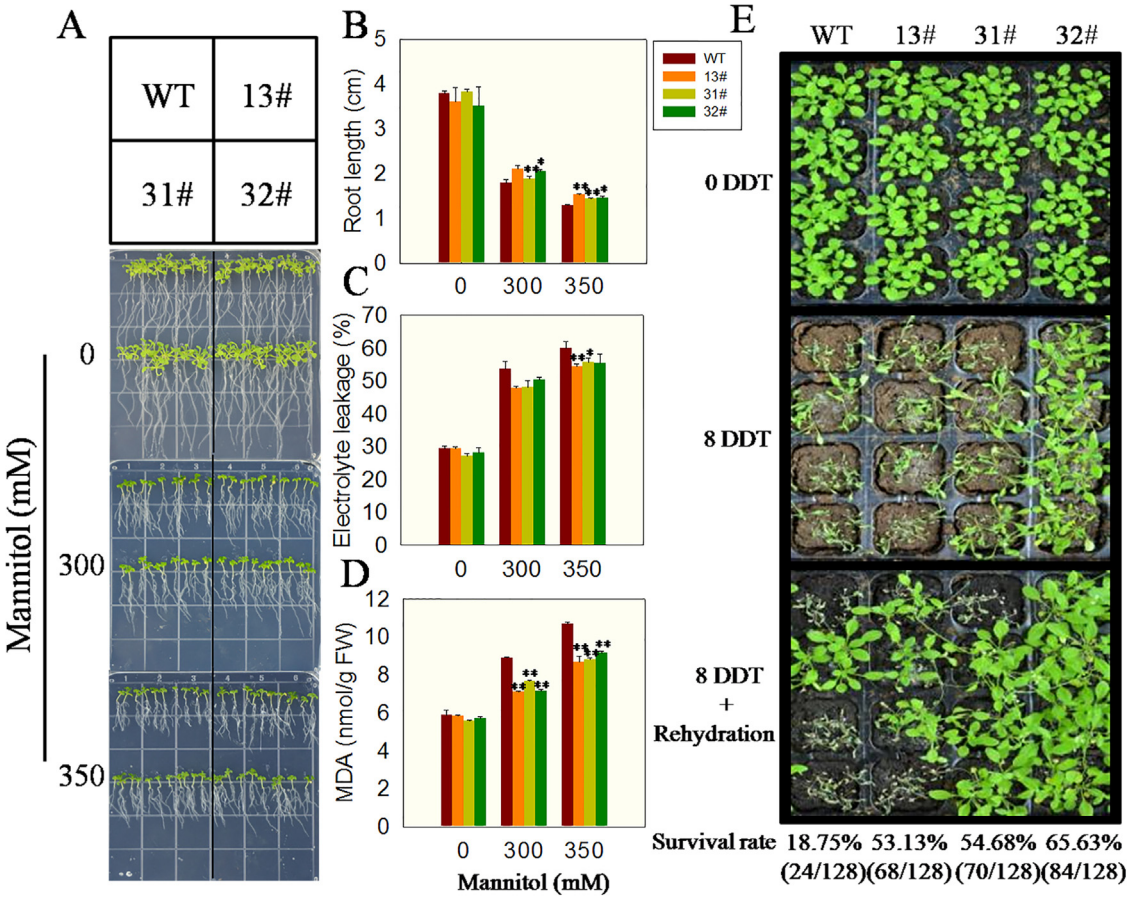


**Fig. 7.** Resistance to osmotic stress in post-germination growth of *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* lines. (A) Photographs of seedlings at 7 d after transfer to Murashige-Skoog (MS) basal medium, or MS basal medium supplemented with 300 and 350 mM mannitol, respectively. Seedlings were 7 d old at the time of transfer. (B) Root length of wild type (WT) and transgenic lines after 7 d of growth with or without 300 and 350 mM mannitol. Three independent experiments were performed using 30 plants per experiment. (C, D) Relative electrolyte leakage (C) and malondialdehyde (MDA) content (D) of transgenic and WT seedlings. 1-week-old transgenic and WT seedlings were transferred from plates into MS medium or MS medium supplemented with various concentrations of mannitol, and grown further for 7 d. (E) Drought tolerance phenotypes and survival rates of transgenic and WT plants grown in soil. 3-week-old plants (upper panel) were dehydrated for 8 d (middle panel) and then rehydrated for 3 d (lower panel). DDT: day of dry treatment. 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.

When plants were watered normally for 3 weeks, no obvious morphological differences between the transgenic and WT plants were observed; however, the performance of 3-week-old transgenic and WT plants subjected to drought stress was different. After 8 d of dehydration, all of the WT plants showed severe wilting symptoms, while only slight wilting was observed in most of the transgenic plants. After 3 d of rehydration, most of the WT plants did not recover, while the transgenic lines rapidly recovered, and after recovery 53–66% of the transgenic lines survived, in contrast to 19% of the WT plants (Fig. 7E).

### 3.5. Overexpression of VlbZIP36 increases the tolerance of A. thaliana to dehydration stress

To examine the effect of *VlbZIP36* overexpression on drought tolerance in more detail, the transgenic lines and WT plants were transferred to dry filter paper under ambient conditions. During the entire period of dehydration, the three transgenic lines showed lower rates of water loss than did the WT plants (Fig. 8C). For example, after 1 h and 3 h of dehydration, the WT plants showed ~6–7% higher water loss rates than the transgenic lines. When the dehydration time was increased to 6 h, WT plants showed more severe wilting (Fig. 8A) and deeper staining with trypan blue (Fig. 8B) than the transgenic lines, suggesting a higher rate of cell death after dehydration. Consistent with the visible phenotypes, WT leaves showed significantly higher electrolyte leakage level (Fig. 8D) and greater MDA levels (Fig. 8E) compared with transgenic lines, indicating that over-expression of *VlbZIP36* in *A. thaliana* led to enhanced dehydration tolerance.

Since many guard cells of drought-tolerant plants are hypersensitive to ABA [4,6], we investigated the ABA sensitivity of the guard cells of transgenic lines. As shown in Fig. 8F, without ABA treatment, no significant difference was observed in the size of stomatal aperture between transgenic and WT plants (data not shown), whereas after 1 h of 10 μM ABA treatment, the stomatal width to length ratio of the transgenic lines decreased considerably more than that of the WT, which is consistent with the fact that the seeds of *VlbZIP36*-overexpression transgenic *A. thaliana* were hypersensitive to exogenous ABA (Fig. 4,6). The result suggested that the dehydration tolerance of the transgenic lines could be attributed to increased sensitivity to ABA.

### 3.6. VlbZIP36 over-expressing lines have higher antioxidant enzyme activities, and a lower abundance of ROS under dehydration stress

Since excess ROS triggered by drought stress can result in progressive oxidative damage to cellular membranes and, ultimately, cell death [46,47], we investigated the levels of two ROS, $O_2^-$ and $H_2O_2$, in WT and transgenic lines before and after dehydration for

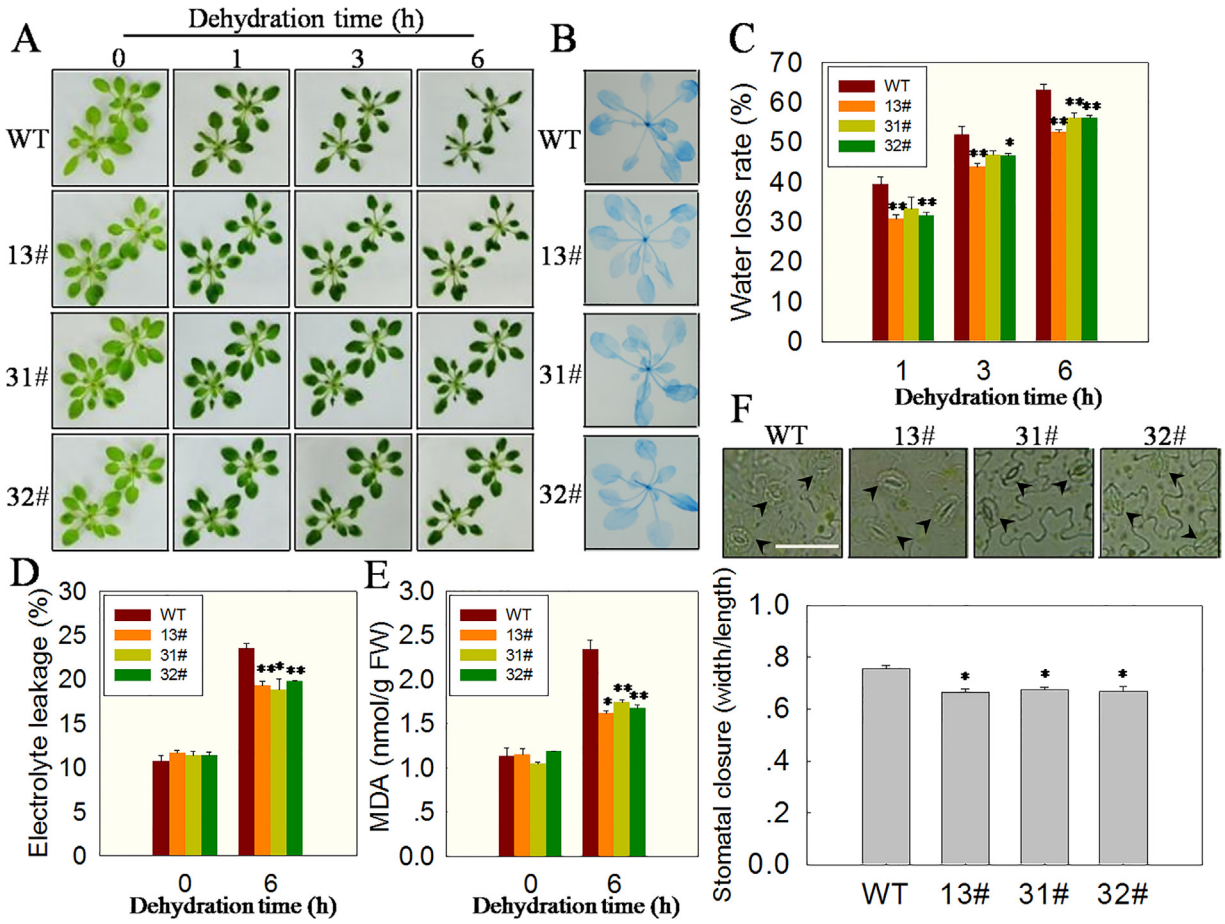


**Fig. 8.** Overexpression of *VlbZIP36* in *Arabidopsis thaliana* conferred enhanced dehydration tolerance. (A) Leaf phenotype of 3-week-old wild type (WT) and transgenic lines before and after dehydration for 1, 3, 6 h. (B) Staining of detached leaves from WT and transgenic lines with trypan blue after 6 h of dehydration. (C) Water loss rates in WT and transgenic lines during 6 h of dehydration. (D, E) Relative electrolyte leakage (D) and malondialdehyde (MDA) content (E) of transgenic and WT plants. Leaves were detached from 3-week-old WT and transgenic lines before and after dehydration for 6 h. (F) Stomatal closure in response to 10 μM exogenous ABA in 3-week-old WT and transgenic plants. Arrows indicate guard cells. Scale bar = 50 μm. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6 h by NBT and DAB histochemical staining, respectively. $O_2^-$ and $H_2O_2$ levels were not significantly different in WT and the transgenic lines before dehydration, but after 6 h of dehydration, the WT leaves showed stronger staining with either NBT or DAB than did leaves of the transgenic lines (Fig. 9A,B).

It is known that efficient scavenging of ROS overproduced during drought stress requires the action of several antioxidant enzymes [46]. We therefore measured the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in plants under the same stress conditions as those described above. The activities of all three enzymes were indistinguishable between WT and transgenic lines before dehydration, but were significantly higher in the transgenic lines than in WT plants after dehydration for 6 h (Fig. 9C–E), which was consistent with the results of the histochemical staining.

### 3.7. Overexpression of VlbZIP36 alters the expression of ABA- and stress-responsive genes in transgenic A. thaliana

The expression of various ABA- and stress-responsive genes was determined by qRT-PCR in 3-week-old WT and transgenic lines dehydrated for 1 or 2 h on dry filter paper (Fig. 10). *AtABI1*, which encodes a protein phosphatase 2C (PP2C), is a co-receptor of ABA and negatively regulate ABA signaling [48,49]. The *abi1-1* mutation is a dominant gain-of-function mutation that cancels the ABA-dependent inhibition of the protein phosphatase activity of *AtABI1* [48,49]. We found that the relative expression levels of *AtABI1* gradually increased after 1 and 2 h of dehydration stress in both WT and the transgenic lines, but that they increased by 1.8–2.4 fold in the transgenic lines compared with the WT after dehydration for 2 h. *AtERA1*, a negative regulator of ABA signaling, is a target of *AtABI1* and acts with or downstream of *AtABI1*, and its mutation results in ABA hypersensitivity with respect to seed germination and stomatal closure [50]. We observed that the expression of this gene gradually decrease by 0.56–0.63 and 0.5–0.6 fold in the transgenic lines compared with the WT after 1 and 2 h of dehydration stress treatment, respectively. *AtABI5* can be negatively regulated by *AtERA1* [50], and its constitutive expression in *A. thaliana* results in hypersensitivity to ABA [51]. Here, we found that compared with the expression in WT, the relative expression levels of *AtABI5* were increased by 2.6–3.1 and 2.3–3 fold in the transgenic lines after 1 and 2 h treatment of dehydration stress, respectively. In addition, the transcript levels of *AtNCED3*, which is involved in ABA biosynthesis during dehydration [52], increased by 1.8–2.3 fold in the transgenic lines after 1 h dehydration stress treatment compared with the WT. Interestingly, the expression of *AtNCED3* was decreased after 2 h of treatment both in WT and transgenic lines, while the transcript levels of *AtNCED3* were also higher in transgenic lines compared with that in WT. The expression of a key TF in the ABA signaling pathway, *AtABF3* [53], gradually increased

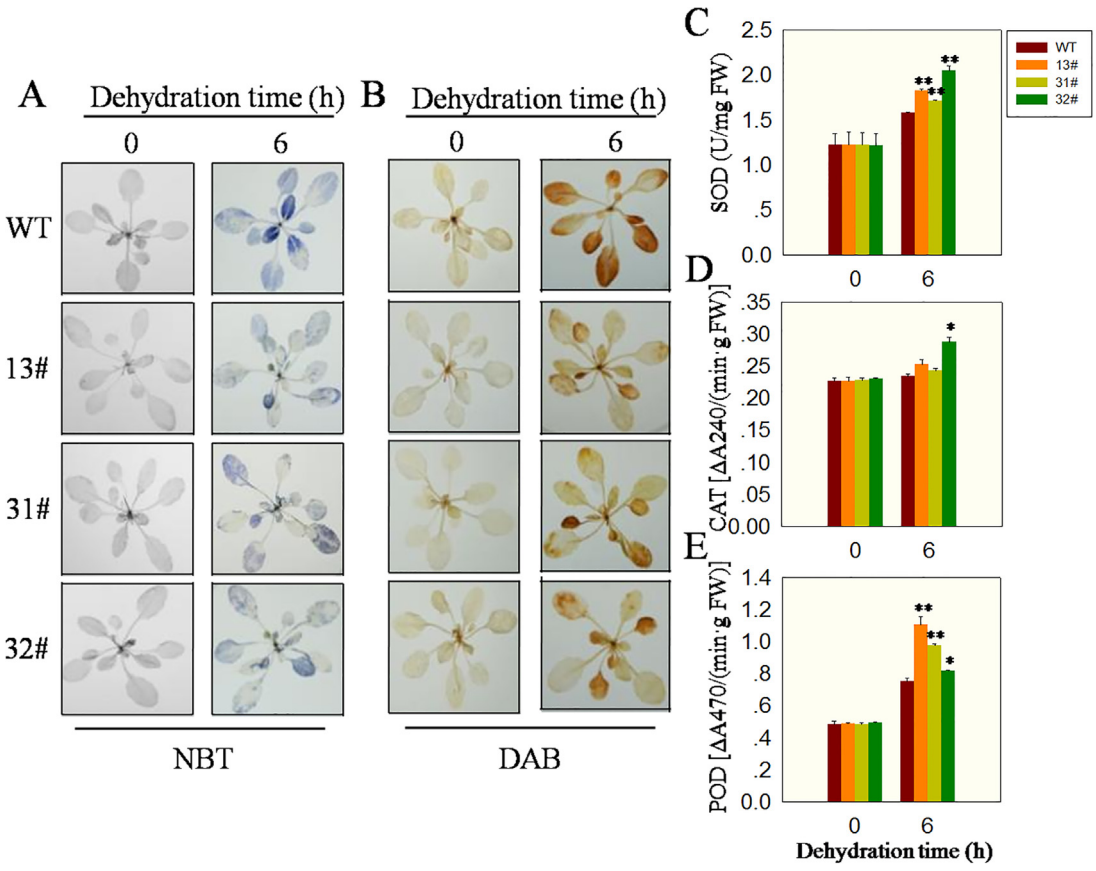


**Fig. 9.** Reactive oxygen species (ROS) levels and oxidative enzyme activities of wild type (WT) and *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* plants. (A, B) Histochemical staining assay detecting $O_2^-$ and $H_2O_2$ accumulation with nitro blue tetrazolium (NBT) (A) and diaminobenzidine (DAB) (B), respectively, in whole (except roots) 3-week-old transgenic and WT plants before and after dehydration for 6 h. The experiment was repeated three times with 5 whole plants. (C-E) Activities of superoxide dismutase (SOD) (C), catalase (CAT) (D) and peroxidase (POD) (E) in the whole (except roots) 3-week-old transgenic and WT plants before and after dehydration for 6 h. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to 1.2–1.5 and 1.6–2 fold in the transgenic lines after 1 and 2 h dehydration stress treatment, respectively, compared to the WT. In addition, we examined the expression of the guard cell ion channel gene, *AtKAT2* [54], which under normal conditions mediates $K^+$ influx, enabling stomatal opening, and is inhibited by ABA and abiotic stresses [53]. Here, we observed that its transcript abundance gradually decreased to ~0.8- and ~0.7-fold of that in the WT in the transgenic lines after 1 and 2 h of dehydration stress, respectively.

Finally, we measured the changes in expression of *AtRD29A*, *AtRD29B* [8] and *AtRAB18* [55], which are key genes in the ABA signaling pathway. Compared with the expression in the WT, the relative expression levels of all the three genes were greater in the transgenic lines after 1 and 2 h of dehydration stress. In addition, since it has been shown that expression of *ANAC103* is directly mediated by *AtbZIP60* [56], the expression of *ANAC103* in the transgenic lines was investigated by qRT-PCR. Although, as far as we know, there are no reports that *ANAC103* is involved in abiotic stress, we found that its transcript levels increased by 1.6–1.7 fold in the transgenic lines after 1 h dehydration stress treatment compared with the WT. Interestingly, while the expression levels of *ANAC103* subsequently decreased after 2 h of desiccation in both WT and transgenic lines, they were 2–2.2 fold higher in the transgenic lines compared with the WT (Supplementary Fig. 1), further suggesting that the expression of *ANAC103* is regulated by *VlbZIP36* in the transgenic plants. Taken together, we concluded that the overexpression of *VlbZIP36* in *A. thaliana* resulted in the altered expression of ABA- and stress-related genes.

## 4. Discussion

Grapevine is one of the most economically important perennial fruit crops globally, due to its diverse uses, which include 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 abiotic stresses, which cause significant losses in yield [57], the expression and function of genes regulating abiotic stress responses in grape are of considerable interest. However, the roles of only a few grapevine (*Vitis vinifera*) bZIP TFs in stress responses have been characterized [58–60]. In this current study, we demonstrated that a group K bZIP gene, *VlbZIP36*, is a component of the ABA-dependent signaling pathway involved in adaptation to dehydration stress.

The defining feature of bZIP TFs is the presence of a DNA binding domain of ~16 amino acid residues containing an invariant (N-X7-R/K) motif and a leucine zipper domain (L-X6-L-X6-L) containing a heptad repeat of leucine residues or other bulky hydrophobic amino acids positioned exactly nine amino acids, based on which they have been divided into 10 [25] or 13 [26] distinct groups in *A. thaliana* and rice (*Oryza sativa*), respectively. VlbZIP36 was classified into group K, which has only one member in grape [19,20]. This classification was supported by a phylogenetic analysis, in which VlbZIP36 clustered in the same clade as AtbZIP60 and OsbZIP74 (Fig. 1A). *AtbZIP60* [16] and *OsbZIP74* [17] have been shown to be an important regulators of the endoplasmic reticulum (ER) stress response. Previously, it has been reported that abiotic

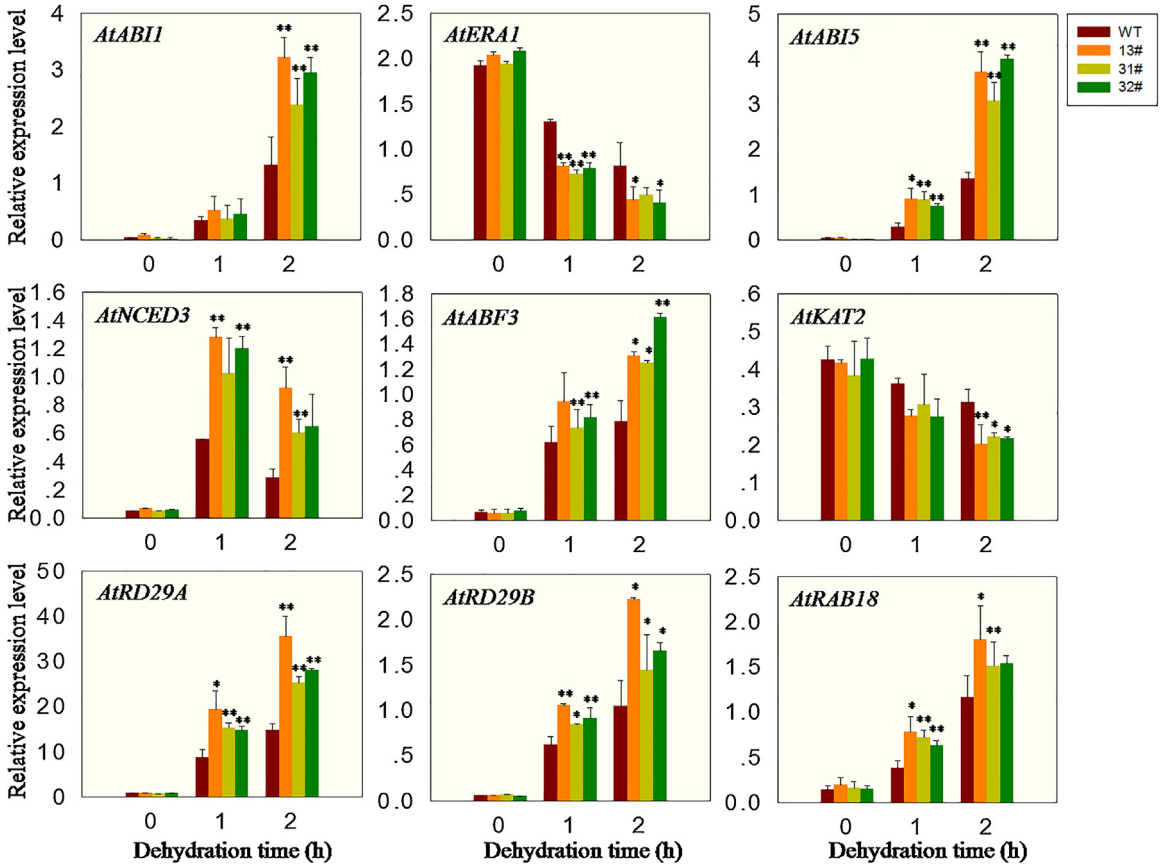


**Fig. 10.** Expression profiles of stress-marker genes in wild type (WT) and *VlbZIP36*-expressing transgenic *Arabidopsis thaliana* plants, analyzed using quantitative real-time PCR. Expression levels were based on total RNA extracted from whole (except roots) 3-week-old transgenic and WT plants that had been dehydrated on dry filter paper for 0, 1, 2 h. The *AtActin2* gene was used as an internal control. 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.

stresses responses are related to ER stress signaling. For example, loss of function in *AtBI1* resultes in hypersensitivity of the *A. thaliana* to the abiotic stresses, whereas over-expressing of *AtBI1*-modulated ER stress suppresses the plant's cell death induced by abiotic stresses [61–63]. Furthermore, Duan et al. [63] suggested that under drought stress, significantly high accumulation of $H_2O_2$ in the root tip of Arabidopsis may interrupt ER normal functions resulting in ER stress. In this current study, the ectopic expression of *VlbZIP36* in *A. thaliana* was shown to enhance drought stress tolerance, likely through the ABA signaling pathway.

Pei et al. [64] showed that ROS, which act as key signaling molecules in guard cells, are required in the ABA-induced signaling cascade. Moreover, ABA was reported to enhance the synthesis of ROS, which is known to serve as a second messenger to promote stomata closure. Excess ROS triggered by drought stress contribute to increased drought sensitivity [46,47], and maintaining low levels of ROS enhances tolerance to drought stress [65]. Here, we observed almost no difference in ROS levels between the WT and transgenic lines before dehydration, whereas after the dehydration treatment the transgenic lines exhibited higher antioxidant enzyme activities, but lower levels of ROS than did those of the WT (Fig. 9). Taken together, these results suggested that *VlbZIP36* may confer dehydration tolerance by eliminating excess ROS triggered by the drought stress, via the modulation antioxidant enzymes, thereby regulating ROS homeostasis.

We analyzed the transcriptional levels of several ABA- and stress-related genes. A large proportion of ABA induced genes (include *AtABI1*, *AtABI5*, *AtNCED3*, *AtABF3*, *AtRD29A*, *AtRD29B*, *AtRAB18*) were up-regulated by *VlbZIP36* over-expression, while the expression of *AtERA1* and *AtKAT2*, which are known to be inhibited by ABA, were down-regulated in the transgenic lines after dehydration treatment (Fig. 10). It should be noted that although *AtABI1* is the negative regulator of the early step of the ABA signaling [66], the expression of *AtABI1* was up-regulated in *AtABF3* over-expressing Arabidopsis [53]. Furthermore, Li et al. [29] suggested that the *AtABI1* can be regulated by downstream ABA signaling to maintain the ABA signal steady state. We therefore concluded that *VlbZIP36* is a transcriptional regulator in the ABA dependent signaling pathway that is activated in response to dehydration stress, and it may be a good candidate gene for improving drought tolerance in crop species.

## Author contribution

Xiping Wang and Mingxing Tu designed the study. Mingxing Tu, Tongying Feng, Xiaomeng Sun contributed to the most of experiments. Xianhang Wang performed the construction of vector, Xianhang Wang, Yaqiong Wang, Li Huang performed data analysis. Min Gao, Yuejin Wang assisted with the analysis of the results. Yuejin Wang and Xiping Wang provided guidance on the study. Mingxing Tu and Xiping Wang wrote the manuscript. All of the authors approved the ultimate manuscript.

## Conflict of interest

There are no competing interests in this paper, and the authors do not have any possible conflicts of interest.

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## 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.plantsci.2016.08.011.

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