Plant Science 287 (2019) 110202

Contents lists available at ScienceDirect

Plant Science

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

VqbZIP1 isolated from Chinese wild *Vitis quinquangularis* is involved in the ABA signaling pathway and regulates stilbene synthesis

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ARTICLE INFO

Keywords:
Chinese wild grape
bZIP transcription factor
Abscisic acid
Stilbenes

ABSTRACT

Resveratrol is an important phytoalexin in grapevine. Not only does it confer increased disease resistance and but as a food component it offers significant benefits in human health. Abscisic acid (ABA) is an important phytohormone involved in many biological processes in plants and can also promote the accumulation of stilbenes. Stilbene synthase (STS) is an important enzyme which catalyzes the last step of resveratrol synthesis. Our study characterizes a basic leucine zipper (bZIP) transcription factor, *VqbZIP1*, isolated from Chinese wild *Vitis quinquangularis* accession Danfeng-2. The results show that *VqbZIP1* encodes 299 amino acids and belongs to the Group A subfamily of the bZIP family. VqbZIP1 showed transcriptional activation activity in yeast and is predicted to be located in the nucleus. The yeast two-hybrid assay and bimolecular fluorescence complementation (BiFC) assay together show that VqbZIP1 interacts with VqSnRK2.4 and VqSnRK2.6. *VqbZIP1*, the *STS* genes, *VqSnRK2.4* and *VqSnRK2.6* can all be induced by ABA treatment. A GUS activity experiment indicates *VqbZIP1* can activate the GUS reporter gene driven by STS promoters. Further studies show that co-expression of *VqbZIP1* with *VqSnRK2.4* or *VqSnRK2.6* can confer higher efficiency than expression of *VqbZIP1* alone in activating the STS promoters. Overexpression of *VqbZIP1* in grape leaves promoted the transcript level of the *STS* genes and the accumulation of stilbenes. Overexpression of *VqbZIP1* in Arabidopsis thaliana can confer ABA sensitivity. In summary, our results suggest *VqbZIP1* participates in the ABA signaling pathway and regulates stilbene synthesis.

1. Introduction

Grapevine is an important fruit crop in many countries both because of its economic contributions and also because of it benefit to human health. Data from the Food and Agriculture Organization (FAO) show that in 2017, world production of grapes was 74 276 583 t. Compared with other perennial fruit crops, it is ranked the third, after bananas (113 918 763 t) and apples (83 139 326 t). In recent years, grapevines have drawn public attention for the content of one of its secondary metabolites, resveratrol. This has been shown not only to protect plants from biotic and abiotic stresses but also to act as a cardioprotective, antitumor and neuroprotective agent when included as part of the human diet [1–5]. Resveratrol was first isolated from *Veratrum grandiflorum*, and then detected in the roots of *Polygonum cuspidatum* [6,7]. Until now, it has been identified in at least 12 families, 31 genera, and 72 species [1]. The biosynthesis of resveratrol is catalyzed by STS via the phenylalanine synthetic pathway. STSs compete with chalcone synthases (CHSs) for the same substrate and catalyze three malonyl-CoA and one p-coumaroyl-CoA to form resveratrol [8–10]. Resveratrol is the basic unite of stilbenes and its derivatives such as piceid and viniferin also have different biological and health protection functions. As important phytoalexin, the biosynthesis of resveratrol is induced by a variety of biotic and abiotic, such as powdery mildew infection [5], wonding [11], UV-C irradiation [12] and application of different hormones like ABA [13,14].

Stilbene synthase, as the key enzyme in the biosynthesis of resveratrol, belongs to the type III polyketide synthase family. It was first isolated in peanuts in 1984 [15]. Until now, *STS* genes have been identified in variety of plants such as peanut, scot pine and grapevine. Among them, grape is the only fruit tree that has completed genome

Abbreviations: ABA, abscisic acid; STS, stilbene synthase; bZIP, basic leucine zipper; BiFC, bimolecular fluorescence complementation; Y2H, yeast two-hybrid assay; PYR, pyrabactin resistance; PP2C, type 2C protein phosphatase; SnRK2, SNF1-related protein kinase 2; SA, salicylic acid; Eth, ethylene; MeJA, jasmonic acid methyl ester; GFP, green fluorescent protein; YFP, yellow fluorescent protein; PEG, poly-ethylene glycol; AbA, aureobasidin A; PCC, Pearson's correlation coefficient

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https://doi.org/10.1016/j.plantsci.2019.110202
Received 14 April 2019; Received in revised form 25 June 2019; Accepted 23 July 2019
Available online 26 July 2019
0168-9452/

sequencing. Along with the publication of the grape genome, 48 *VvSTS* gene sequences were identified from the PN40024 genome [16–18]. Cluster analysis divides the 33 *STS* genes which contain complete coding sequences (CDS) to three groups, group A, group B and group C. All the six *STS* genes belonging to group A are located on chr10 and members of group B and group C are located on chr16 [18]. Different stress treatments, such as fungal infection, wounding and UV−C treatment, lead to the expression of *STS* genes. In recent years, a number of transcription factors regulating *STS* genes expression and accumulation of stilbenes have been reported. In the first report, MYB14 and MYB15 were demonstrated to participate in regulating stilbene synthesis and in the following year, MYB14 was reported to bind directly to the promoter of *STS* [19,20]. Next, Wong et al. (2017) reported that, other transcription factor families such as WRKY, ERF, bZIP are probably involved in regulating the *STS* genes [21]. Later, MYB13, WRKY03, WRKY43 and WRKY53 were reported to participate in regulating *STS* genes expression [22,23]. VvWRKY8, which is the same as WRKY03 was reported to interact directly with VvMYB14 to repress the *STS* genes [24]. In addition, overexpression of *VvABF2* can increase the accumulation of resveratrol and piceid in grape cells [25]. However, improving the transcriptional regulatory network of stilbenes requires further research.

The hormone ABA plays a number of significant roles in plants and the mechanisms of ABA signaling transduction is well studied. During ABA signal transduction, when endogenous ABA is up-regulated, the PYR/PYL/RCAR proteins bind to ABA and interact with PP2Cs, releasing SnRK2s from a phosphorylation state by PP2Cs. The SnRK2 proteins can then phosphatase and activate the downstream transcription factors, especially the bZIP type transcription factors [26–30]. Studies before found that exogenous ABA can increase the accumulation of resveratrol in grape berries. But, the regulation mechanism of stilbenes in response to ABA signaling is still unclear.

The bZIP transcription factor family is present in many animals, yeasts and plants [31]. The bZIP proteins contain a conserved bZIP domain which consists of a basic region and a leucine zipper motif. The bZIP domain has the function of recognizing the ACGT core motif [32]. Currently, 75 bZIP members have been identified in *Arabidopsis* [31], 89 in rice [33] and 47 in grapevine [34]. In *Arabidopsis*, bZIP family members are divided into 10 groups (groups A–S). Members of group A have been demonstrated to play roles in the ABA signaling pathway. For example, *ABI5* participates in early seed development regulated by ABA [35,36]. *AREB/ABF/ABI5* are the main target genes for SnRK2 proteins which are a core component in the ABA signaling pathway [37,38]. In grape, *VvABF2* is thought to participate in the ABA signaling pathway and to affect grape berry ripening [25]. Overexpression of *VqbZIP39* in Arabidopsis promotes tolerance to multiple abiotic stresses by the ABA pathway [39]. *VlbZIP36* responds to drought and ABA signaling and increases plant tolerance to drought stress by regulating ABA and stress-related genes [40]. *VlbZIP30* promotes plant tolerance to drought by regulating genes involved in ABA signaling pathways [41].

China as one of the centers of origin of grapevine possess a range of wild resources. The Chinese wild *V. quinquangularis* accession Danfeng-2 has been shown contain high levels of resveratrol compared with other species [42]. Later, proteomic sequencing was carried out and analyzed using *V. quinquangularis* accession Danfeng-2 and *Vitis vinifera* L. cv. Cabernet Sauvignon to determine why there is such a high level of resveratrol in the ripe berries of Danfeng-2 [43]. Meanwhile, our group has conducted transcriptome sequencing of four different berry development stages in these two grape cultivars. When we conducted co-expression analysis using data from transcriptome, a bZIP type transcription factor was identified co-expressed with the *STS* genes. In this study, we report the function of *VqbZIP1*, which participates in the ABA signaling pathway and regulates *STS* genes expression and stilbene synthesis.

2. Materials and methods

2.1. Plant materials

Tissues include stems, leaves, inflorescences, berries and tendrils of Danfeng-2 were collected during 2017 from the grape Germplasm repository of Northwest A & F University, Yangling, Shaanxi, China (34°20′N, 108°24′E). Plants of tobaccos (*Nicotiana benthamiana*) were grown in the growth chamber at 25 °C and a photoperiod of 16 h. *Arabidopsis* Columbia wild type were grown in a growth chamber for transgenic *Arabidopsis*.

2.2. Cloning of VqbZIP1 and sequence analysis

VqbZIP1 was isolated from the cDNA of Danfeng-2 berries using primers VqbZIP1-F and VqbZIP1-R. The coding sequence (CDS) of *VqbZIP1* was submitted to GenBank (accession NO. AXN75965.1). The nucleotide sequence was used for a Blat-Search in the Grape Genome Browser to predict the location of *VqbZIP1*. DNAMAN software was used for amino-acid sequence alignment with bZIP proteins in different species. A phylogenetic tree was built with MEGA 5.0 software. The promoters of *VqbZIP1* and *STS* genes were cloned from the genomic DNA of Danfeng-2. Homologous cloning was used to obtain the promoter of *VqbZIP1* and *VqSTSs*. The online analysis tool Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to analyze the cis-elements in the promoters.

2.3. Powdery mildew inoculation and various treatments

The method used for inoculation of powdery mildew was as described previously [44]. Conidia of *U. necator* were resuspended in sterilized solution with 0.78% glucose and sprayed on leaves. Samples were collected after inoculation at 0, 12, 24, 48, 72, 96 and 120 h. Leaves of Dangfeng-2 were treated with 100 μM ABA and the samples were collected after 0, 0.5, 1, 2, 6, 12 and 24 h of treatment. Other hormones including 100 μM salicylic acid (SA), 100 μM ethylene (Eth), 100 μM jasmonic acid methyl ester (MeJA) and signaling molecules including 5 mM $CaCl_2$ and 1% H_2O_2 were sprayed on to Danfeng-2 leaves and samples were collected after 0, 0.5, 1, 2, 6 and 10 h of treatment.

2.4. Subcellular localization

To demonstrate subcellular localization of VqbZIP1, VqSnRK2.4 and VqSnRK2.6, the CDSs without stop codon of these three genes were amplified from the cDNA of Danfeng-2 and introduced into pCAMBIA2300-35S-GFP to generate 35S-VqbZIP1-GFP, 35S-VqSnRK2.4-GFP and 35S-VqSnRK2.6-GFP fusion vectors. At the same time, AtHY5 (AT5G11260), which had been reported as a nuclear location protein, was cloned from the cDNA of Arabidopsis and inserted into the pCAMBIA2300-mCherry vector to generate 35S-AtHY5-mCherry fusion vector. The fusion vectors 35S-VqbZIP1-GFP, 35S-VqSnRK2.4-GFP, 35S-VqSnRK2.6-GFP and 35S-AtHY5-mCherry were generated to a concentration of 2000 ng/μl using the Plasmid Mini Kit (OMEGA). Then the fusion vectors 35S-VqbZIP1-GFP, 35S-VqSnRK2.4-GFP and 35S-VqSnRK2.6-GFP were seperately co-transformed with 35S-AtHY5-mCherry into protoplasts of Arabidopsis using the PEG-mediated method [45]. Co-transformation of 35S-GFP and 35S-AtHY5-mCherry was used as control. The GFP and mCherry signals were observed after transformation for 20 h with a confocal laser microscopy (LSM 510, Zeiss, Oberkochen, Germany). In order to distinguish from the red fluorescence of mCherry, we changed the red chlorophyll signal to blue using the LAS-AF-Lite software. Primers used here were listed in the Supplementary Table S1.

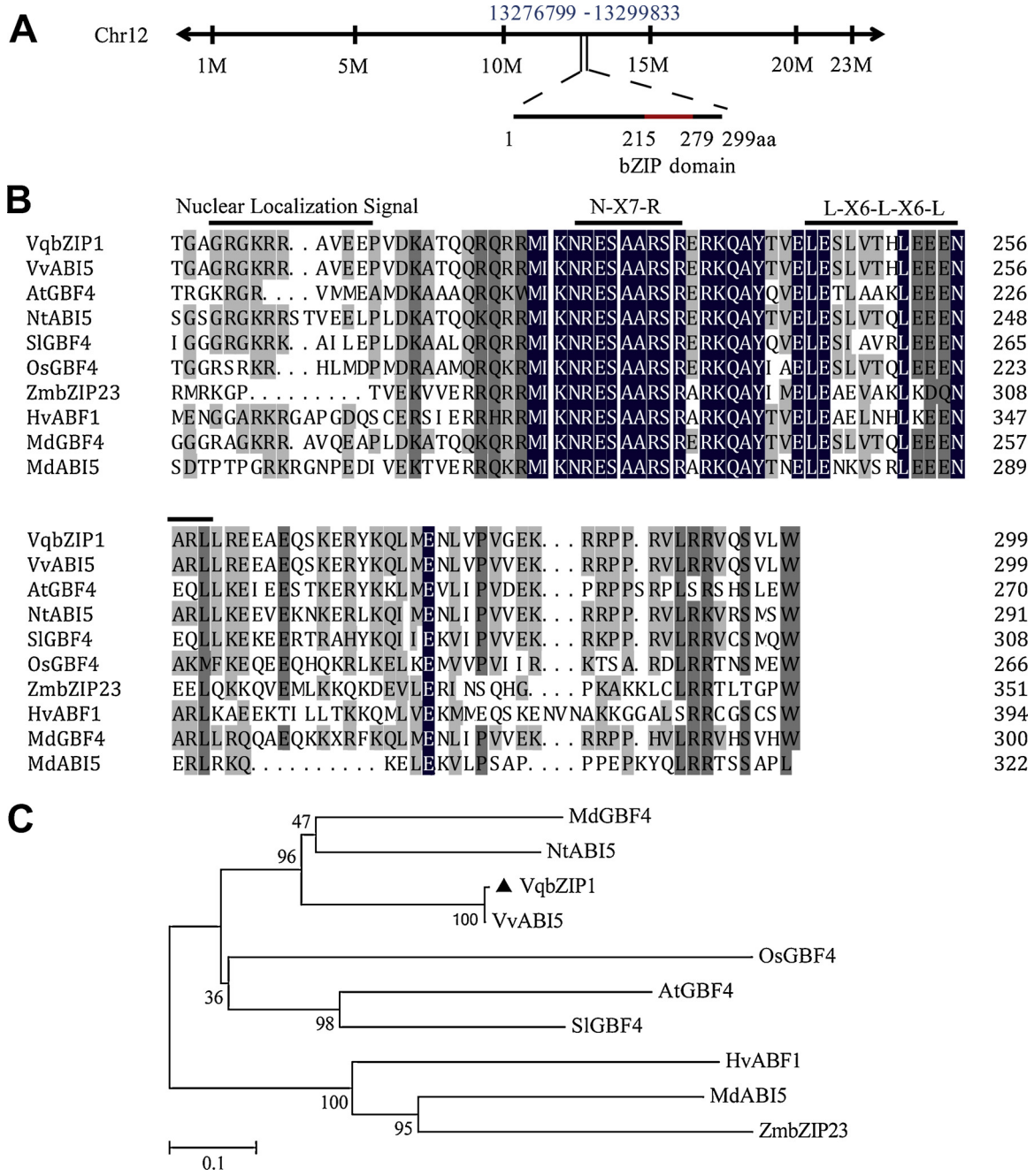

Fig. 1. Sequence analysis of the bZIP transcription factor *VqbZIP1* from Chinese wild *Vitis quinquangularis*. (A) Chromosomal location of *VqbZIP1*. *VqbZIP1* was located on chromosome 12, the bZIP domain includes 65 amino acid from 215 to 279aa. (B) Alignment of VqbZIP1 with its homologous genes in different species. The conserved bZIP domain is shown (black line). The accession numbers of genes used here are listed below: VvABI5 (XP_002266344.1), AtGBF4 (BAF00453.1), NtABI5 (XP_016498628.1), SlGBF4 (XP_004237796.1), OsGBF4 (XP_015612904.1), ZmbZIP23 (XP_008659130.1), MdABI5(NP_001280812.1), MdGBF4 (XP_008365275.1), HvABF1 (ABH05131.1). (C) Cluster analysis of VqbZIP1 with its homologs. VqbZIP1 is highlighted (black disk).

2.5. Yeast two-hybrid assay (Y2H assay)

The yeast two-hybrid assay was carried out using the yeast system from Clontech. The online website STRING was used to predict protein-protein interaction. The CDS of *VqbZIP1* was inserted into the vector pGADT7 and the CDSs of *VqSnRK2.4* and *VqSnRK2.6* were inserted into the vector pGBKT7. The fusion vector pGBKT7-VqSnRK2.4-BD and pGBKT7-VqSnRK2.6-BD were separately co-transformed with pGADT7-VqbZIP1-AD into Y2H gold strain. At the same time, co-transformation of pGADT7-T with, respectively, pGBKT7-p53 and pGBKT7-lam were used as positive and negative controls. The Y2H train harboring different vector combinations was diluted with water and dropped on to the SD/-Trp/-Leu/-Ade/-His with 200 ng/ml AbA and 40 μg/ml X-α-Gal media at 28℃ for three days.

2.6. BiFC assay

To confirm the interaction between VqbZIP1 and VqSnRK2.4 or VqSnRK2.6, the BiFC assay was carried out. The full-length CDSs of *VqSnRK2.4* and *VqSnRK2.6* were inserted into the pSPYNE vector to generate pSPYNE-VqSnRK2.4 and pSPYNE-VqSnRK2.6 fusion vectors. The coding sequence of *VqbZIP1* without termination codon were cloned into pSPYCE vector to generate pSPYCE-VqbZIP1 fusion vector. The fusion vector pSPYCE-VqbZIP1 was seperately co-transformed with

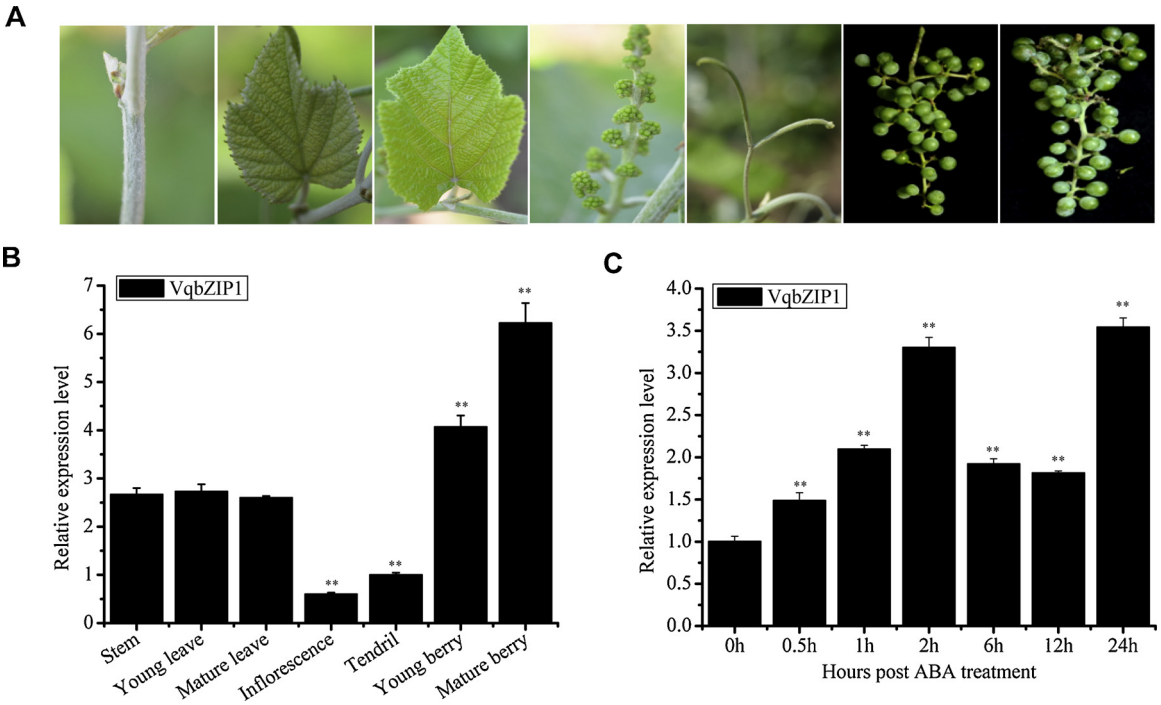

Fig. 2. VqbZIP1 expression analysis in various tissues and following ABA treatment. (A) Stems, young leaves, mature leaves, inflorescences, tendrils, young berries and mature berries from Chinese wild *Vitis quinquangularis* accession Danfeng-2. (B) qRT-PCR analysis of the transcript level of *VqbZIP1* in various organs of Danfeng-2. (C) Expression analysis of *VqbZIP1* in response to ABA treatment. Grapevine *VvGAPDH* was used as internal standard. The significance was analyzed using *Tukey* test (*P < 0.05; **P < 0.01) with SPSS. Error bars indicate the SD from three biological replicates.

pSPYNE-VqSnRK2.4 and pSPYNE-VqSnRK2.6 into protoplasts of *Arabidopsis* using the PEG-mediated method [45]. Different combinations including pSPYCE-VqbZIP1 with pSPYNE empty vector, pSPYNE-VqSnRK2.4 with pSPYCE, pSPYNE-VqSnRK2.6 with pSPYCE were co-transformed protoplasts of *Arabidopsis* as negative control. The YFP signal was observed after transformation for 20 h with a confocal laser microscopy (LSM 510, Zeiss, Oberkochen, Germany). Here, the chlorophyll signal was red. Primers used here were listed in the Supplementary Table S1.

2.7. Transient overexpression experiments in grapevine

An agrobacterium strain harboring the vector 35S-VqbZIP1-GFP was cultured in LB liquid medium and the OD600 was adjusted to 0.6 with MES resuspension solution [46]. Leaves of Danfeng-2 used for transient transformation were collected from the grape germplasm repository the same day. A vacuum infiltration method was used as described previously [46]. After treatment, leaves were placed in the incubator (at 25 °C with a 16 h photoperiod) for three days and then collected for use.

2.8. GUS activity measurement

The promoter of *STS* genes (*STS6*, *STS16* and *STS20*) was inserted into the pC0380-GUS vector. The fusion vectors P_{STS}-GUS were transformed into the GV3101 strain and pC0380-GUS empty vector was transformed into GV3101 as negative control. The OD_{600} of GV3101 was adjusted to 0.6 and infiltrated into tobacco leaves. The tobacco was cultured in incubator at 25°C with a 16 h photoperiod for three days. The GUS activity was detected using the method previously reported [47]. An Infinite 200 PRO Microplate Reader (TECAN, Switzerland) was used to detect the GUS activity. The BSA and 4-MU (methyl umbelliferone) were used as the standard. Three independent experiments were carried out in this experiment and the error bars were calculated from three biological replicates.

2.9. Arabidopsis transformation

The ORF of *VqbZIP1* was constructed into the pCAMBIA2300 vector. The agrobacterium strain GV3101 harboring the pCAMBIA2300-35S-VqbZIP1-GFP was grown in a liquid Luria broth (LB) medium with 50 mg/mL kanamycin and 60 mg/mL gentamycin [48]. The bacterial suspension was centrifuged at 5000 rpm for 5 min to form a pellet. The bacteria were then suspended in MES re-suspension medium to OD_{600} nm 0.4. The resuspended bacterial suspension was used to infect the flowers of Arabidopsis [49]. The T0 seeds were screened on MS agar medium 50 mg/mL kanamycin. The T3 homozygous lines 5#, 8# and 16# with highly-expressed *VqbZIP1* genes, were chosen for the subsequent experiment.

2.10. Quantitative real-time PCR (qRT-PCR)

The Omega Plant RNA Kit (Omega, Norcross, Georgia) was used for RNA extraction. The FastKing RT Kit (TIANGEN, Beijing, China) was used for cDNA first-stand synthesis [50]. SYBR Premix Ex TaqTM (Bioer Technology, Hangzhou, China) and the Bio-Rad IQ5 RT-PCR system (Bio-Rad Laboratories, Hercules, CA, USA) was used for qRT-PCR. The volume used for qRT-PCR was 20 µl including 0.8 µl primer-F, 0.8 µl primer-R, 1 µl sample cDNA, 10 µl SYBR and 7.4 µl H2O. *VvGAPDH* (GenBank accession no. GR883080) and *AtActin* (AT3G18780) were used as reference genes [50]. Three independent experiments were carried out in these experiments and the error bars were calculated from three biological replicates. The $2^{-\triangle\triangle c(t)}$ method was used to calculate the relative expression of target genes [51]. Primers used here are listed in the Table S1.

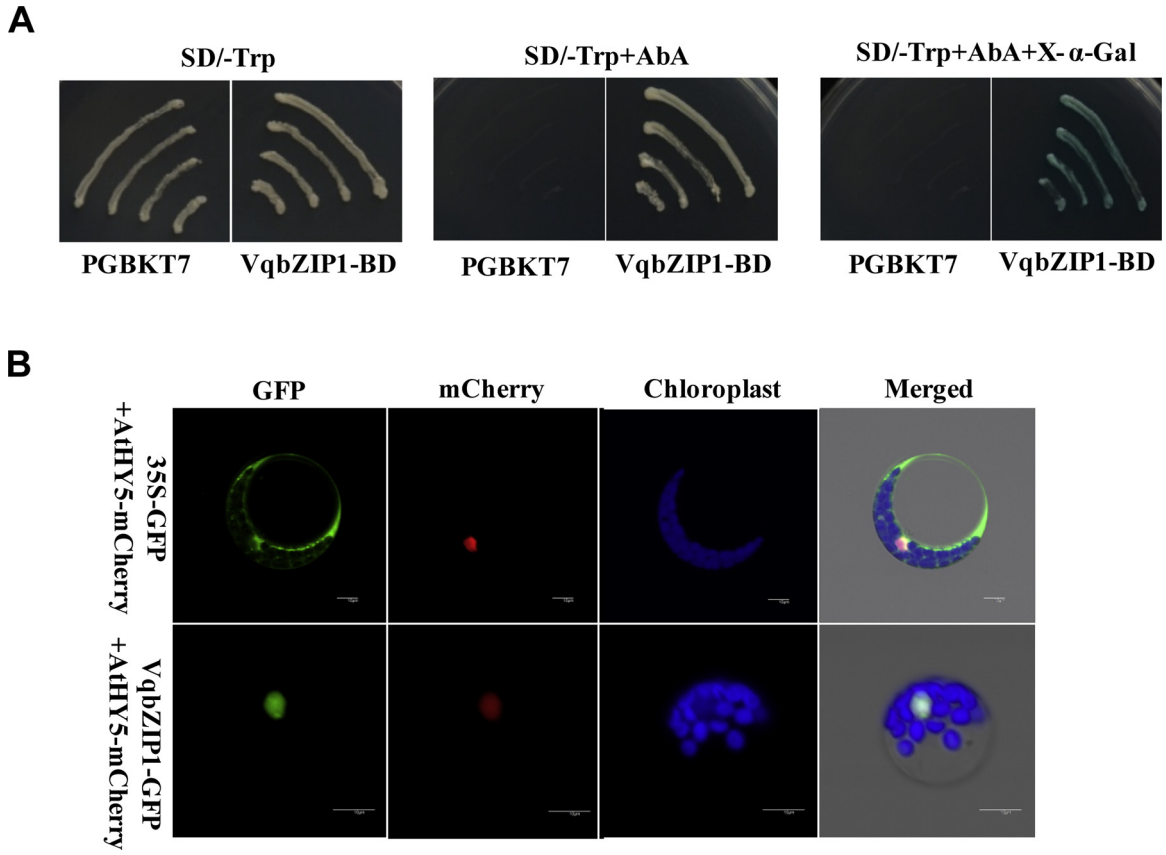

Fig. 3. Transactivation analysis and subcellular localization of VqbZIP1. (A) Transactivation experiment with VqbZIP1 in yeast. The pGBKT7 empty vector was used as control. The vector pGBKT7-VqbZIP1 and pGBKT7 empty vector were separately transformed into the Y2H yeast strain. The transformed yeast was streaked on SD/-Trp, SD/-Trp + AbA and SD/-Trp + AbA + X-α-Gal plate and cultured at 28°C for 3 days. (B) Subcellular localization of VqbZIP1in Arabidopsis thaliana protoplasts. The GFP signal was observed using confocal laser microscopy. The pCAMBIA2300 empty vector was used as control. The A. thaliana nuclear protein AtHY5 combined with mCherry were used as marker genes. Here the chlorophyll signal was marked in blue. Bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3. Results

3.1. Identification a bZIP transcription factor VqbZIP1 co-expressed with STS genes

Co-expression analysis was carried out using data from our transcriptome of four different stages of berry development in Danfeng-2 and Cabernet Sauvignon. The Pearson's correlation coefficient (PCC) value which is a linear correlation coefficient that reflects the degree of linear correlation between two variables was used to analyze the co-expression relationship [52]. A bZIP type transcription factor *VqbZIP1* (GenBank accession NO. AXN75965.1) co-expressed with *STS* genes (Fig. S1 A). Among these, three *STS* genes, STS6, STS16 and STS20 showed high PCC values (> 0.9) with *VqbZIP1*. Cluster analysis of the co-expressed *STS* genes was carried out and we found they were distributed among three STS subfamilies, Group A, Group B and Group C (Fig. S1 B).

The coding sequence of *VqbZIP1* was isolated from the cDNA of Danfeng-2. According to the blat-search in the grape genome, *VqbZIP1* was predicted to be located on chromosome 12. The CDS of *VqbZIP1* contained 900 bp and encoded 299 amino acids with a bZIP domain (residues 215–279 aa) (Fig. 1A, B). Using NCBI blast analysis, VqbZIP1 shared 99% amino acid identity with a predicted protein VvABI5 of the Pinot noir genotype, 52% with MdGBF4 and 48% with NtABI5 (Fig. 1C). At the same time, cluster analysis of the *VqbZIP1* gene with the Arabidopsis bZIP family and grape bZIP family indicated that *VqbZIP1* belongs to the Group A subfamily (Fig. S2).

The 1000 bp promoter fragment of *VqbZIP1* was cloned from gDNA of Danfeng-2. Cis-elements in the promoter were analyzed using PlantCARE. There was one ABRE element (abscisic acid responsive), two G-box, two Box 4, one I-box and two GA-motifs (light responsiveness), one CGTCA-motif (MeJA-responsive), one HSE (heat stress responsive), one TC-rich (defense and stress responsive) and one TCA-element (salicylic acid responsive) (Fig. S3).

3.2. VqbZIP1 was expressed in different organs and responded to ABA treatments

To know more about the expression of *VqbZIP1* in grape, qRT-PCR was carried out in different organs of Danfeng-2 including stems, leaves, inflorescences, tendrils and berries (Fig. 2A). The results show that *VqbZIP1* was expressed in all the organs tested with the highest transcript level in the mature berries (Fig. 2B). The differential expression of *VqbZIP1* between young and mature fruits suggests that it may respond to hormone induction, such as ABA. To further analysis, leaves of Danfeng-2 were sprayed with 100 μM ABA. When treated with ABA, *VqbZIP1* responded quickly at 0.5 h and reach a peak at 2 h with a 3.3 folds increase. After treatment for 24 h, the transcript level of *VqbZIP1* reach another peak (Fig. 2C).

To further analyze the expression of *VqbZIP1* under different treatments, leaves of Danfeng-2 were infected with powdery mildew and treated with hormones of 100 μM MeJA, SA, Eth and signaling molecules including 5 mM $CaCl_2$ and 1% H_2O_2. We found *VqbZIP1* responded to powdery mildew infection and the transcript level reached the highest at 48 hpi (Fig. S4 A). After treatment with MeJA, the transcript level of *VqbZIP1* increased at 1 h and reached a maximum at

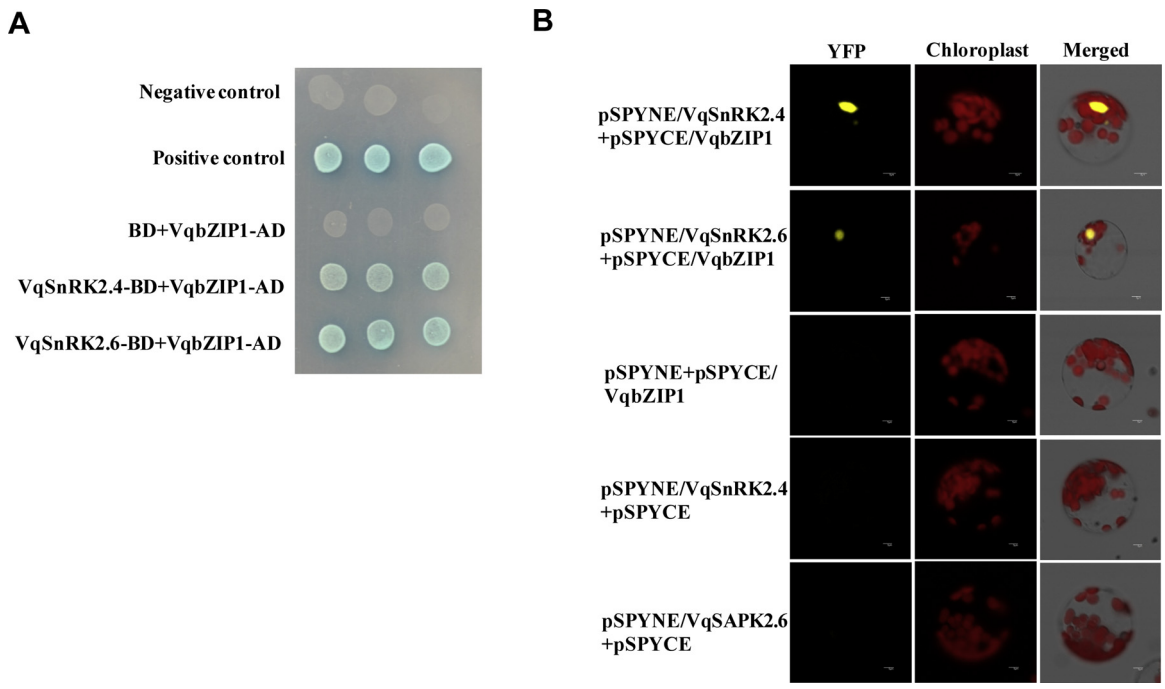

Fig. 4. Interaction between VqbZIP1 and VqSnRK2.4, VqSnRK2.6 in yeast and Arabidopsis protoplasts. (A) Yeast two-hybrid assay used to demonstrate interaction between VqbZIP1 and VqSnRK2.4, VqSnRK2.6. Yeast Y2H strain harboring pGADT7-T and pGBKT7-Lam was used as negative control, with pGADT7-T and pGBKT7-p53 as positive control. Yeast Y2H strain carrying pGADT7-VqbZIP1 and pGBKT7-VqSnRK2.4 or pGBKT7-VqSnRK2.6 were cultured on medium SD/–Trp/–Leu/–Ade/–His + AbA + X-α-Gal at 28 ℃ for three days. (B) VqbZIP1 interacts with VqSnRK2.4 and VqSnRK2.6 in the BiFC assay. Different plasmid combinations (chosen from plasmid pSPYNE/VqSnRK2.4, pSPYNE/VqSnRK2.6, pSPYNE, pSPYCE/VqbZIP1, pSPYCE) were co-transformed into Arabidopsis protoplasts using the PEG-method. The YFP fluorescence was observed with confocal laser microscopy after transformation for 20 h. Here the chlorophyll signal was marked in red. Bars = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2 h (Fig. S4 B). *VqbZIP1* showed a slight response to SA treatment (Fig. S4 C). The expression of *VqbZIP1* increased at 6 h after Eth application (Fig. S4 D). The expression of *VqbZIP1* was also induced by $CaCl_2$ and H_2O_2 signaling (Fig. S4 E, F).

3.3. VqbZIP1 functions as a transcriptional activator and is located in the nucleus

The transcription activation assay was carried out in yeast. The fusion vector pGBKT7-VqbZIP1-BD was transformed into the Y2H yeast strain. We found that the yeast strain harboring VqbZIP1 grew on the SD/-Trp and SD/-Trp + AbA media, but the negative control harboring pGBKT7 empty vector grew only on the SD/-Trp medium (Fig. 3A). So, VqbZIP1 had transcription activation function in yeast.

To confirm the subcellular location of VqbZIP1, the CDS sequence of *VqbZIP1* was inserted into the pCAMBIA2300-GFP vector to generate pCAMBIA2300-35S-VqbZIP1-GFP vector which could be driven by CaMV 35S promoter and fused with GFP tag at the C-terminus. The empty pCAMBIA2300-GFP vector was used as control. At the same time, *AtHY5* was chosen as a nuclear localization marker gene to co-transform with *VqbZIP1*. The different plasmids were transformed into *Arabidopsis* protoplasts. The result showed that, as with the marker gene *AtHY5*, VqbZIP1 was also located in the nucleus (Fig. 3B).

3.4. VqbZIP1 interacts with VqSnRK2.4 and VqSnRK2.6

Sequence analysis shows that VqbZIP1 is an ABI5-like protein, which had been reported to participate in the ABA signaling pathway. To further study the function of *VqbZIP1*, the online analysis tool STRING was used to find the interaction proteins of this gene. Seven genes were predicted to interact with VqbZIP1 (Table S2). The Y2H assay was first carried out to verify the protein interaction. We found that among the predicted proteins, two SnRK2 kinases, SnRK2.4 (VIT_07s0031g03210) and SnRK2.6 (VIT_03s0063g01080) interacted with VqbZIP1 in yeast (Fig. 4A). The interaction was further confirmed by the BiFC assay. Compared with the control group, the YFP fluorescence could be observed only at co-expression pSPYCE/VqbZIP1 and pSPYNE/VqSnRK2.4 or pSPYNE/VqSnRK2.6 in *Arabidopsis* protoplasts (Figs. 4B, S5). These two results both confirmed the interaction between VqbZIP1 and VqSnRK2.4, VqSnRK2.6.

3.5. Sequence analysis and subcellular location of VqSnRK2.4 and VqSnRK2.6

To learn more about *VqSnRK2.4* and *VqSnRK2.6*, sequence analysis was conducted. The coding sequences of *VqSnRK2.4* and *VqSnRK2.6* were 1068 bp and 1092 bp, respectively. The two genes encoded 355 and 363 amino acids, respectively. Using blast in the Grape Genome Brower, *VqSnRK2.4* and *VqSnRK2.6* were predicted to be located on chromosomes 7 and 3 (Fig. 5A). The SnRK2 family is divided into three subclasses (I, II and III). Multiple sequence alignment and cluster analysis was carried out using the amino acid sequences of the two VqSnRK2 proteins, all of the six VvSnRK2 proteins and ten AtSnRK2 proteins. *VqSnRK2.4* was classified into subclass I and *VqSnRK2.6* into subclass III (Fig. 5B, C). To determine the subcellular locations of VqSnRK2.4 and VqSnRK2.6, the CDSs of *VqSnRK2.4* and *VqSnRK2.6* were inserted into the pCAMBIA2300 vector to generate pCAMBIA2300-35S-VqSnRK2.4-GFP and pCAMBIA2300-35S-VqSnRK2.6-GFP fusion vectors. The empty vector pCAMBIA2300 was transformed as control. When observed under the laser scanning confocal microscope, we found both VqSnRK2.4 and VqSnRK2.6 were in the membrane, cytoplasm and nucleus (Fig. 5D).

3.6. Expression profiles of VqSTSs and VqSnRK2s under ABA treatment

We further investigated the expression profiles of *VqSTS6*, *VqSTS16*,

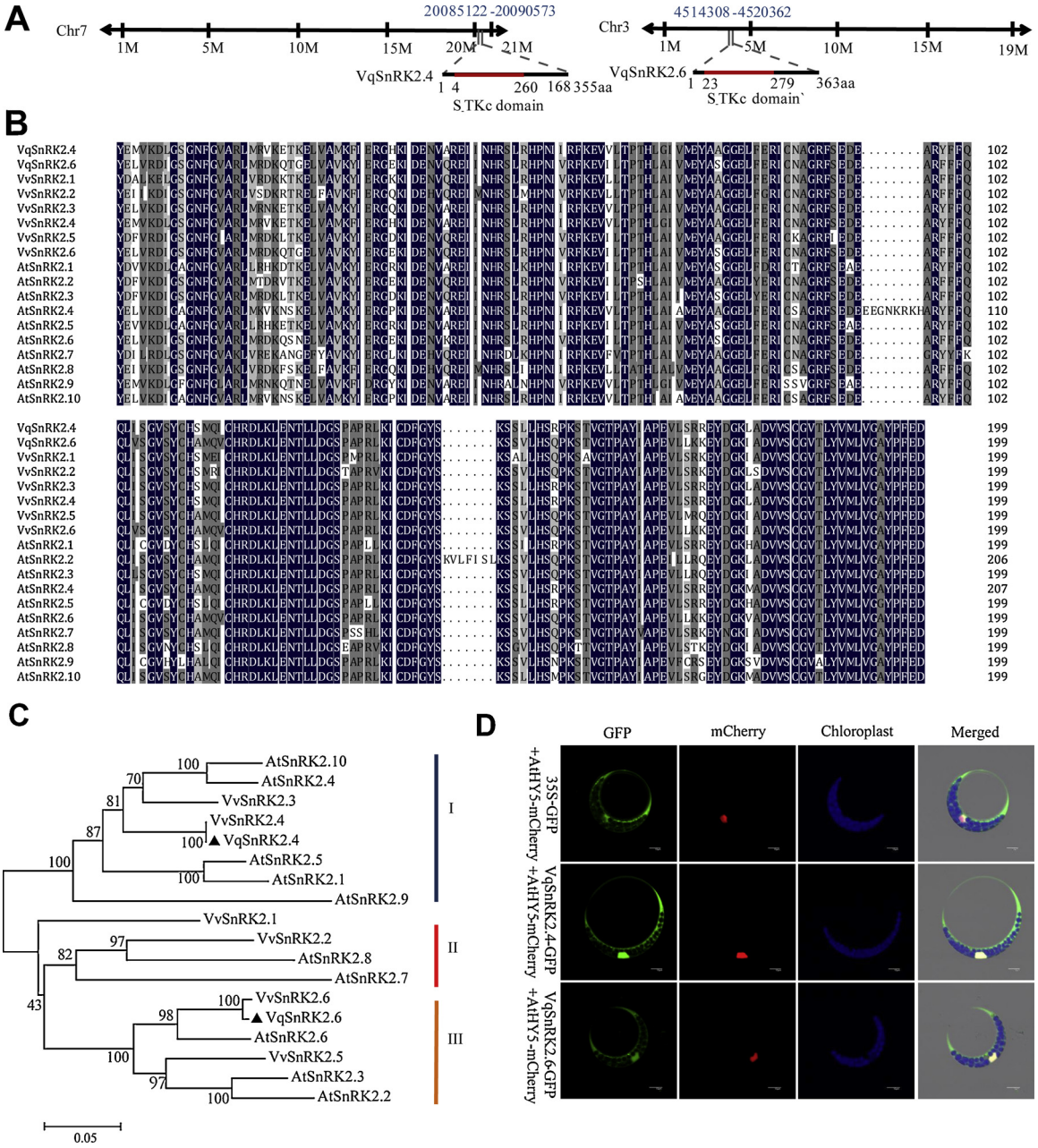

Fig. 5. Sequences analysis and subcellular localization of VqSnRK2.4 and VqSnRK2.6. (A) Chromosomal location of *VqSnRK2.4* and *VqSnRK2.6*. *VqSnRK2.4* was located on chromosome 7 with a S-TKc conserved domain from 4 to 260 aa. *VqSnRK2.6* was located on chromosome 3 with a S-TKc conserved domain from 23 to 279 aa. (B) Alignment of VqSnRK2.4 and VqSnRK2.6 with SnRK2 members from *Vitis vinifera* and *Arabidopsis thaliana*. The accession number of genes used here are listed below: VvSnRK2.1 (XM_002262690.3); VvRnRK2.2 (XM_003634430.3); VvSnRK2.3 (XM_002269185.3); VvSnRK2.4 (XM_002267886.2); VvSnRK2.5 (XM_002264136.3); VvSnRK2.6 (XM_002284923.4); AtSnRK2.1 (AT5G08590); AtSnRK2.2 (AT3G50500); AtSnRK2.3 (AT5G66880); AtSnRK2.4 (AT1G10940); AtSnRK2.5 (AT5G63650); AtSnRK2.6 (AT4G33950); AtSnRK2.7 (AT4G40010); AtSnRK2.8 (AT1G78290); AtSnRK2.9 (AT2G23030); AtSnRK2.10 (AT1G60940). (C) Cluster analysis of VqSnRK2.4 and VqSnRK2.6 with SnRK2 family of *Vitis vinifera* and *Arabidopsis thaliana*. MEGA5.0 was used to construct the tree. (D) The fusion plasmids 35S-VqSnRK2.4-GFP and 35S-VqSnRK2.6-GFP were transformed into Arabidopsis protoplasts. The GFP signal was observed using confocal laser microscopy after transfomation for three days. The 35S-GFP empty vector was used as positive control. Here the chlorophyll signal was marked in blue. Bars = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

VqSTS20, *VqSnRK2.4* and *VqSnRK2.6* in grapevine after ABA treatment. The results showed that the three *STS* genes all respond to ABA treatment. The expression of *VqSTS6* reached 3.8 folds after ABA treatment for 0.5 h, then declined to a low level. The transcript level of *VqSTS6* reached another peak at 24 h (Fig. 6A). The expression of *VqSTS16* and *VqSTS20* genes respectively significantly up-regulated at 12 h and 24 h after ABA treatment and reached 3.3 folds and 7.4 folds at 24 h (Fig. 6B,C). At the same time, we detected the expressions of *VqSnRK2.4* and *VqSnRK2.6* after ABA treatment. The result show that *VqSnRK2.4* responded to ABA after treatment for 2 h and increased slowly over the next few hours. After treatment for 6 h, the expression level of *VqSnRK2.4* reached 2.8-fold. Compared with *VqSnRK2.4*, *VqSnRK2.6* responded to ABA treatment more quickly at 0.5 h. After treatment for 6 h, the expression level of *VqSnRK2.6* also reached 2.8-fold (Fig. 6D, E).

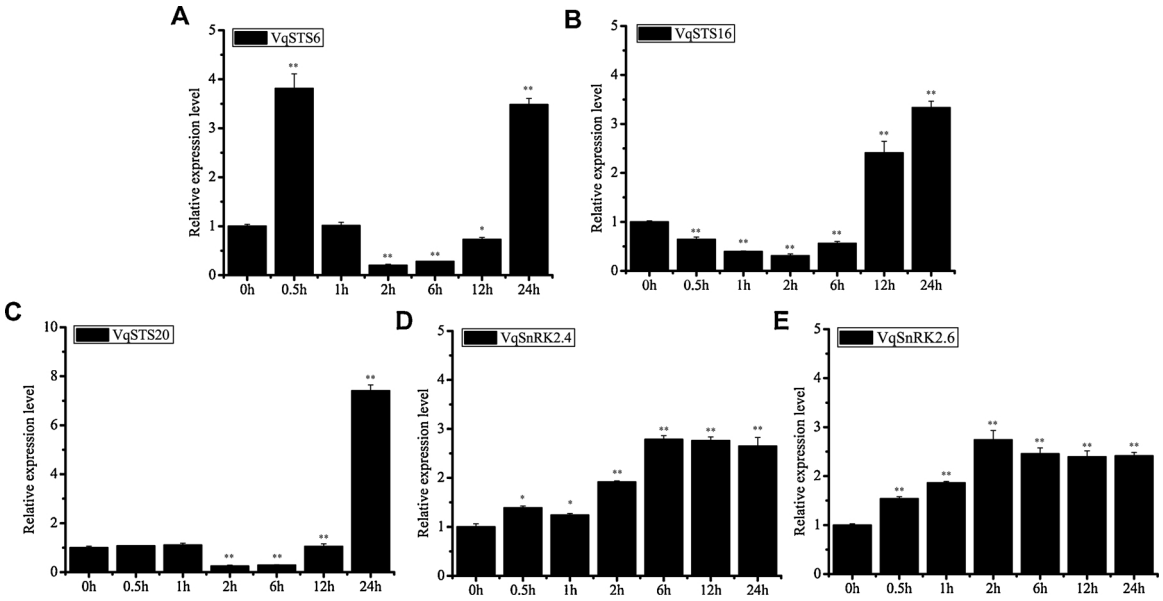

Fig. 6. Expression analysis of *STS* genes and *VqSnRKs* in response to ABA treatment. Leaves of Danfeng-2 were treated with ABA (100 μM) and samples were collected after 0, 0.5, 1, 2, 6, 12 and 24 h. qRT-PCR analysis of the expression profiles of *VqSTS6* (A), *VqSTS16* (B), *VqSTS20* (C), *VqSnRK2.4* (D) and *VqSnRK2.6* (E). Grape *VvGAPDH* was used as internal refernce gene. Error bars (SD) were calculated from three biological experiments. The significance was analyzed using *Tukey* test (*P < 0.05; **P < 0.01) with SPSS.

3.7. VqbZIP1 can activate the expression of the STS genes and stilbene accumulation

To test whether the promoters of *STS* genes can be activated by *VqbZIP1*, a GUS activity experiment was conducted. Three *STS* genes (*STS6*, *STS16*, *STS20*), whose PCC values are higher than 0.9, were chosen to study the relationship between *VqbZIP1* and the *STS* genes. The promoters of these *STS* genes were cloned from the genome DNA of Danfeng-2 using the homology-based cloning method. First, we analyzed the promoter sequence of these three *STS* genes using the online website PlantCARE. We found that the three *STS* promoters all contain the ABRE cis-element, which is reported to be related to the ABA response and the binding site of bZIP type transcription factors (Fig. 7A). Next, the STS promoters were constructed to pC0380-GUS vector to generate P_{VqSTS}-GUS. Co-transformed the pCAMBIA2300-35S-VqbZIP1-GFP vector with P_{VqSTS}-GUS into tobacco leaves and the GUS activity measured. The results show that compared with the control (empty vector of pCAMBIA2300 with P_{VqSTS}-GUS) the fusion vector P_{VqSTS}-GUS was detected with stronger expression in co-transformation with *VqbZIP1*. The result suggests *VqbZIP1* activated the promoter of *VqSTS6*, *VqSTS16* and *VqSTS20* (Fig. 7B). To demonstrate the influence of *VqSnRK2.4* and *VqSnRK2.6* on *VqbZIP1*, GUS activity was also detected. The co-transformation *VqbZIP1* and P_{VqSTS}-GUS was used as control. The result show that co-expression of *VqSnRK2.4* or *VqSnRK2.6* with *VqbZIP1* can enhance GUS activity driven by *STS* promoters compared with the control. This implied *VqSnRK2.4* and *VqSnRK2.6* can promote *VqbZIP1* activating *STS* promoters (Fig. 7B).

To determine whether the expression level of *VqbZIP1* can influence stilbene accumulation, a transient overexpression assay was conducted in leaves of Danfeng-2. The results show that, compared with the empty vector (EV) control, the transcript level of *STS* genes including *VqSTS6*, *VqSTS16* and *VqSTS20* were up-regulated in the overexpression lines (OE) (Fig. 7C). Meanwhile, we detected the transcript level of *VqSnRK2.4* and *VqSnRK2.6*. We found that *VqSnRK2.4* and *VqSnRK2.6* were down regulated in the overexpressing leaves (Fig. 7C). In parallel, the stilbene contents including trans-resveratrol, trans-piceid and ε-viniferin were detected using High Performace Liquid Chromatography (HPLC). As a result, in the overexpressing lines the concentrations of trans-resveratrol and trans-piceid were higher than in the control but the content of ε-viniferin showed no significant change (Fig. 7D).

3.8. Overexpression of VqbZIP1 in A. thaliana increased ABA sensitivity

To learn more about the function of *VqbZIP1*, further studies were carried out in transgenic *Arabidopsis* overexpressing *VqbZIP1*. The transgenic seedlings were screened to T3 generation, and the lines OE#5, OE#8 and OE#16 were identified using semi-quantitative PCR and RT-PCR tests (Fig. S7). Seeds of the wild-type and transgenic lines were cultivated on MS medium for four days and then transferred to MS medium with ABA in different concentrations (0, 0.5, 5 and 50 μM) for 10 days. The results show that on MS medium without ABA treatment, transgenic lines showed better growth than wild-type plants with greater elongation of the roots. In the presence of ABA, the growth of both the transgenic lines and the wild-type plants become weaker. Compared with the wild type, the roots of the transgenic *Arabidopsis* were shorter (Fig. 8A, B). This phenomenon implies that overexpression of *VqbZIP1* in *A. thaliana* increases ABA sensitivity. Furthermore, three ABA receptors PYR1 (AT4G17870), PYL1 (AT5G46790) and PYL2 (AT2G26040), two PP2C proteins ABI1 (AT4G26080) and ABI2 (AT5G57050), four SnRK2 proteins SnRK2.2 (AT3G50500), SnRK2.3 (AT5G66880), SnRK2.4 (AT1G10940) and SnRK2.6 (AT4G33950) and a bZIP type transcription factor ABI5 (AT2G36270), were selected to conduct the following qRT-PCR analysis. The *VqbZIP1* transgenic line OEbZIP1#8 and Col-0 were sprayed with 100 u M ABA for 0, 1, 2 and 4 h. Samples were collected for qRT-PCR. The results showed that, after ABA treatment, compared with the Col-0 line, the expression levels of *PYR1*, *PYL1*, *PYL2*, *ABI1*, *ABI2*, *ABI5*, *SnRK2.2*, *SnRK2.3*, *SnRK2.4* and *SnRK2.6* were all higher in the transgenic lines (Fig. 8C). Moreover, compared with normal conditions, ABA treatment caused greater up-regulation of these genes, especially of *ABI5*. We conclude that over-expression of *VqbZIP1* promotes the expression of ABA signaling core component genes in Arabidopsis.

4. Discussion

Grapevine, as an important fruit tree crop in the world contributes huge economic benefits. Resveratrol is a phytoalexin in grapevine that arouses wide public interest because of its benefits to human health

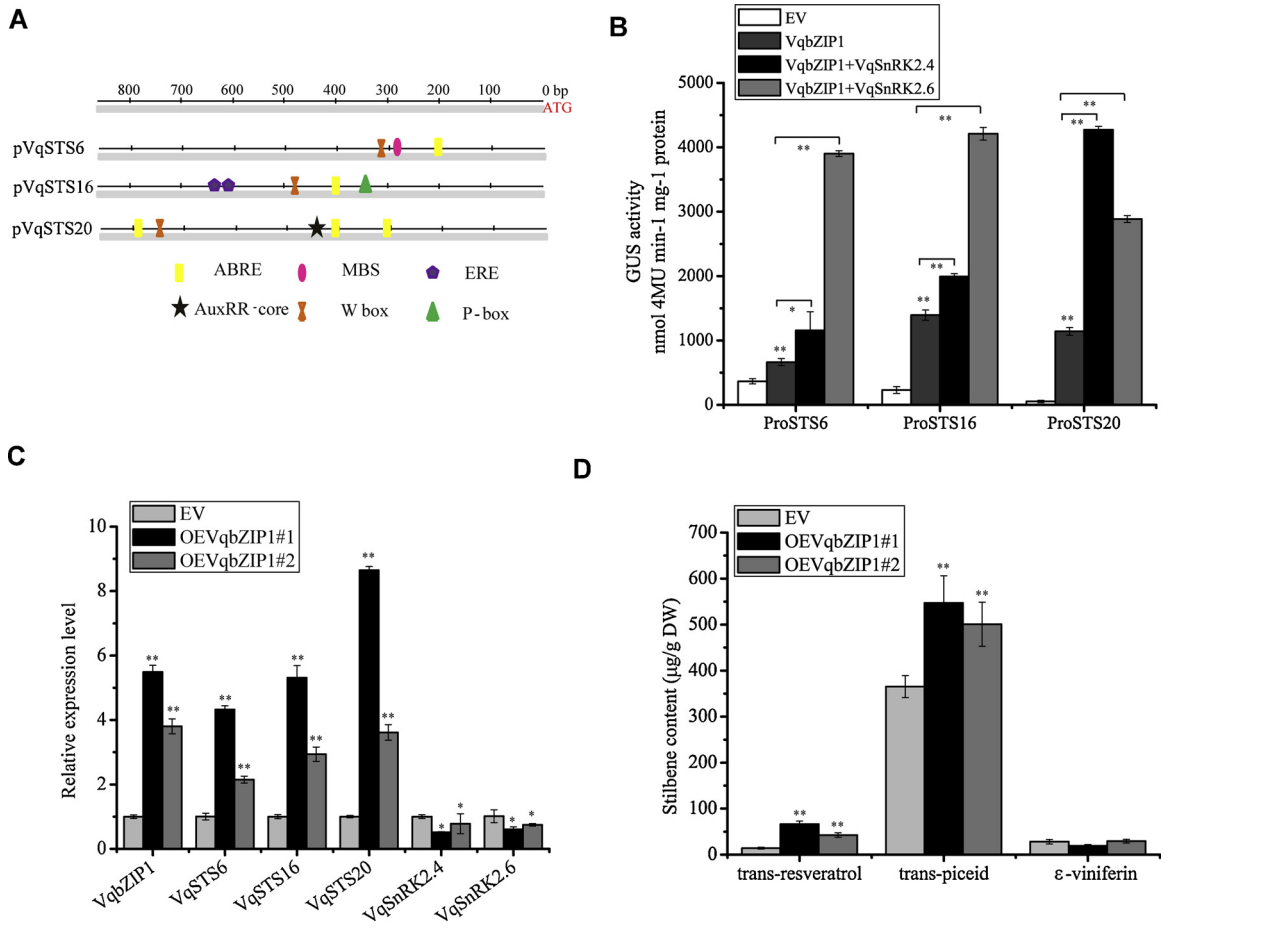

Fig. 7. *VqbZIP1* increased the transcript level of *STS* genes and the content of stilbenes. (A) Cis-element analysis of *VqSTS6*, *VqSTS16* and *VqSTS20* promoters. Different cis-elements are labeled with different shapes. ABRE is involved in abscisic acid responsiveness; MBS is the MYB transcription factor binding domain; W box is the WRKY transcription factor binding domain. (B) The vector P_{VqSTS6}-GUS, $P_{VqSTS16}$-GUS and $P_{VqSTS20}$-GUS were seperately transformed into tobacco leaves with various conbinations (the empty vector pCAMBIA2300, VqbZIP1, VqbZIP1 + VqSnRK2.4,VqbZIP1 + VqSnRK2.6). The microplate spectrophotometer was used for measuring GUS activity. (C) Transient overexpression of *VqbZIP1* was conducted in leaves of Danfeng-2. The transcript levels of *VqbZIP1*, *VqSTS6*, *VqSTS16*, *VqSTS20* and *VqSnRK2.4*, *VqSnRK2.6* was detected using qRT-PCR. *VvGAPDH* was used as internal control. Transient of empty vector (EV) was used as control. (D) The content of stilbenes including trans-resveratrol, trans-piceid and ε-viniferin was detected using HPLC. The error bar (SD) was calculated from three biological replicates. The significance was analyzed using *t* test (*P < 0.05; **P < 0.01) with SPSS.

[53,54]. The biosynthesis of resveratrol is via phenylalanine pathway and induced by many factors including ABA treatment. Recently, transcription factors belonging to MYB and WRKY families have been demonstrated participating in the regulation of stilbene biosynthesis [19,20,24]. Other transcription factor families, such as ERF, bHLH, and bZIP family, are predicted to be involved in the regulation of stilbene synthesis by co-expression analysis with *STS* genes [55,56]. However, only one ABA-induced bZIP transcription factor, *VvABF1*, was reported promoting resveratrol accumulation [25]. Therefore, the founding of new transcription factors makes sense for improving the regulation network of stilbenes. Here, we identified a bZIP type transcription factor *VqbZIP1* from Chinese wild *V. quinquangularis* which showed high co-expression relationship with *STS* genes especially *STS6*, *STS16* and *STS20* (Fig. S1). We investigated *VqbZIP1* involved in the ABA signaling pathway and participating in stilbene synthesis.

The bZIP transcription factors are widely found in eukaryotes and bZIP proteins contain a conserved bZIP domain. The bZIP domain has two characteristics, a 16 amino acid residues containing an N-X7-R/K motif and a leucine zipper (L-X6-L-X6-L) [31]. *VqbZIP1* isolated here was considered to be a bZIP transcription factor with a conserved bZIP domain (Fig. 1A, B). Sequence analysis revealed that the bZIP domain of VqbZIP1 contained a nuclear localization signal (Fig. 1B), and further studies had shown that VqbZIP1 was located in the nucleus (Fig. 3B). This may indicate that VqbZIP1 performs transcriptional regulation function in the nucleus. In a previous study in Arabidopsis, the bZIP family were divided into 10 subgroups (A–S) [31]. The 47 bZIP proteins from grapevine have been divided into 13 subgroups [57]. The *VqbZIP1* gene was homologous of *AtbZIP40*, *AtbZIP13* and *VvbZIP26* which belonged to Group A (Fig. S2). Previous studies found that bZIP members belonging to Group A play important roles in the ABA signaling pathway. For example, *VvABF1* and *VvABF2* responded to different treatments including exogenous ABA treatment [58]. Here, we found *VqbZIP1* also responded to ABA treatment (Fig. 2C) and this implied that *VqbZIP1* may be involved in the ABA signaling pathway.

Studies before demonstrated that bZIP transcription factors can recognize the ACGT motif in the promoter region to regulate downstream genes expression [32]. When analyzing the promoters of *VqSTS6*, *VqSTS16* and *VqSTS20*, we found the three *STS* promoters all contained the ABRE cis-element which contained the ACGT motif (Fig. 7A). This indicates that *VqbZIP1* may participate in regulating the expression of *VqSTS6*, *VqSTS16* and *VqSTS20*. Further studies demonstrated that over-expression of *VqbZIP1* up-regulated the expression level of *VqSTS6*, *VqSTS16* and *VqSTS20* and promoted the accumulation of resveratrol and piceid (Fig. 7C, D). The bZIP transcription factors belonging to Group A, have been reported in many studies requiring phosphorylation by SnRK2 proteins for transcriptional activation. In

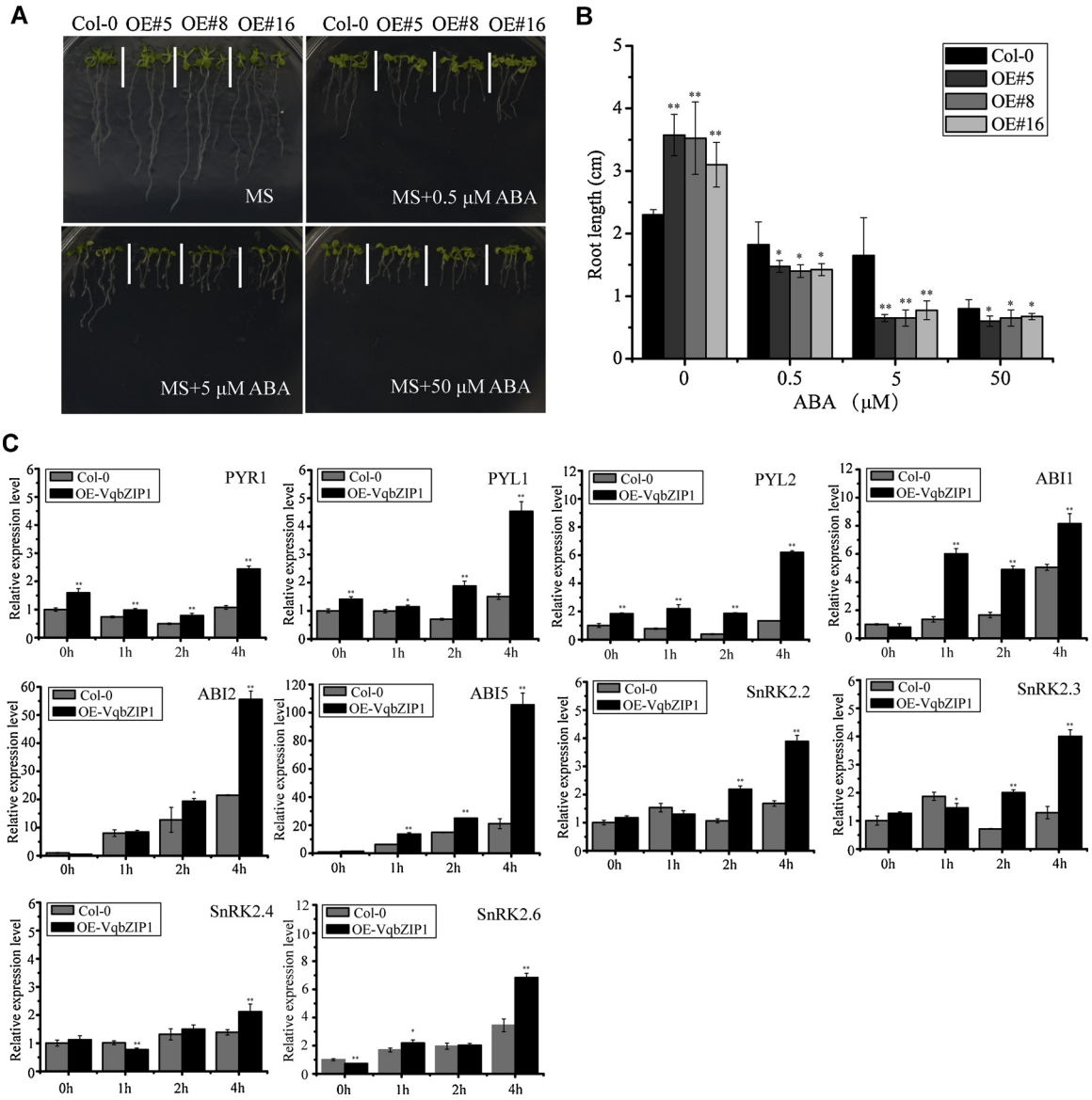

Fig. 8. Overexpression of VqbZIP1 in Arabidopsis increases plant ABA sensitivity. (A) Phenotype of wild-type *A. thaliana* (Col-0) and three *VqbZIP1* overexpression lines (OE#5, OE#8 and OE#16) seedlings were grown on MS agar medium with ABA at different concentrations (0, 0.5, 5 and 50 μM) for 10 days. (B) Measurement of the root length of wild-type *A. thaliana* (Col-0) and three *VqbZIP1* overexpression lines under different ABA concentration. (C) qRT-PCR analysis of ABA-related genes in *VqbZIP1* transgenic Arabidopsis thaliana after ABA treatment. Seedlings grown on MS medium for 10 days were transferred into soil for 30 days and then used for ABA (100 u M) treatment. Samples were collected at four times (0, 1, 2 and 4 h). The *AtActin* gene was used here as internal control. Error bars (SD) were calculated from three biological replicates and the significance was analyzed using *t* test (*P < 0.05; **P < 0.01) with SPSS.

Arabidopsis, *AREB1* could not regulate the downstream genes when phosphorylated by SnRK2 proteins such as SnRK2D, SnRK2E and SnRK2I [59]. A BiFC assay was used to identify the interaction between ABRE1 and SRK2D/E/I in the nucleus [60]. Later, SnRK2.2 and SnRK2.3 were shown to play major roles in phosphorylating and activating ABF1, ABF2 and ABI5 [61]. In grapevine, VvABF1, VvABF2 and VvABI5 have been shown to interact with different VvSnRK2s [58]. Here, we identified that VqbZIP1 interacted with VqSnRK2.4 and VqSnRK2.6 in the nucleus (Fig. 4A, B). Further study confirmed that co-expression of *VqSnRK2.4* or *VqSnRK2.6* with *VqbZIP1* can enhance GUS activity driven by *STS* promoters compared with expression of *VqbZIP1* only (Fig. 7B). This indicated that *VqbZIP1* showed the same characteristic with other Group A bZIP transcription factors published before. R/K-x-x-S/T and S/T-x-x-D/E were two key phosphorylation sites in bZIP proteins [59,62,63]. When we analyzed the protein sequence of VqbZIP1, contains two TxxD, three TxxE and four RxxS conserved sites (Fig. S6). This indicates that VqbZIP1 protein is likely to be phosphorylated by VqSnRK2.4 and VqSnRK2.6. But this requires further confirmation.

SnRK2 proteins play important roles in ABA signal transduction in plants. The SnRK2 families have been widely studied in various plants. In *Arabidopsis*, 10 *SnRK2s* (*SnRK2.1-SnRK2.10*) have been isolated [64], and in grape, there are six *SnRK2s* (*SnRK2.1-SnRK2.6*) [58]. An earlier study divided the SnRK2 family into three subclasses (I, II and III) [65]. Here, *VqSnRK2.4* and *VqSnRK2.6* were, respectively, divided into subclasses I and III (Fig. 5C). Studies in *Arabidopsis* indicate that only the SnRK2s belonging to subclasses II and III can response to ABA while members of subclass I cannot [65,66]. Among the SnRK2 subclass III members, *SnRK2.2*, *SnRK2.3* and *SnRK2.6* can be strongly and quickly activated by ABA in 30 min [67]. Unlike the *SnRK2s* from Arabidopsis, SAPK3 from subclass II and SAPK4, SAPK6 and SAPK7 from subclass I in rice can be upregulated by ABA [65]. In our studies, we found *VqSnRK2.4* and *VqSnRK2.6* could both be activated by ABA. Compared with *VqSnRK2.4*, *VqSnRK2.6* responded to ABA more strongly and

quickly. *VqSnRK2.6* can be upregulated in 30 min and this result is similar to the SnRK2s of subclass III from *Arabidopsis* (Fig. 6D, E). At the same time, the expression profiles of *VqSTS6*, *VqSTS16* and *VqSTS20* were detected using qRT-PCR (Fig. 6A–C). Compared with *VqbZIP1*, *VqSnRK2.4* and *VqSnRK2.6*, the response of *STS* genes to ABA signal is significantly delayed. This may be because the *STS* genes located at downstream of the ABA signaling pathway.

In *Arabidopsis*, several genes have been identified as participating in ABA signal transduction. *ABI5* was shown to positively regulate ABA sensitivity and affect seed germination and seedling growth [68–71]. *SnRK2.2* and *SnRK2.3* were activated by the ABA signal and phosphorylate the ABA-responsive bZIP transcription factors including *ABI5* [37,60]. In our study, overexpression of *VqbZIP1* in A. thaliana conferred ABA sensitivity which is reflected on shorter roots (Fig. 8A, B). The qRT-PCR analysis showed the transcript levels of *ABI5*, *SnRK2.2* and *SnRK2.3* were higher in the transgenic lines after ABA treatment compared with in the wild-type plants (Fig. 8C). We hypothesize that in the transgenic lines, because of overexpression of *VqbZIP1*, the ABA signaling pathway genes may accumulate to higher levels than in the wild-type plants and this may contribute to greater sensitivity to ABA in the overexpression lines. On the other hand, compared with the expression level of genes at 0 h after ABA treatment, their transcript level was significantly increased after ABA treatment for 4 h (Fig. 8C). This also implied that ABA treatment could activate the transcriptional regulatory function of *VqbZIP1*.

In conclusion, this study identified a bZIP type transcription factor *VqbZIP1* from Chinese wild *V. quinquangularis*. We charactized the function of *VqbZIP1* involved in ABA signaling pathway and the biosynthesis of stilbenes. According to our study, we hypothesize a possible pathway from ABA signal to the accumulation of stilbenes. When ABA content increases in grapevine, the expression of *VqbZIP1*, *VqSnRK2.4* and *VqSnRK2.6* are activated. VqbZIP1 interacts with VqSnRK2.4 and VqSnRK2.6 and during this process VqbZIP1 may be phosphorylated by VqSnRK2.4 and VqSnRK2.6 which need furhter study. Then *VqbZIP1* activates the expression of *VqSTS6*, *VqSTS16*, *VqSTS20* and further promotes the accumulation of stilbenes. This study improves the regulation network of stilbenes and provides new insight to clarify how resveratrol accumulation is induced by ABA signal.

Author contributions

Y. Wang conceived this study and reviewed the manuscript. D. Wang carried out the experiments, analyzed the data and wrote the first draft of the manuscript. C. Jiang helped with the experimental works. R. Li carried out the bioinformatics analyses. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest

Acknowledgements

This work was supported by State Key Laboratory of Crop Stress Biology in Arid Areas, Northwest A&F University, Yangling 712,100, Shaanxi, China. The research was funded by the National Science Foundation of China (Grant No. 31672129). The authors specifically thank Dr Alexander (Sandy) Lang from RESCRIPT Co. (New Zealand) for useful comments and language editing, which have greatly improved the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2019.110202.

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