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ORIGINAL ARTICLE

VyUSPA3, a universal stress protein from the Chinese wild grape *Vitis yeshanensis*, confers drought tolerance to transgenic *V. vinifera*

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Abstract

Key message **VyUSPA3 from the Chinese wild grape Vitis yeshanensis interacts with ERF105, PUB24 and NF-YB3, and overexpression of the VyUSPA3 gene in V. vinifera cv. 'Thompson Seedless' confers drought tolerance.**

Abstract Drought is a major abiotic stress factor that seriously affects the growth and yield of grapevine. Although many drought-related genes have been identified in *Arabidopsis* and other plants, the functions of only a few of their counterparts have been revealed in grape. Here, a universal stress protein (USP) A from the Chinese wild grape *Vitis yeshanensis*, VyUSPA3, was identified and its function was subsequently characterized by overexpressing or silencing the *VyUSPA3* gene in *V. vinifera* cv. 'Thompson Seedless' via *Agrobacterium*-mediated genetic transformation. After 21 d of the drought treatment, most leaves of the untransformed (UT) 'Thompson Seedless' lines wilted, yet UT lines were less damaged compared to the RNAi-*VyUSPA3* lines, nonetheless, the OE-*VyUSPA3* lines were mostly unaffected. Meanwhile, OE-*VyUSPA3* lines showed smaller stomatal aperture, more developed roots, higher leaf relative water content, proline content, and antioxidant enzyme activities, as well as lower malondialdehyde, H_2O_2 and $O_2^{\bullet-}$ accumulation than UT lines, but this response pattern was reversed in the RNAi-*VyUSPA3* lines. Besides, the transcript levels of four drought-related genes (*RD22*, *RD29B*, *DREB2A*, and *NCED1*) in OE-*VyUSPA3* lines were greater than those in the RNAi-*VyUSPA3* and UT lines. In addition, a yeast two-hybrid assay and a bimolecular fluorescence complementation assay confirmed that VyUSPA3 interacted with ERF105, PUB24, and NF-YB3, respectively. This study revealed that *VyUSPA3* improved drought tolerance in transgenic grapevines possibly through interaction with the hormone signaling, ubiquitination system, ethylene-responsive element binding factor and nuclear factors.

Keywords *Vitis yeshanensis* · Drought tolerance · VyUSPA3 · Genetically modified grapes · Interacting protein

Introduction

Grape (*Vitis vinifera*) is a widely cultivated fruit crop with high nutritional value and economic benefits. As one of the world's major grape producers, China ranks first for total grape production and second for vineyard area in the world (https://www.fao.org/home/). Arid and semi-arid northwest China is one of the five main grape-growing regions of China (Cheng et al. 2022), and soil water deficit caused by sparse rain is a critical factor restricting the development of the grape industry in these regions. Drought stress affects the distribution, growth, and yield of grapevines worldwide (Fraga et al. 2016). Apart from some management practices, such as water-saving irrigation, spraying anti-transpiration agents, and covering the soil surface (with grass, straw, or a mulching film), the fundamental way to resolve this problem is to breed drought-tolerant grape varieties. Notably, wild grape germplasms are relatively tolerant to drought compared to cultivated grape varieties (Wang et al. 2004; Zhang et al. 2012). Therefore, it is important to explore key genes related to drought tolerance in wild grape resources for the molecular breeding of drought tolerance.

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In the last few decades, many drought-related genes have been discovered in model plants (Chhaya et al. 2021; Yadav et al. 2021). For example, *RESPONSIVE TO DEHYDRATION 22* (*RD22*) and *RD29* are typical dehydration response genes, which are up-regulated in *Arabidopsis* under drought stress conditions (Yamaguchi-Shinozaki and Shinozaki 1993); *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN 2A* (*DREB2A*) is strongly induced in the roots and leaves of *Arabidopsis* under drought and high salt stress (Sakuma et al. 2002). Moreover, the concentration of ABA was positively correlated with the expression of *9-CIS-EPOXYCAROTENOID DIOXYGENASES* (*NCED1*) gene in grapevine, and the expression of *NCED1* gene in leaves was a good marker for drought stress (Lehr et al. 2022). The discovery of drought-related genes provides valuable genetic resources for improving drought tolerance of grapevine via molecular breeding techniques.

In plants, the universal stress protein (USP) was first found in rice (*Oryza sativa*), and *OsUSP1* functions in ethylene-mediated tolerance to waterlogging stress (Sauter et al. 2002). Some studies have confirmed that overexpression of *USP* genes could improve drought tolerance of transgenic plants by promoting root growth and ABA-induced stomatal closure, thereby reducing transpiration rate and leaf water loss but increasing photosynthesis, proline, total chlorophyll and soluble sugar content (Loukehaich et al. 2012; Yang et al. 2019; Hassan et al. 2021). The interaction between AnnSp2 and SpUSP suggests that the stomatal closure induced by *SpUSP* probably involves the Ca^{2+} signaling pathway since annexins have been considered targets of Ca^{2+} signals (Laohavisit et al. 2009; Loukehaich et al. 2012). Beside drought tolerance, USP proteins can also improve the ability of plants to resist other abiotic stresses, such as high salinity (Udawat et al. 2014; Gou et al. 2020), oxidative stress (Jung et al. 2015; Gou et al. 2020), osmotic stress (Udawat et al. 2016; Gou et al. 2020), and low temperatures (Melencion et al. 2017; Gou et al. 2020). Also, USP proteins have antifungal (Park et al. 2017) and antiparasitic (Espinola et al. 2018) functions. In addition, the *USPA* gene of *Arabidopsis* is involved in the processes of seed germination and post-germination growth (Gorshkova et al. 2018; Gorshkova and Pojidaeva 2021). Notably, the exogenous application of some hormones, such as ethylene (Sauter et al. 2002), ABA (Gorshkova et al. 2018) and gibberellin (Zahur et al. 2009) can induce the expression of *USPA* in plants.

USPs are small molecular weight proteins that exist in *Arabidopsis* in various forms, such as monomer, dimer, trimer, and oligomer, however, USPs are transformed to a high molecular weight complex once plants are subjected to an external stress factor (Jung et al. 2015). SlRd2, an ATP-binding protein containing the USP domain in tomato, forms a homodimer in plants and is phosphorylated via interaction with CIPK6, thus acting as a target of CIPK6 to regulate CIPK6-mediated reactive oxygen species (ROS) production under oxidative stress (Gutiérrez-Beltrán et al. 2017). Although the *USPA* genes have been studied in several plant species, to the best of our knowledge, no relevant reports yet exist for grapes.

In our previous study, the drought-responsive gene *USPA* was found as a differentially expressed gene of *Vitis yeshanensis* and *V. riparia* by transcriptome sequencing analysis (Cui et al. 2020). *V. yeshanensis*, which originated in the arid sunny slopes of Tashan Mountain, Hebei province, Northern China, is highly resistant to drought and cold (Wang et al. 2004; Zhang et al. 2012). In contrast, *V. riparia* is native to eastern North America, where soil moisture is higher, and thus *V. riparia* is more sensitive to drought (Knipfer et al. 2015). The qRT-PCR analysis showed that the transcript level of *USPA* was always higher in the Chinese wild *V. yeshanensis* accession 'Yanshan-1' than in the *V. riparia* acc. 'He'an (♀)' during the drought treatment (Cui et al. 2020), and it was named *USPA3* after a genome-wide analysis. Heterologous expression of the *USPA3* gene could increase the growth rate of *E. coli* under PEG, mannitol, and NaCl stress treatments (Cui et al. 2021). In this study, our aim was to overexpress and silence the *VyUSPA3* gene in *V. vinifera* cultivar 'Thompson Seedless', which is a relatively drought-sensitive grape variety (Pouzoulet et al. 2020), to further confirm the function of *VyUSPA3* in drought tolerance, and to screen and verify its interacting proteins. The findings thus provide a sound basis for understanding the regulation of *VyUSPA3* in drought tolerance and thus contributing to drought tolerance molecular breeding of grape.

Materials and methods

Plant materials and treatments

Tobacco (*Nicotiana benthamiana*) and *Arabidopsis* (*Arabidopsis thaliana* acc. Col-0 and *AtERF6* gene mutant) plants were grown in a greenhouse at the State Key Laboratory of Crop Stress Biology in Arid Areas of Northwest A&F University, Yangling, Shaanxi, China. The growth conditions inside the greenhouse were 25/23°C air temperature with a 16-h/8-h photoperiod (day/night). Potted seedlings (one seedling per pot, the diameter of the pot was 12 cm, 20 plants overall) of *V. vinifera* cv. 'Thompson Seedless' were maintained in the Grapevine Germplasm Resources Orchard of Northwest A and F University, Yangling, Shaanxi, China. Five-week-old seedlings of 'Thompson Seedless' grape were kept under normal conditions or treated with drought for 21 d by withholding water supply, and then the roots, stems, and leaves were collected for tissue-specific analyses. To investigate the expression pattern of *USPA3*, potted seedlings of 'Thompson Seedless' were treated with 4°C, 40°C,

and 200 mM NaCl, and leaves of 'Thompson Seedless' were sprayed with 20 mM H_2O_2 or 100 µM abscisic acid (ABA), and leaf samples were collected after 0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h.

To confirm the biological function of *VyUSPA3*, the untransformed (UT), overexpressed (OE-*VyUSPA3*), and silenced (RNAi-*VyUSPA3*) lines of 'Thompson Seedless' grape seedlings were subjected to drought treatment. For this, previously well-watered plants were initially watered to runoff (i.e., the relative water content of the soil was about 60%, Fig. 4b), and then water supply was withheld for 21 d (i.e., the relative water content of the soil was maintained below 5%, Fig. 4b). Phenotypes of the experimental plants were observed and photographed just before the withholding of water and again 21 d later. Similarly, drought treatment was applied to wild-type (WT) and the *erf6* mutants of *Arabidopsis* for 14 d.

Isolation and sequence analysis of the *VyUSPA3* gene

Based on the sequence of *VvUSPA* of *V. vinifera* cv. 'Pinot Noir' (accession number XM_002283354), the full-length CDS of *VyUSPA3* was isolated from the cDNA of the leaves of 'Yanshan-1' grape by homologous cloning. The Grape Genome Browser (https://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat) was used to predict the chromosome location of *VyUSPA3*. A conserved domain analysis of the VyUSPA3 protein was conducted by utilizing SMART (http://smart.embl-heidelberg.de), and its secondary structure was analyzed with SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl). The software tool DNAMAN (Lynnon Biosoft, San Ramon, CA, USA) was used to align the amino acid sequences of VyUSPA3 to several homologous ones from *V. vinifera* 'Pinot noir' and 'Thompson Seedless', *V. riparia*, model plant *A. thaliana*, other economical crops like *Hordeum vulgare*, *Castanea mollissima*, and *Juglans regia*. The primers used are shown in Supplementary Table S1.

Plasmid construction

The coding sequence (CDS) without the stop codon of *VyUSPA3* was inserted into the *Kpn* I and *Bam*H I sites of the pCAMBIA2300-GFP vector as the overexpression vector (Fig. 2a). For the RNAi vector, the specific fragments of *VyUSPA3* were cloned from the CDS of *VyUSPA3* with *Xba* I/*Bam*H I and *Kpn* I/*Eco*R I, respectively, and inserted into the vector pKANNIBAL (Wesley et al. 2001) to generate the plasmid pKANNIBAL-*VyUSPA3*. Then, the interfering cassette was removed with *Not* I from the vector pKANNIBAL-*VyUSPA3* and cloned into the site for *Not* I in the vector pART27 (Gleave 1992) to generate the vector pART27-*VyUSPA3*.

Subcellular localization of VyUSPA3

The pCAMBIA2300-*USPA3*-GFP and non-modified control pCAMBIA2300-GFP were introduced into *Agrobacterium tumefaciens* GV3101 and the transformed bacteria were then separately injected into *N. benthamiana* leaves (Wang and Wang 2019). The two plasmids (pCAMBIA2300-*USPA3*-GFP and pCAMBIA2300-GFP) were transformed into *Arabidopsis* protoplasts by implementing the polyethylene glycol-mediation method (Zhao et al. 2016); these transformed protoplasts were cultured at room temperature for 20 h before the observation. The distribution of GFP was visualized under a laser confocal microscope (TCS SP8SR, Leica, Germany). The primers used are shown in Supplementary Table S1.

Agrobacterium-mediated genetic transformation of the grape callus and identification of transgenic plants

Agrobacterium-mediated genetic transformation (Shu et al. 2021) was carried out using the callus from stem segments of 'Thompson Seedless' grape, which was preserved in our laboratory. GV3101, carrying the plasmid pCAMBIA2300-*USPA3*-GFP or RNAi-*USPA3*, was activated and adjusted to $OD_{600} = 0.8$. The callus was then cut into 0.5 mm^3 pieces before they were infected in the bacterial suspension for 10 min. Then, each infected and sterilized callus was cultivated on the Murashige & Skoog Basel Medium (MS) with 30 g/L sucrose, 4 mg/L benzylaminopurine (6-BA), 0.02 mg/L indole-3-butyric acid (IBA), 7 g/L agar, 300 mg/L ceftiofur, 200 mg/L carbenicillin, and 75 mg/L kanamycin for 2 months and thereafter sub-cultured monthly. The resistant callus was then induced into seedlings on the Lloyd & McCown wood plant basal medium (WPM) (2.41 g WPM + 30 g/L sucrose + 0.2 mg/L 6-BA + 0.2 mg/L IBA + 7 g/L agar + 1.5 g/L activated carbon).

Real-time fluorescence quantitative PCR (qRT-PCR) and western blot analyses were used to identify the positive seedlings of transgenic plants. We used the Plant RNA Kit (Omega) for the total RNA extraction from samples of each grape plant. Next, 1 µg RNA was used to synthesize the first strand of cDNA with the FastKing RT Kit (Tiangen, Beijing, China). Oligonucleotide primers (Table S1) for qRT-PCR were designed online at the NCBI website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_ LOC = BlastHome) and synthesized by the Shaanxi Zhongke Yutong Biotechnology Co., Ltd (Xi'an, China). The qRT-PCR reaction volume was 20 µL, consisting of 1 µL cDNA template, 0.8 µL of each forward and reverse

primer, 10 µL of SYBR Green fluorescent dye, and 7.4 µL of ddH_2O. The reaction profile was as follows: 95°C for 1 min, followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s. Three biological replicates were used for each treatment and their relative gene expression levels were determined by the $2^{-\Delta\Delta CT}$ method. Grape *Actin7* gene (accession no. XM_002282480) served as an internal reference. The positive plants identified by qRT-PCR were confirmed using western blot. From each sample, its total protein was extracted following Méchin (2006), resolved by a 10% SDS-PAGE gel, then transferred to a PVDF membrane, and detected with the anti-GFP antibody (ABclonal). The primers used are shown in Supplementary Table S1.

Measurement of stomatal aperture

After 21 d of drought treatment, the detached leaves of drought-treated grapevines were incubated in a buffer solution (10 mM MES-KOH, pH 6.15; 10 mM KCl; 50 µM $CaCl_2$) for 2 h under light conditions. Then its Ca^{2+} concentration was increased to 2 mM and the incubation period was extended for another 2 h. To observe the stomatal aperture, the transparent sticky-tape method (Wang et al. 2020) was applied under a microscope (ECLIPSE 50i, Nikon, Japan). The Nano Measurer software tool was used to measure stomatal pore diameter. The average of nine pore diameters from three individual plants was recorded, i.e. three stomata per leaf and one leaf per plant.

Observation of roots

After 21 d of imposed drought stress, the potted seedlings of the UT, OE-*VyUSPA3*, and RNAi-*VyUSPA3* lines were carefully pulled out from their pots together with the substrate, and the substrate was removed. The roots were carefully cleaned under running water to ensure their integrity, then scanned by a root scanner (Epson Perfection V700 Photo, Epson, Japan) and analyzed by WinRHIZO (Regent, Canada) software program.

Quantification of physiological and biochemical indicators

The leaf relative water content was measured by the method of Zhang et al. (2022), and the content of proline was determined by the acidic-ninhydrin method (Ábrahám et al. 2010), while malondialdehyde (MDA) content was determined by the thiobarbituric acid method (Zhang et al. 2020). Electrolyte leakage was measured using a previously described method (Campos et al. 2003). POD activity was assayed by the method of Fang and Kao (2000), SOD activity was determined by measuring the reduction of nitro blue tetrazolium at 560 nm, and CAT activity was analyzed by measuring the H_2O_2 consumption at 240 nm (Mellacheruvu et al. 2016). The H_2O_2 content was quantified by the titanium sulfate method (Wang et al. 2009), and the $O_2^{\bullet-}$ content was measured by applying the method of Elstner and Heupel (1976).

DAB and NBT staining

Grape leaves were immersed in diaminobenzidine (DAB) (1 mg/mL, pH 3.8) and nitro blue tetrazolium (NBT) (5 mg/mL) dye under dark conditions for 8 h, and then transferred to 95% ethanol for de-staining and subsequent imaging.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed according to the method of Chen et al. (2021) as follows. The CDS of *VyUSPA3* was transformed into the vector pGBKT7 to form a bait vector, which was co-transformed into Y2HGold strain with yeast cDNA library, and obtained three stress-related interacting proteins: ERF105 (accession no. XM_002281876), PUB24 (accession no. XM_002265021), and NF-YB3 (accession no. XM_003635491). The corresponding CDS of *ERF105*, *PUB24*, and *NF-YB3* was separately inserted into the vector pGADT7, and then pGBKT7-*VyUSPA3* was co-transformed with pGADT7-X (the X denoting genes encoding the three interacting proteins). In parallel, the co-transformation of pGADT7-T with pGBKT7-VyUSPA3 was used as a negative control. The transformed Y2HGold strain was spread onto SD/-Trp/-Leu/-Ade/-His/AbA/X-α-Gal medium containing 200 ng/mL AbA and cultured at 28°C for 3 d. The primers used are shown in Supplementary Table S1.

Bimolecular fluorescence complementation assay to verify protein interactions

Bimolecular fluorescence complementation (BiFC) assay was carried out according to the method of Chen et al. (2021) as follows. The CDS of *VyUSPA3* was inserted separately into the vectors pBI221NE, pSPYNE, and pSPYCE. The corresponding CDS of candidate genes *ERF105*, *PUB24*, and *NF-YB3* was inserted into the pBI221CE vector, respectively. Next, pBI221NE-*VyUSPA3* was co-transformed with pBI221CE-X into *Arabidopsis* protoplasts, while co-transformation of pBI221NE-*VyUSPA3* with pBI221CE was carried out as a control. The transformed protoplasts were cultured and wrapped in tinfoil at 22°C in an incubator for 20 h, and their yellow fluorescent protein (YFP) fluorescence was observed under a laser confocal microscope (TCS SP8 SR, Leica, Germany). Both pSPYNE-*VyUSPA3* and pSPYCE-*VyUSPA3* were each transformed into GV3101, separately, and the recombinant bacteria were then injected

into *N. benthamiana* leaves. YFP was observed under a laser confocal microscope (TCS SP8 SR, Leica, Germany) after 48 h. The primers used are shown in Supplementary Table S1.

Identification and functional analysis of the *ERF6 Arabidopsis* mutant

To reveal the biological function of *VvERF105*, we procured the *Arabidopsis AtERF6/103* mutant AT4G17490 (donor stock no. SALK_087356.10.b) from AraShare (https://www.arashare.cn/index/Product/index.html) because *AtERF6/103* is the homologous gene of *VvERF105* and they have a 39.06% amino acid identity. Genomic DNA was extracted from leaves of WT and the mutant *Arabidopsis* by the CTAB method (Murray and Thompson 1980), and PCR-amplified using the primers LBb1.3, LP, and RP. Then, qRT-PCR was performed to determine the expression of *AtERF6* in the two types of lines using primers qRT-*AtERF6*-F, and qRT-*AtERF6*-R, for which *Arabidopsis Actin2* gene (accession no. NM_001338359) served as an internal reference. The primers used are shown in Supplementary Table S1. After 14 d of drought treatment, phenotypic and physiological and biochemical changes including proline content, MDA content, electrolyte leakage and antioxidant enzyme activity (POD, SOD, and CAT) of WT and *ERF6* mutant were assessed.

Statistical analysis

Three biological replicates were performed for each set of data, and the results were expressed as mean ± standard error (SE). The SPSS 23.0 software (IBM, New York, USA) was used for the statistical analysis of data. Significant differences among means were determined by one-way ANOVA followed by Tukey's-b (K) test ($P < 0.05$).

Results

Cloning and sequence analysis of *VyUSPA3*

VyUSPA3 is located on chromosome 1 (Fig. 1a) and has a length of 1931 bp. The CDS of *VyUSPA3* is 495 bp in length and encodes 164 amino acids (aa); the carboxyl terminal (residues 13–161 aa) of VyUSPA3 contains a USP-like domain. VyUSPA3 has an amino acid sequence identical to its homologue in *V. vinifera* cv. 'Pinot Noir', yet one amino acid difference from that of *V. riparia* acc. 'He'an (♀)', and two of *V. vinifera* cv. 'Thompson Seedless', which is highly similar to that for USPA of *C. mollissima*, *J. regia*, *H. vulgare*, and *A. thaliana* (Fig. 1b). Secondary structure analysis revealed that VyUSPA3 has five β-folds separated by four α-helices as well as an ATP-binding site sequence G-(2X)-G-(9X)-G(S/T), indicating that VyUSPA3 is a type of ATP-binding protein (Fig. 1b).

VyUSPA3 is localized to the nucleus and cytoplasm, and may exist in plants as a homodimer

Green fluorescent protein (GFP) appeared throughout the cells of tobacco leaves and protoplasts of *Arabidopsis* transformed with the empty vector pCAMBIA2300, but appeared only in the nucleus and cytoplasm when transformed by the recombinant plasmid *VyUSPA3*-pCAMBIA2300 (Fig. 2). This confirms that VyUSPA3 is localized to the nucleus and cytoplasm. Yeast two-hybrid (Fig. S1a) and BiFC assays (Fig. S1b, c) showed that VyUSPA3 interacts with itself and may form a homodimer in plants.

Expression pattern of *USPA3*

As shown in Fig. 3, *USPA3* was expressed in the roots, stems, and leaves of 'Thompson Seedless', but its transcript level in leaves was significantly higher than that in roots or stems under normal and drought conditions. The transcript level of *USPA3* peaked after 2 h of the low temperature (4°C) treatment, suggesting that *USPA3* is potentially an early response gene to low temperature, while its expression remained higher at all sampling times under high temperature (40°C) than that of control (25°C). The transcript level of *USPA3* increased with the prolongation of salt stress, peaking at 12 h. Both oxidative stress and the plant hormone abscisic acid (ABA) were also able to induce the expression of *USPA3*, although the expression level was almost unchanged in the early stage of the treatments. In a previous study, we analyzed the promoter of the *USPA3* gene and found the presence of an ABA response element (ABRE) (Cui et al. 2021). Based on these results, it can be concluded that *USPA3* responds to temperature, salt, oxidative stress and ABA treatment, and thus it appears to be a general stress-response gene, and may be related to the ABA signaling pathway.

VyUSPA3 improves drought tolerance of transgenic 'Thompson Seedless' grape

Agrobacterium-mediated genetic transformations were carried out using the meristematic callus of the 'Thompson Seedless' as the recipient material (Fig. S2a). Three overexpression lines OE-60, OE-66, and OE-68, and two RNAi-silencing lines RNAi-7 and RNAi-15 were identified by qRT-PCR and the western blot (Fig. S2b, c). To explore the relationship between *VyUSPA3* and drought tolerance, the UT, OE-*VyUSPA3*, and RNAi-*VyUSPA3* 'Thompson Seedless' lines with comparable and robust growth were selected

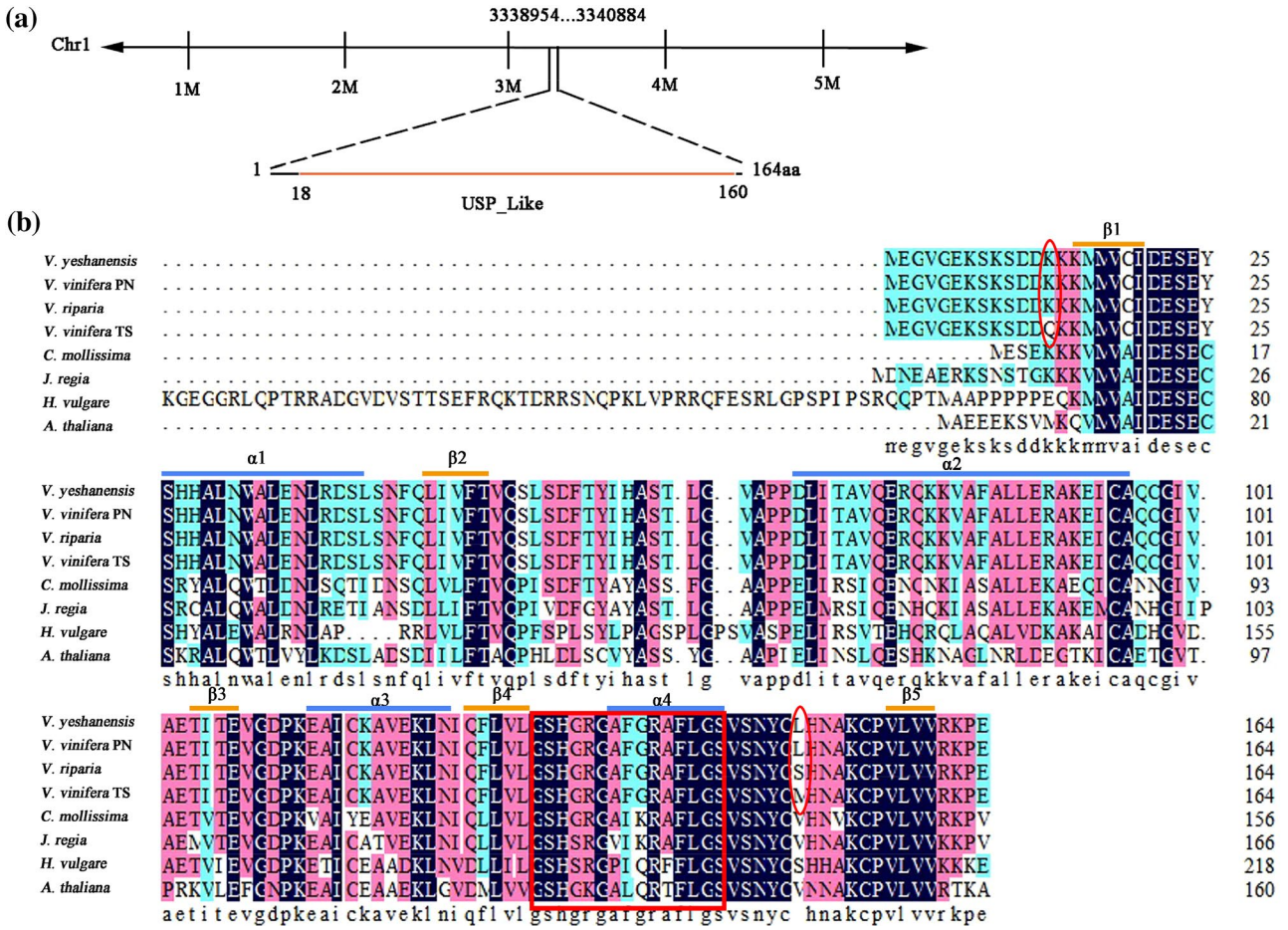

Fig. 1 Sequence analysis of *VyUSPA3*. **a** Chromosome location analysis of *VyUSPA3*; its location was predicted at 3,338,954 to 3,340,884 on chromosome 1. The USP-like domain is situated from 13 to 161 aa. **b** Multiple sequence alignments between VyUSPA3 and its homologous proteins from other plant species. Protein information for the sequence alignments: *V. vinifera* cv. 'Pinot Noir' (PN) VvUSPA (XM_002283354), *V. riparia* VrUSPA (XM_034828214), *V. vinifera* cv. 'Thompson Seedless' (TS) VvUSPA (SRP026420), *C. mollissima* CmUSPA (KAF3949527), *J. regia* JrUSPA (XP_018806489), *H. vulgare* HvUSPA (ADB54810), and *A. thaliana* AtUSPA (NP_564927). Differential amino acids between different grapes are represented by red ovals. The α-helix and β-fold components are indicated above the sequence as blue and orange lines, respectively, with ATP-binding sites in red boxes

and subjected to 21 d of a drought treatment (Fig. 4a). There was no significant difference in soil relative water content among the grape genotypes before and after the drought treatment (Fig. 4b). After drought stress, nearly all the leaves of UT lines appeared withered, while the overexpressed lines displayed negligible or no damage, in stark contrast to the severe leaf wilting and curling of RNAi-*VyUSPA3* lines (Fig. 4a). Also, the leaf relative water content was similar among all genotypes before the drought treatment, but after 21 d of the drought treatment, the leaf relative water content of OE-*VyUSPA3* lines was significantly higher than that of RNAi-*VyUSPA3* lines and the UT lines (Fig. 4c). Stomatal observations after 21 d of drought stress revealed a smaller stomatal aperture in the OE-*VyUSPA3* lines than in the UT lines and a bigger stomatal aperture in the RNAi-*VyUSPA3* lines than in the UT lines (Fig. 4d, e), indicating that the *VyUSPA3* gene can induce a decrease in stomatal aperture under drought stress conditions. We also found that the OE-*VyUSPA3* lines had more developed roots than the RNAi-*VyUSPA3* lines and UT lines (Fig. 4f–i), indicating that the *VyUSPA3* gene activity and protein product are potentially related to root growth under drought stress. Overall, these results suggested that *VyUSPA3* improves the drought tolerance of grape plants, possibly by reducing the stomatal aperture and promoting root growth.

VyUSPA3 alleviates oxidative damage under drought stress

There was no significant difference in the proline content among the grape genotypes before the drought stress treatment. After 21 d of treatment, the proline content increased,

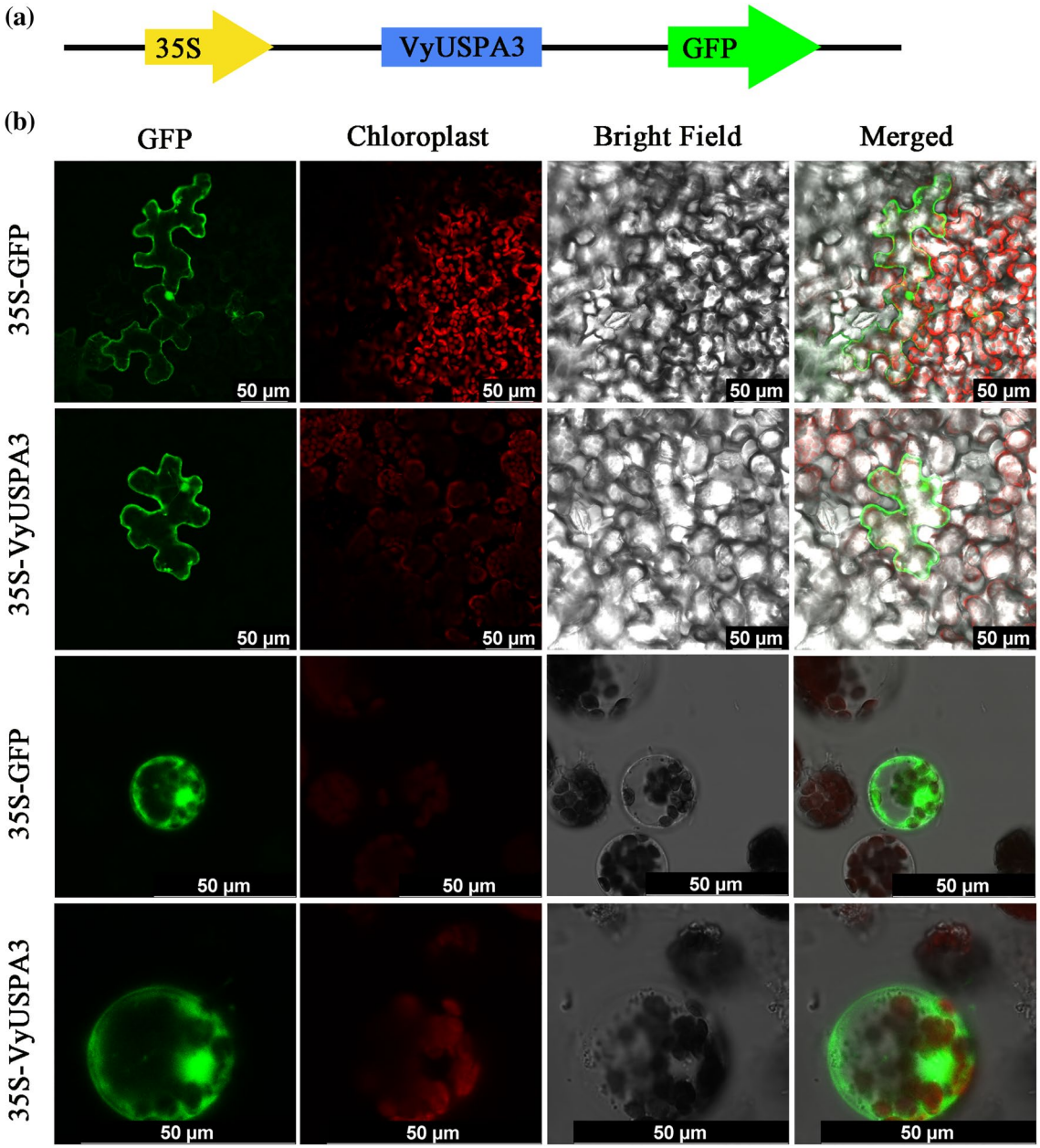

Fig. 2 Subcellular localization analysis of VyUSPA3. **a** Schematic diagram of the *VyUSPA3*-pCAMBIA2300 vector construction. **b** Using 35S-GFP as the control, localization of *VyUSPA3*-pCAMBIA2300 in tobacco cells and *Arabidopsis* protoplasts was observed by a laser confocal microscopy. Scale bars = 50 μm

but it was significantly higher in the OE-*VyUSPA3* lines than in RNAi-*VyUSPA3* lines and UT lines (Fig. 5a). Before the drought treatment, except for the OE-*VyUSPA3* line OE-66, which had a lower MDA content, there were no significant differences among the other lines. But after the drought treatment, the MDA content of RNAi-*VyUSPA3* line RNAi-15 was significantly higher than that of other genotypes, while OE-*VyUSPA3* lines had the lowest MDA content (Fig. 5b). The POD, SOD, and CAT activities of all plants had no or little difference just prior (0 d) to the drought treatment. By contrast, after 21 d of drought treatment, POD and SOD activities of the OE-*VyUSPA3* lines significantly surpassed those of the UT lines, with the significantly lower in the RNAi-*VyUSPA3* lines (Fig. 5c, d). There was lower CAT activity in the RNAi-*VyUSPA3* lines than either in the UT lines or OE-*VyUSPA3* lines at 21 d of drought treatment (Fig. 5e). Before incurring drought stress, the contents of H_2O_2 and $O_2^{\bullet-}$ were similar among all genotypes, however, after drought stress, ROS content was significantly lower in the OE-*VyUSPA3* lines than in the RNAi-*VyUSPA3* lines

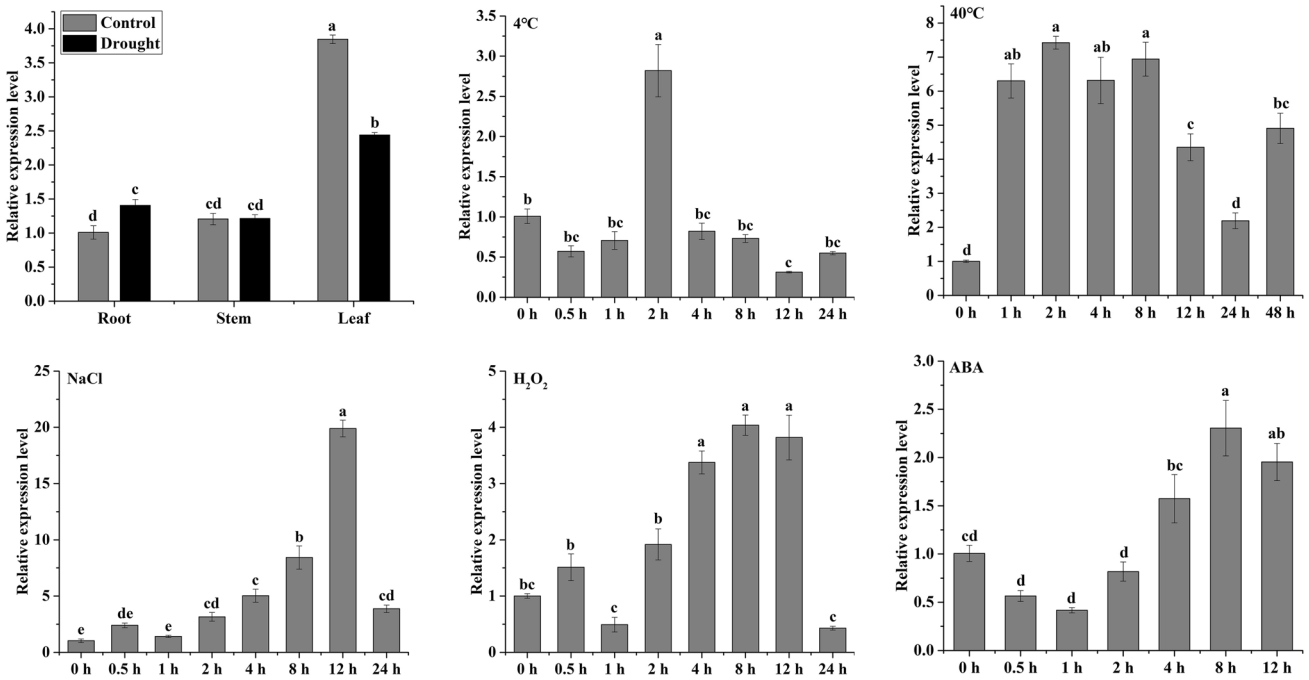

Fig. 3 Transcript quantification of *USPA3* in different plant tissues and treatments. The *USPA3* gene expression was analyzed in roots, stems, and leaves of 'Thompson Seedless' grape under normal conditions and after 21 d of drought treatment by qRT-PCR. Potted seedlings of 'Thompson Seedless' were treated with 4℃, 40℃, or 200 mM NaCl, and leaves were sprayed with 20 mM H_2O_2 and 100 µM ABA and collected at 0, 0.5, 1, 2, 4, 8, 12, 24, and 48 h post-treatment. The analysis employed qRT-PCR with the grapevine *Actin7* gene (accession no. XM_002282480) as an internal control. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Data are the mean (±SE) of three biological replicates. Significant differences among means were determined by one-way ANOVA followed by Tukey's-b (K) test ($P < 0.05$)

and the UT lines, except for the content of $O_2^{\bullet-}$ in OE-66 (Fig. 5f, g). The results of DAB and NBT staining were consistent with the H_2O_2 and $O_2^{\bullet-}$ contents. Leaf ROS accumulation as observed in the DAB and NBT staining was the lowest in the OE-*VyUSPA3* lines and highest in the RNAi-*VyUSPA3* lines (Fig. 5h, i). The above results indicated that the *VyUSPA3* gene is responsible for promoting the accumulation of proline and the activity of antioxidant enzymes while reducing the accumulation of ROS and MDA under drought stress.

Overexpression of *VyUSPA3* up-regulates the transcript levels of some drought-related genes

In this experiment, the transcript levels of four drought-related genes (*RD22*, *RD29B*, *DREB2A*, and *NCED1*) in the UT, OE-*VyUSPA3* and RNAi-*VyUSPA3* lines before and after drought treatment were quantified. The results showed that the four genes were all up-regulated after the drought treatment in the UT and OE-*VyUSPA3* lines, with their transcript levels significantly higher in OE-*VyUSPA3* plants than in the UT and RNAi-*VyUSPA3* lines, especially *RD29B* (Fig. 6), further indicating a role for *VyUSPA3* in drought stress response.

VyUSPA3 interacts with ERF105, PUB24, and NF-YB3 in yeast and *Arabidopsis* protoplasts

The pGADT7-*ERF105*, pGADT7-*PUB24*, and pGADT7-*NF-YB3* were each co-transformed with pGBKT7-*VyUSPA3* into the Y2HGold strain, with co-transformed pGBKT7-*VyUSPA3* and pGADT7 as the negative control. Except for the negative control, other co-transformed strains were able to grow on the SD/-Trp/-Leu/-Ade/-His/AbA/X-α-Gal medium containing 200 ng/mL AbA; that is, VyUSPA3 was found to interact with ERF105, PUB24, and NF-YB3 in yeast (Fig. 7a). Next, pBI221NE-*VyUSPA3* and pBI221CE-X (Fig. 7b) were each co-transformed into *Arabidopsis* protoplasts. We found that YFP (Fig. 7c) appeared in all protoplasts co-transformed with recombinant plasmids except for the negative control after 20 h of culture, confirming that VyUSPA3 interacted with each of the above three proteins in *Arabidopsis* protoplasts. Moreover, the interaction between VyUSPA3 and ERF105 or PUB24 primarily occurred in the nucleus, while interaction with NF-YB3 occurred mainly in the cytoplasm (Fig. 7c).

To further study the interactions between the above-mentioned interacting proteins and VyUSPA3, the expression patterns of *ERF105*, *PUB24*, and *NF-YB3* under drought

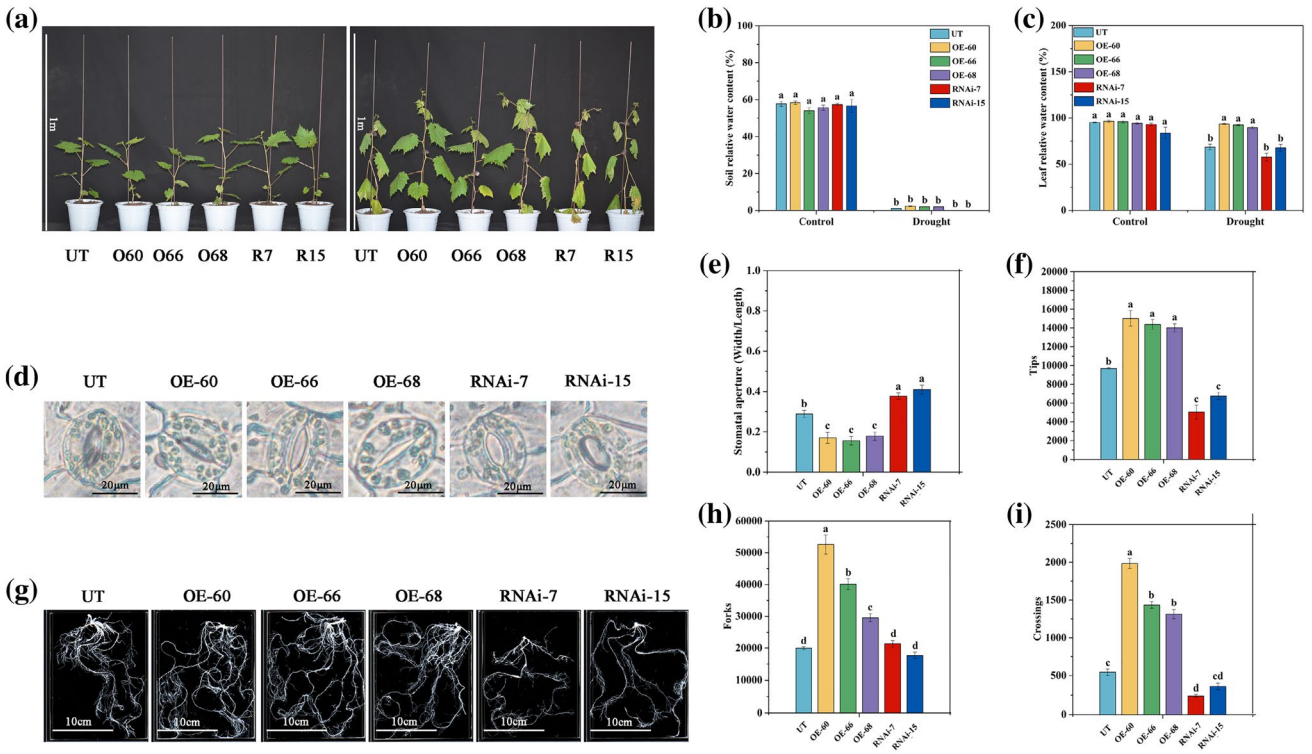

Fig. 4 Plant phenotypes, soil relative water content, leaf relative water content, stomatal aperture, and root morphology of the untransformed (UT), overexpression (OE-*VyUSPA3*) and silenced (RNAi-*VyUSPA3*) grape lines before (0 d) and after (21 d) the drought treatment. **a** Plant phenotypes, **b** soil relative water content, and **c** leaf relative water content in UT lines, OE-*VyUSPA3* and RNAi-*VyUSPA3* grapes at 0 and 21 d under drought treatment. **d, e** Stomata, **f** root tips, **g** roots, **h** root forks, and **i** root crossings of UT, OE-*VyUSPA3* and RNAi-*VyUSPA3* grapes after 21 d of drought treatment. Data are the mean (± SE) of three biological replicates. Significant differences among means were determined by one-way ANOVA followed by Tukey's-b (K) test ($P < 0.05$)

stress were analyzed based on the results of previous transcriptome data (Cui et al. 2020). From the results shown in Fig. S3, we can see that *NF-YB3* and *USPA3* showed the same expression pattern under drought stress, and the expression levels were up-regulated after drought treatment and reached a peak at 16 d. In contrast, *ERF105* and *PUB24* showed downregulation in response to drought stress.

ERF6 negatively regulates drought tolerance in *Arabidopsis*

Since *AtERF6/103* is a homologous gene of *VvERF105*, we revealed the biological function of *VvERF105* by detecting the changes in phenotype and some physiological and biochemical indexes of *Arabidopsis erf6* mutant under drought stress. Genomic DNA and qRT-PCR assays confirmed the veracity of the *erf6* mutant and that it is a homozygous mutant (Fig. S4a-c). After incurring the drought stress treatment, almost all leaves of the WT plants wilted at 14 d, whereas the *erf6* mutant grew vigorously (Fig. S4d, e). In addition, compared with WT, the *erf6* mutant had a higher proline content (Fig. S4f), lower MDA content (Fig. S4g) and electrolyte leakage (Fig. S4h), and higher antioxidant enzyme activity (Fig. S4j-k), implying that *ERF6* aggravates drought-induced oxidative stress in *Arabidopsis*. Taken together, these results suggest that the *ERF6* gene is a negative regulator of drought tolerance in *Arabidopsis*.

Discussion

In this study, a differentially expressed gene, *USPA*, was cloned from the extremely drought-resistant Chinese wild grape *V. yeshanensis* acc. 'Yanshan-1' based on our previous transcriptome data (Cui et al. 2020). According to the distribution of that gene on the chromosome, it was named *VyUSPA3* (Cui et al. 2021). Heterologous expression of the *VyUSPA3* gene in *E. coli* can improve the tolerance of the strain TOP10 to PEG, mannitol, and NaCl (Cui et al. 2021). These results coupled with previous research results (Yang et al. 2019; Gou et al. 2020; Hassan et al. 2021) motivated us to infer that *VyUSPA3* is a drought-related gene. To verify the function of the *VyUSPA3* gene in drought tolerance, *VyUSPA3* overexpression and RNAi-silencing 'Thompson Seedless' grape lines were executed in the present study. After a 21-d drought

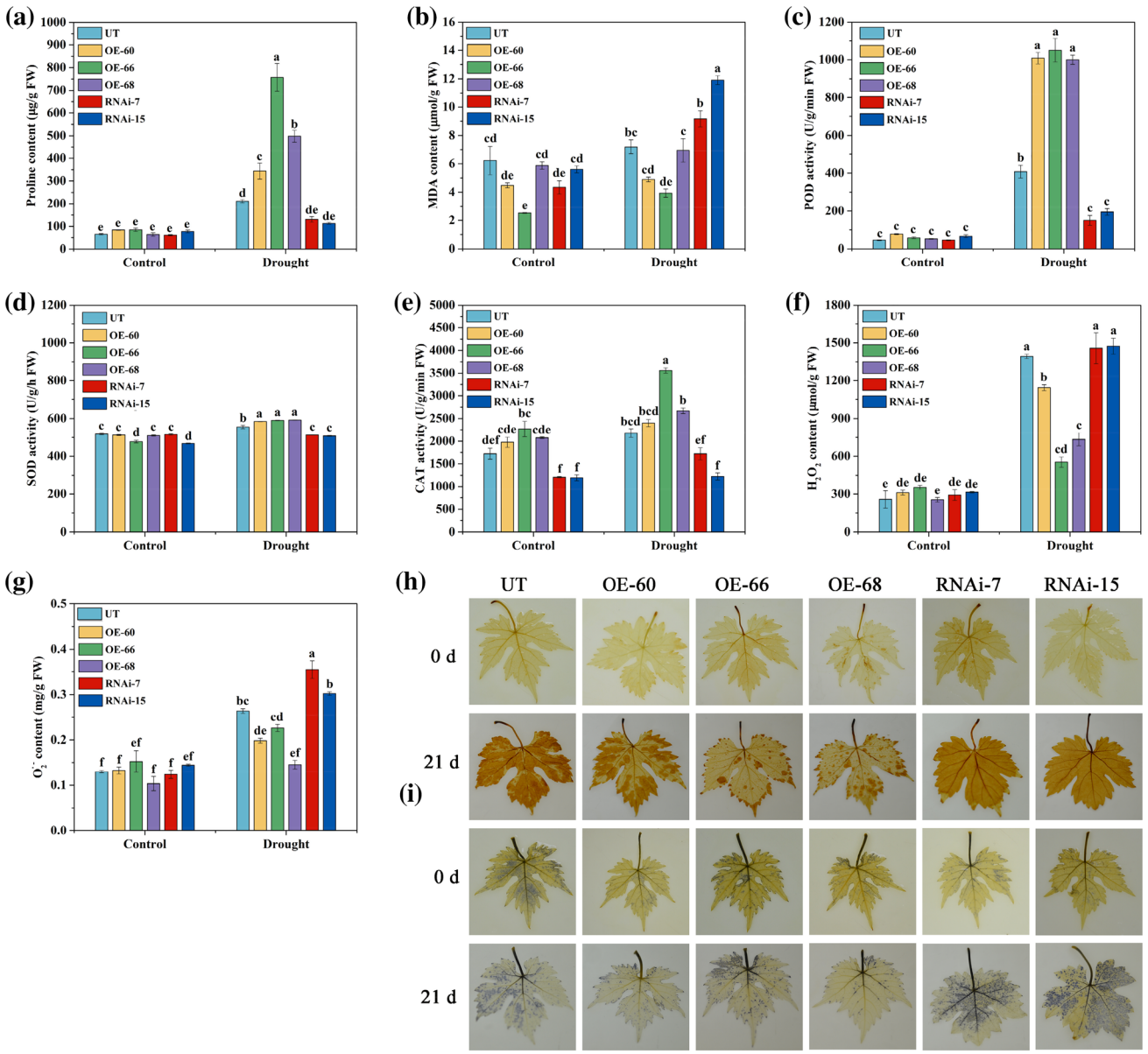

Fig. 5 Changes in osmoregulatory substances, lipid peroxidation, antioxidant enzyme activity and ROS accumulation in the UT, OE-*VyUSPA3* and RNAi-*VyUSPA3* grape lines before (0 d) and after (21 d) the drought treatment. **a** Proline content. **b** MDA content. **c** POD activity. **d** SOD activity. **e** CAT activity. **f** H_2O_2 content. **g** $O_2^{\bullet-}$ content. **h** DAB staining and **i** NBT staining of UT, OE-*VyUSPA3* and RNAi-*VyUSPA3* grape leaves at 0 and 21 d under drought stress. Data are the mean (±SE) of three biological replicates. Significant differences among means were determined by one-way ANOVA followed by Tukey's-b (K) test ($P < 0.05$)

treatment, we found that the OE-*VyUSPA3* lines feature the best growth status, while both the RNAi-*VyUSPA3* lines and the UT lines presented wilting phenotype, and the wilting degree of the former was slightly higher than that of the latter. The non-significant difference between the RNAi-*VyUSPA3* lines and the UT lines may be due to the existence of multiple genes similar in structure and function to *VyUSPA3* in the grape genome, which might compensate *VyUSPA3* to maintain drought tolerance in grapes when the expression of *VyUSPA3* is suppressed. However, overexpression of *VyUSPA3* increased drought tolerance in the 'Thompson Seedless' grape cultivar.

Roots and leaves are essential organs for maintaining water balance and many of their traits are closely related to plant growth and stress resistance. Plants with longer and denser root systems are more likely to survive in the face of drought stress (He et al. 2021). Leaf water loss is mainly determined by transpiration, and stomatal conductance is closely related to plants' transpiration rate and water use efficiency (Egea et al. 2011). Overexpressing the *Malus*

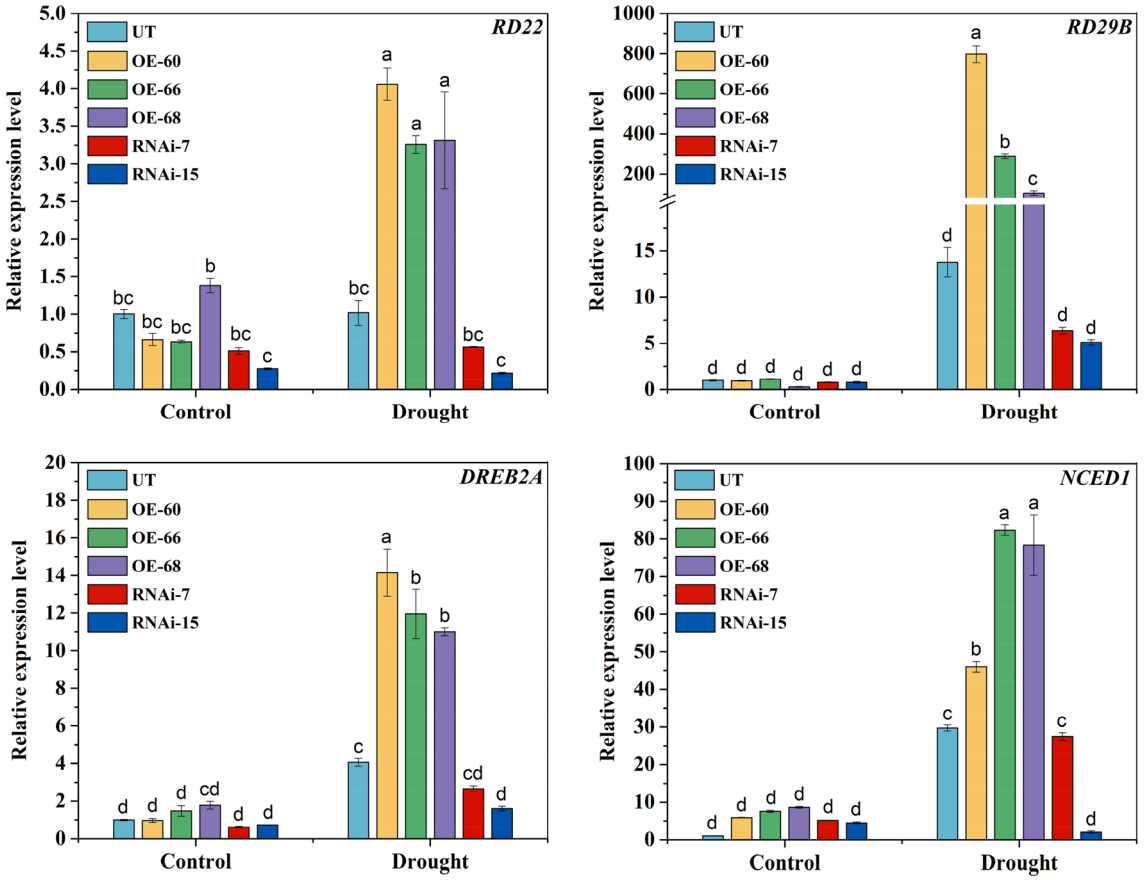

Fig. 6 Expression of four stress-responsive genes (*RD22*, *RD29B*, *DREB2A*, and *NCED1*) in the UT, OE-*VyUSPA3* and RNAi-*VyUSPA3* lines, before (0 d) and after (21 d) the drought treatment. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. Quantitative data are the mean (±SE) of three biological replicates. Significant differences among means were determined by one-way ANOVA followed by Tukey's-b (K) test ($P < 0.05$)

sieversii MsUSPA gene in *Arabidopsis* induces longer roots, and a more compact leaf cell structure than UT lines under extreme drought conditions (Yang et al. 2019). Similarly, overexpression of *Gossypium arboreum GUSP1* gene in *G. hirsutum* can increase the leaf water content, chlorophyll content, stomatal conductance, and root length of transgenic cotton plants under drought stress (Hassan et al. 2021). Our study also yielded similar results. Compared with the UT lines or RNAi-*VyUSPA3* lines, the OE-*VyUSPA3* lines had smaller stomatal aperture, more developed roots and higher leaf relative water content. This indicates that the *VyUSPA3* gene could improve drought tolerance of grapes by promoting root growth and reducing stomatal openings and delaying leaf water loss.

Under drought stress, the changes in some important physiological and biochemical indexes, such as proline content, MDA accumulation and antioxidant enzyme activity in plants can reflect the drought tolerance ability of plants (Kar and Mishra 1976; Davey et al. 2005; Shi et al. 2018). Our results showed that the proline content in the OE-*VyUSPA3* lines was much higher than either UT lines or RNAi-*VyUSPA3* lines after the drought treatment, and the MDA content was lower in the OE-*VyUSPA3* lines. Proline, a crucial osmotic regulator, accumulates quickly in plants in response to drought stress (Shi et al. 2018). Under drought stress, a higher accumulation of proline can lower cell osmotic potential and decrease leaf water loss, thus enhancing the drought tolerance of the plant. MDA accumulation reflects the level of lipid peroxidation of the plant cell membrane, and thus a lower MDA content is a signature of the stability of the cell membrane (Sudhakar et al. 2001). Moreover, excessive ROS accumulation can be toxic to plant cells, nonetheless, a higher antioxidant enzyme activity can ensure a stronger ROS scavenging capacity (Kapoor et al. 2019). Compared with the UT or RNAi-*VyUSPA3* lines, the contents of H_2O_2 and $O_2^{\bullet-}$ in the OE-*VyUSPA3* lines were lower and the activities of POD, SOD, and CAT in the OE-*VyUSPA3* lines were higher under drought treatment. The insignificant difference in CAT activity between the two OE-*VyUSPA3* lines and the UT lines

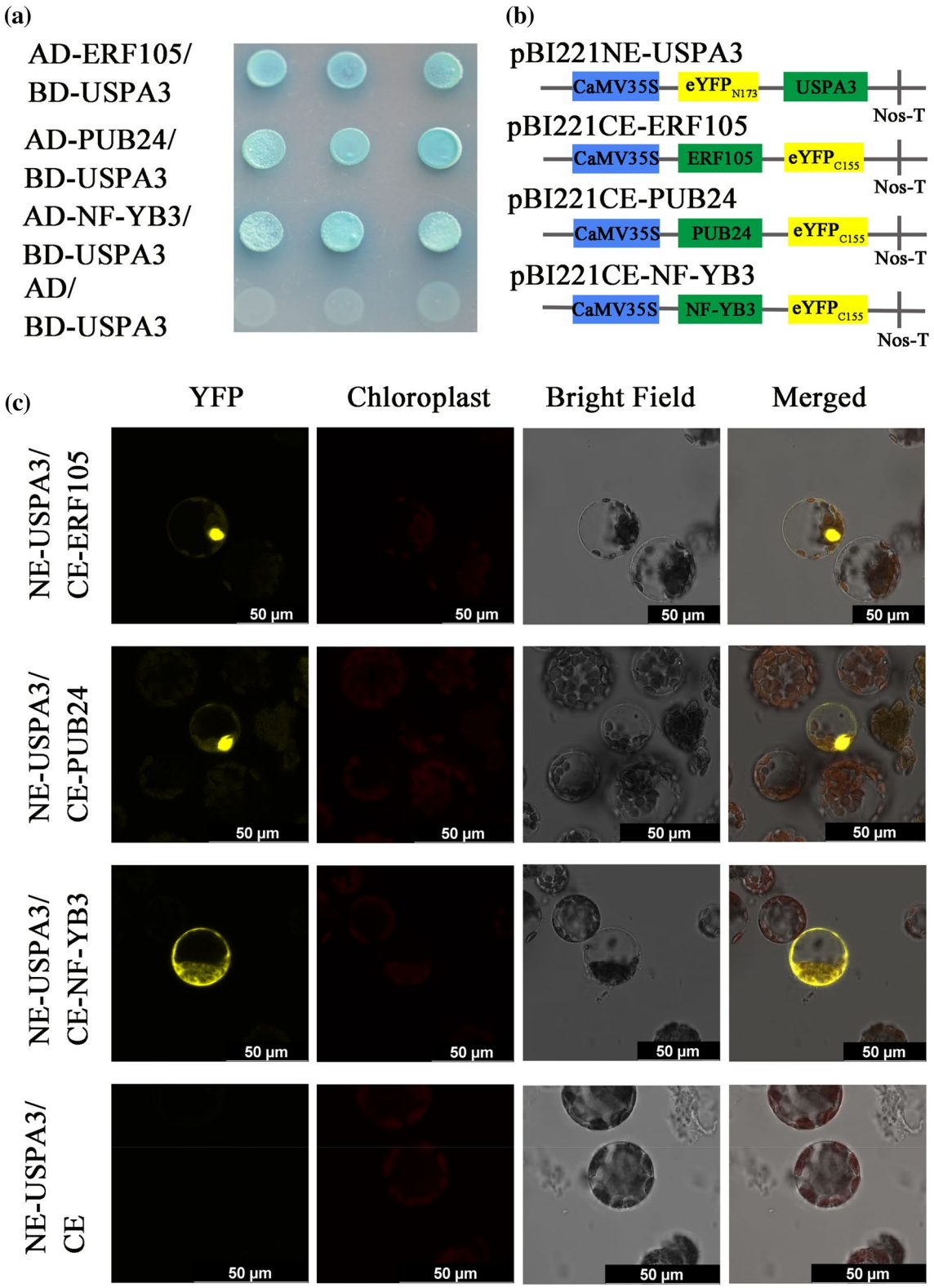

Fig. 7 The interactions of VyUSPA3 with ERF105, PUB24, and NF-YB3 were verified by yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. **a** Yeast two-hybrid. The pGBKT7-*VyUSPA3* and pGADT7 vectors were co-transformed as the controls. **b** Depicted genetic construction used for BiFC. **c** BiFC assay. Scale bars = 50 μm

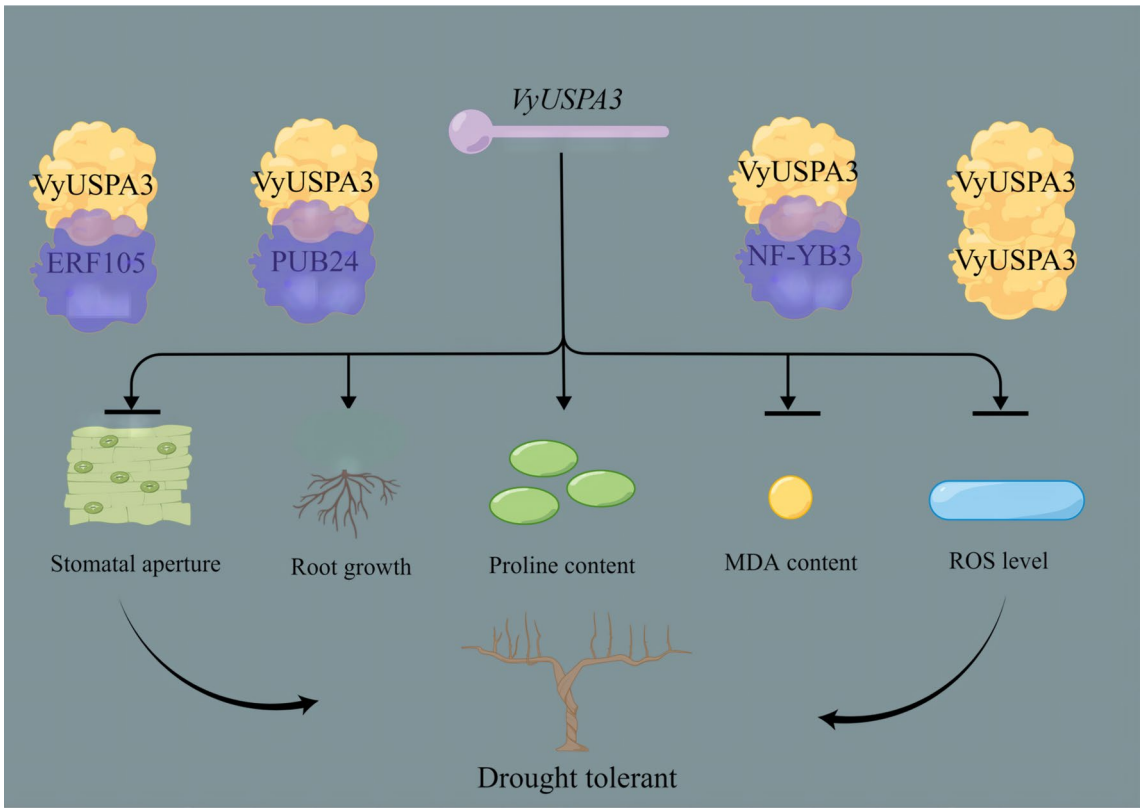

Fig. 8 A hypothetical working model of VyUSPA3 regulating and enhancing drought tolerance. On the one hand, *VyUSPA3* can accelerate root growth and the accumulation of osmoregulatory substance proline, reduce harmful malondialdehyde accumulation, and increase the activity of antioxidant enzymes (POD, SOD and CAT) to remove ROS. On the other hand, *VyUSPA3* can up-regulate the transcription of genes involved in ABA synthesis or signaling pathways and decrease the stomatal aperture. In addition, VyUSPA3 can physically interact with ERF105, PUB24, NF-YB3 and itself to regulate the drought tolerance in grape

may be attributed to the overlapping functions of CAT and POD in certain contexts. The main role of SOD is to dismutate $O_2^{\bullet-}$ into H_2O_2 (Kapoor et al. 2019), while the main function of POD and CAT is to remove H_2O_2, and ascorbate peroxidase also catalyzes the production of H_2O from H_2O_2 (Nakano and Asada 1981; Kapoor et al. 2019). These results show that the *VyUSPA3* gene functions in removing ROS by increasing the activity of antioxidant enzymes. Apart from physiological and biochemical processes, plants can also increase their resistance to stress by regulating the expression of certain stress-related genes. The transcript levels of *RD22*, *RD29B*, *DREB2A*, and *NCED1*, especially *RD29B* were always greater in OE-*VyUSPA3* plants than in the UT and RNAi-*VyUSPA3* lines. Previous studies have shown that these genes are strongly associated with ABA to varying degrees, for example, *RD22* and *RD29B* are ABA-dependent (Virlouvet et al. 2014), *NCED1* is involved in ABA synthesis (Liu et al. 2016), and *DREB2A* is induced by ABA (Kim et al. 2011). Our results suggest that *VyUSPA3* improved the drought tolerance of transgenic grapes probably by regulating ABA signaling pathway.

To further clarify the drought tolerance mechanism of *VyUSPA3*, we carried out yeast two-hybrid and BiFC experiments, and uncovered three proteins such as ERF105, PUB24, and NF-YB3 that interacted with VyUSPA3. ERF105 is a member of the AP2/ERF family, and ERF transcription factors are known to be involved in the regulation of various developmental processes and biotic and abiotic stresses (Joo et al. 2013; Bolt et al. 2017; Xie et al. 2019). *Arabidopsis ERF6/ERF103* is the ortholog of *VvERF105*, and responds to oxidative stress (Vermeirssen et al. 2014) and cold (Xin et al. 2007), but its involvement in drought resistance has not been reported. We found that *erf6* mutants of *Arabidopsis* are more tolerant to drought stress than the UT lines, indicating that *AtERF6* negatively regulates drought tolerance in *Arabidopsis*. This study suggests that *VvERF105* may negatively regulate the drought tolerance of grapes as well. It is highly likely that some regulatory relationships between USPA3 and ERF105 may exist except for physical interaction. It is also possible that the presence of other interacting proteins influences their binding. PUB24 is a U-box type E_3 ubiquitin ligase. The ubiquitination system

plays a prominent role in plant growth and development, immune regulation, and resistance to abiotic stresses (Gong et al. 2020). So far, *PUB* genes have been reported to positively regulate the resistance of plants to cold and bacterial pathogen *Xanthomonas euvesicatoria* (Yao et al. 2017; Liu et al. 2021), while certain *PUB* genes act as negative regulators of drought stress tolerance (Cho et al. 2008; Seo et al. 2021). The Nuclear Factor (NF) belongs to the CCAAT-box binding factor (CBF) and consists of three subunits, namely NF-YA, NF-YB, and NF-YC (Kim et al. 1996). Overexpression of the *Picea wilsonii NF-YB3* gene enhances the tolerance of *Arabidopsis* to salt and drought stress by regulating the expression of genes that depend on the CBF pathway (Zhang et al. 2015). Overexpressing the wheat *NF-YB3:1* gene in tobacco improves the tolerance of transgenic tobacco to drought stress by regulating the ABA signaling pathway (Yang et al. 2017). Based on the expression pattern of the three genes under drought stress and the relationship between the three interacting proteins and plant drought resistance, we speculate that VyUSPA3 cooperates with NF-YB3 and antagonizes ERF105 and PUB24 to regulate the drought tolerance of grape plants.

In summary, the function of the *VyUSPA3* gene from *V. yeshanensis* was confirmed by analyzing the OE-*VyUSPA3* and RNAi-*VyUSPA3* grape lines in the current study. The results indicate that the *VyUSPA3* gene from Chinese wild grapes can positively regulate drought tolerance in cultivated grapes. Furthermore, VyUSPA3 interacted physically with ERF105, PUB24, and NF-YB3, thus regulating the drought tolerance of the grapevine (Fig. 8). These findings advance our current understanding of the regulation network of drought tolerance and provide new insights into promising genetic resources for grapevine breeding with drought tolerance.

Author contributions statement ZJX designed the experiments and revised the manuscript. CXY carried out the experiments and drafted the manuscript. ZPY and CCC performed the experiments. All authors read and approved the manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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