R2R3-MYB TRANSCRIPTION FACTOR, *MYB6*, FROM GRAPES CONFERS ENHANCED SALT STRESS TOLERANCE IN TRANSGENIC TOBACCO

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Abstract. The R2R3-MYB family genes encode numerous highly conserved transcription factors that are critical for the plant development and stress response, but there is currently little information on the role of this genes in grape salt tolerance. Salt stress limits grape production, resulting in considerable annual reduction in both yield and quality. The identification and analysis of the stress-related genes from grapes could lead to the development of cultivars that can be stress-tolerant. We identified *MYB6*, a R2R3-MYB transcription factor from grape 'Yatomi Rose' (*Vitis vinifera* L.), and characterized the stress response phenotypes of tobacco lines overexpressing this gene. It was found that the root length, fresh weight, and height of *VvMYB6*-overexpression (*VvMYB6*-OE) tobacco was significantly different from that of the empty vector-transformed (EV) tobacco under 0.1 M NaCl conditions. Under salt stress, the blue-brown patches of transgenic tobacco leaves with *VvMYB6*-OE were less than those of EV-transformed tobacco, and the color was lighter than that of EV tobacco, indicating a reduction in reactive oxygen species (ROS). *VvMYB6*-OE tobacco plants showed less electrolyte leakage, whereas an increase in the chlorophyll and proline content was observed. Additionally, the *VvMYB6*-OE tobacco plants exhibited reduced malondialdehyde (MDA) and H_2O_2 content due to the increase in the activity of the antioxidant enzymes. Taken together, these findings significantly improve the current understanding of the role of the R2R3-MYB transcription factors in plant salt stress tolerance and could lead to the development of more stress tolerant lines.
Keywords: grape, salt stress, MYB6, physiological and biochemical indicators, gene functional verification

Introduction

European grape (*Vitis vinifera* L.) is one of the most economically important fruit trees worldwide, with the second-largest production area and output in the global fruit tree industry (Yu et al., 2020). Soil salinization is an important environmental factor affecting the development of crop industry and a serious problem faced by crop cultivation all over the world. According to the data of the second national soil census, the total area of saline soil in China is about 36 million hm^2, accounting for 4.88% of the country's available land area. Grape is a non-halophytic plant, has a certain ability to tolerate salt and alkali, but the salt damage degree exceeds a certain threshold, its growth and development will be inhibited. Currently, European grape cultivars represent the vast majority of cultivated grapes; Currently, European grape cultivars represent the vast majority of cultivated grapes; however, various environmental factors, such as water scarcity and soil salinization present significant negative impacts on its yield and quality (Yang et al., 2007; Li et al., 2021). The identification of abiotic

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stress tolerance genes could lead to the development of robust cultivars that can cope with these adverse conditions (Vannozzi et al., 2018).

Salt stress response pathways in plants are influenced by the R2R3-MYB transcription factors. Many MYB transcription factors linked to stress tolerance have been cloned from plants to date. For example, the overexpression of the apple *MdSIMYB1* gene has showed an improvement in the tolerance to a variety of stresses in transgenic plants (Wang et al., 2014). Additionally, salt stress has been found to increase the expression level of mangrove *AmMYB1*, and high constitutive expression of the *AmMYB1* gene in tobacco, thus resulting in salt stress tolerance (Ganesan et al., 2012). *BcMYB1* is known to be upregulated in *Boea crassifolia* during drought and is also affected by saline-alkali stress (Chen et al., 2005). During abscisic acid (ABA), salt, drought, or low temperature treatments, the expression of *GmMYB76*, *GmMYB92*, and *GmMYB177* genes in soybean were found to be up-regulated (Liao et al., 2008). The amount of mRNA transcribed from the *GmMYBJ6* gene increased dramatically after salt stress treatment, demonstrating that the transition of the *GmMYBJ6* gene from DNA to mRNA and then to protein was strongly related to the salt stress (Yang et al., 2009). Taken together, these findings highlight the multiple roles that plant MYB transcription factors play in response to environmental stress.

MYB transcription factors are a family of DNA-binding proteins that play a variety of roles in plants, including growth and development, organ formation, and response to abiotic stress (Sun et al., 2014). This family regulates the expression of a number of other genes, affecting the morphology of plant tissues in response to environmental stimuli. MYB transcription factor genes have been studied extensively across many plant species (Tamura et al., 2011; Ganesan et al., 2012). However, there have been few reports on the role of this gene family in grape salt stress responses. In order to further explore and identify more MYB transcription factors affecting the growth and development of grapes, this study employed the European grape ‘Yatomi Rose’ as the research object using a combination of bioinformatics and molecular biology techniques to obtain a MYB member containing R2R3-MYB domain, named *VvMYB6*. After *VvMYB6* gene was overexpressed in the model plant tobacco, the function of *VvMYB6* gene in the overexpressed tobacco plant was analyzed in terms of abiotic stress, so as to provide an important theoretical basis for further improving the cultivation of new cultivars of grape stress resistance by genetic engineering.

Materials and methods

Plant material and treatments

Four-year old ‘Yatomi Rose’ (*Vitis vinifera* L.) (Red table grape cultivar) was grown in the Henan Institute of Science and Technology, Xinxiang, Henan, China. Tobacco plants (*Nicotiana tabacum* ‘NC89’) were grown *in vitro* on MS medium under a 16 h/8 h light/dark photoperiod at 25°C. Tobacco (*Nicotiana tabacum* ‘NC89’) was used for gene function verification. *Escherichia coli* DH5α and *A. tumefaciens* GV3101 were utilized (Shanghai Sangon Biotech Co., Ltd.) as vectors.

RNA extraction, detection, and reverse transcription studies

RNA and DNA was extracted from the grape leaves by SDS/phenol and CTAB methods, respectively (Doyle and Doyle, 1987; Wang et al., 2004). The first strand of

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cDNA was synthesized using Promega's M-MLV reverse transcriptase. Primers were designed based on the 'Pinot Noir' [*Vitis vinifera* (Pinot Noir.)] genome by Primer 5.0 software ("The French-Italian Public Consortium for Grapevine Genome Characterization", 2007). The primers used to amplify *MYB6* were: ATGGGAAGAGCTCCCTGTTG (forward) and TTAAGCAGATAGCGATTCCACT (reverse).

VvMYB6-overexpression vector

A PCR cycle system was used to amplify and recover the *VvMYB6* fragment and cloned into the vector pMD18-T. The *E. coli* cells were transformed, and the positive monoclonal bacteria were selected via the elimination method. The plasmid DNA was extracted by expanded propagation culture of the bacteria solution. The plasmid was incised by *SalI and BstEII* enzymes for detection, and was detected by electrophoresis on a 1% concentration agar gel. Subsequently, the sequencing results and gene protein sequence were analyzed (Shanghai Sangon Biotech Co., Ltd.).

Bioinformatics analysis

The BLAST program from NCBI (http://www.ncbi.nlm.nih.gov/) was used to search for protein sequences with high similarity to the *VvMYB6* gene. Bioinformatics analysis, including the localization of *VvMYB6* gene in the grape genome, phylogenetic tree analysis, sequence analysis, and subcellular localization, are available in our previously published study (Zhu et al., 2021). We obtained the *VvMYB6* gene sequence of 816 bp, including 1 intron (96 bp) and 2 exons (133 bp and 683 bp), located on chromosome 2 of the genome and subcellular on the nucleus, which is a typical MYB transcription factor of the R2R3 type.

Genetic transformation of tobacco and acquisition of transgenic plants

The pMD18-T vector clone was digested with *SalI* and *BstEII* endonucleases and inserted into the overexpression vector pCAMBIA1301, resulting in the final overexpression vector pCAMBIA1301: *VvMYB6*. The vector plasmid was transferred into *Agrobacterium* GV3101 by electroporation, and the *VvMYB6* gene was transformed into tobacco via the leaf disk method of Horsch et al. (2005). Positive plants (T0) were screened by PCR (*Tables 1* and *2*). After self-pollination of T0 generation plants, their seeds (T1) were collected. Transgenic homozygous T2 generation seeds were utilized for subsequent experiments. Three independent lines of T3 seeds were ultimately obtained and used for all subsequent experiments. T2 lines that produced 100% kanamycin-resistant plants in the T3 lines were considered as homozygous transformants. In each experiment, the EV-transformed and *VvMYB6*-OE tobacco line (OE#1, OE#2, OE#3) was selected for further analysis.

Table 1. *PCR reaction system (20 μl) Screening system*

Reaction component	Reaction dosage
2×Tag PCR Master Mix II	10 μl
Primer (10 μM)	2 μl
Template	1 μl
ddH_2O	7 μl

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Table 2. *Screening reaction procedure*

Steps	Reaction temperature	Reaction time
Step 1 Pre-denaturation	95℃	5 min
Step 2 Pre-denaturation	95℃	30 s
Step 3 Annealing	55℃	1 min
Step 4 Extension	72℃	2 min
Step 5 Extension	72℃	10 min
Step 6 Extension	16℃	5 min
From step 2 to 4, 35 cycles were employed		

Determination of germination rate of tobacco seeds under salt stress

Tobacco seeds were sterilized with 10% NaClO and sterile water and sown on a plastic Petri dish (6 cm × 6 cm) containing MS solid medium with different concentrations of NaCl (0.1 M and 0.15 M) (Two concentration gradients of set after search literature and pre-experiment) (Murashige and Skoog, 1962; Feng, 2014). The blank MS solid medium was used as control. The germination rates of EV-transformed and *VvMYB6*-OE tobacco line (OE#1, OE#2, OE#3) seeds were calculated after 7 days at 25°C, 16 h light and 8 h dark.

$$\text{Germination rate}\,(\%) = \frac{\text{number of germinated seeds}}{\text{total number of seeds sown}} \times 100\% \qquad \text{(Eq.1)}$$

Measurement of growth indices of transgenic tobacco under salt stress

The sterilized EV-transformed and *VvMYB6*-OE tobacco line seeds were inoculated on plastic Petri dishes (6 cm × 6 cm) containing MS solid medium with different concentrations of NaCl (0.1 M and 0.15 M). After sealing, the seedlings were cultured at 25°C, 16 h light and 8 h dark. After 7 d, the root length, height, and fresh weight of EV-transformed and *VvMYB6*-OE tobacco line (OE#1, OE#2, OE#3) was measured under salt stress conditions.

Measurement of changes in reactive oxygen species (ROS) content of tobacco under salt stress

For DAB staining, the EV-transformed and *VvMYB6*-OE tobacco seedlings under different salt stress treatments were immersed in 1% DAB solution in 50 mM Tris-HCl buffer (pH 6.5). After 30 min vacuum infiltrating, the immersed leaves were incubated in the dark for 20 h at room temperature. Subsequently, the leaves were bleached by bath in boiling ethanol until the brown spots appeared clearly. The area of brown spots is represented the DAB reaction degree to H_2O_2. EV-transformed and *VvMYB6*-OE tobacco seedlings under different salt stress treatments were immersed in 1% NBT solution in 10 mM potassium phosphate buffer (pH 7.8). After 15 min vacuum infiltrating, the immersed leaves were incubated in the dark for 20 h at room temperature. The leaves were then fixed and cleared in alcoholic lacto-phenol (2:1:1, 95% ethanol: lactic acid: phenol) at 65°C for 30 min, rinsed with 50% ethanol, and then rinsed with water. When NBT interacts with superoxide, the blue precipitate form in leaves can be seen under a microscope, which was photographed (Nikon, Tokyo, Japan) (Jiang et al., 2016).

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Determination of chlorophyll content, proline content, malondialdehyde (MDA) content, and electrolyte leakage (EL)

Tobacco plants were cultured on MS medium, at 25 ± 1°C, 16 h/8 h light/dark photoperiod and 70 ± 5% relative humidity. During 0.1 M NaCl treatments, plants overexpressing *VvMYB6* or transformed with an EV were cultured for 7 d, and were transplanted to soil. After 21 d, the plants were placed in a growth chamber and grown under normal conditions (16 h/8 h light/dark cycle, 25°C/18°C day/night). For experiments using salt stress, the plants were watered with 0.1 M NaCl for up to 15 d, following which these plants were started to be watered with normal water again. The recovered tobacco plants were used to calculate the survival rate.

Chlorophyll content was determined by the spectrophotometric method using testing kits (BC0990; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Proline content was determined by the method of Maghsoudi et al. (2018). The content of malondialdehyde (MDA) was determined by thiobarbituric acid method (Li et al., 2013). EL was measured by the method of Chen et al. (2015).

Determination of antioxidant enzyme activity and H_2O_2 content

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were determined spectrophotometrically using testing kit (BC0170, BC0220, and BC0200; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The content of H_2O_2 was determined by titanium sulfate colorimetry (Estrada-Melo et al., 2015).

Statistical analysis of data

All the data shown represent the mean value of 3 replicates, and all data are shown as mean ± standard error with one-way analysis of variance (one-way ANOVA) conducted by the SPSS 20 software. The p-value in the figure legends is shown as: $*P < 0.05$; $**P < 0.01$.

Results and analysis

Acquisition and verification of the VvMYB6-overexpressed tobacco plants

The T0 generation of *VvMYB6*-overexpressed (*VvMYB6*-OE) tobacco plants were obtained through a series of steps such as pre culture, infection, co culture, bacterial washing and differentiation. The homozygous lines of T2 and T3 generations were obtained by multi generation inbreeding. The *VvMYB6* gene was detected in all the transgenic tobacco plants and resulted in a band with the expected size of 816 bp, while no band was found in the EV-transformed plants (*Fig. 1*).

Effect of VvMYB6-overexpression on seed germination of tobacco plants under salt stress

In order to understand the function of *VvMYB6* in response to salt stress, we conducted the seed germination experiments (Zhu et al., 2021). In the blank media (MS medium), there was no significant difference in the germination of EV-transformed and *VvMYB6*-OE seeds. Seed germination of *VvMYB6*-OE lines (OE#1, OE#2, OE#3) was better than that of EV-transformed tobacco lines under NaCl stress (*Fig. 2A*). The

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germination rate of *VvMYB6*-OE lines (OE#1, OE#2, OE#3) grown on MS medium containing 0.1 M NaCl for 7 d was significantly higher than that of EV-transformed tobacco, reaching 41.67%, 73.33%, and 30.00%, respectively (*Fig. 2B*). The germination rate of *VvMYB6*-OE lines (OE#1, OE#2, OE#3) grown on MS medium containing 0.15 M NaCl for 7 d was higher than that of EV-transformed tobacco, reaching 26.67%, 25.00%, and 24.44%, respectively, whereas the germination rate of EV-transformed tobacco seeds was only 17.2% (*Fig. 2B*). Taken together, these results demonstrate that *VvMYB6*-OE gene increased the germination rate of tobacco seeds during salt stress.

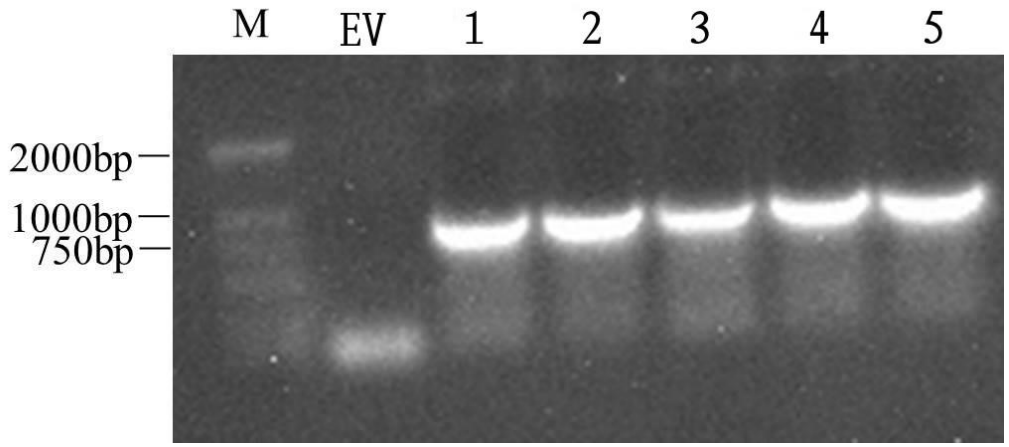

Figure 1. *Detection of VvMYB6 expression in transformed tobacco plants by PCR. M: Yez-2000 DNA marker; EV: EV-transformed; 1, 2, 3, 4, 5: Transgenic lines*

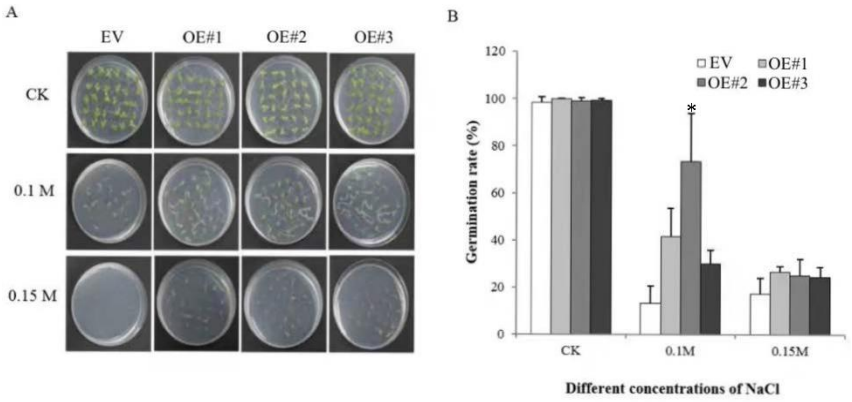

Figure 2. *Effects of different concentrations of NaCl on seed germination. (A) Seed germination of EV-transformed and VvMYB6-OE lines (OE#1, OE#2, OE#3) under NaCl salt stress, CK is EV-transformed. (B) Germination rate of EV-transformed and VvMYB6-OE lines (OE#1, OE#2, OE#3) under NaCl salt stress, CK is EV-transformed. Data presented as the mean ± SD; level of significance indicated by **, $P < 0.05$*

Effect of VvMYB6-overexpression on the growth of tobacco plants under salt stress

It was observed that the *VvMYB6*-OE lines (OE#1, OE#2, OE#3) grew faster than the EV-transformed tobacco lines under NaCl salt stress (*Fig. 3A*). After growing on MS

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medium containing 0.1 M NaCl for 7 d, the root length and plant height of EV-transformed tobacco seedlings was 13.457 mm and 2.59 mm, respectively, which was significantly higher than compared with *VvMYB6*-OE tobacco, while the fresh weight of the seedlings was lower than that of *VvMYB6*-OE tobacco. The root length of the three *VvMYB6*-OE tobacco seedlings was 2.226 mm, 2.202 mm and 3.88 mm, respectively, after growing on MS medium containing 0.15 M NaCl for 7 d, and the fresh weight was 0.0153 g, 0.013 g and 0.0165 g, respectively, which was significantly higher than the EV-transformed seedlings. These results indicate that high levels of NaCl reduced the growth of both EV-transformed and *VvMYB6*-OE tobacco lines (*Fig. 3*). Under salt stress, the *VvMYB6*-OE lines (OE#1, OE#2, OE#3) grew well, but as the level of salt stress increased, the growth of both EV-transformed and *VvMYB6*-OE lines (OE#1, OE#2, OE#3) was reduced.

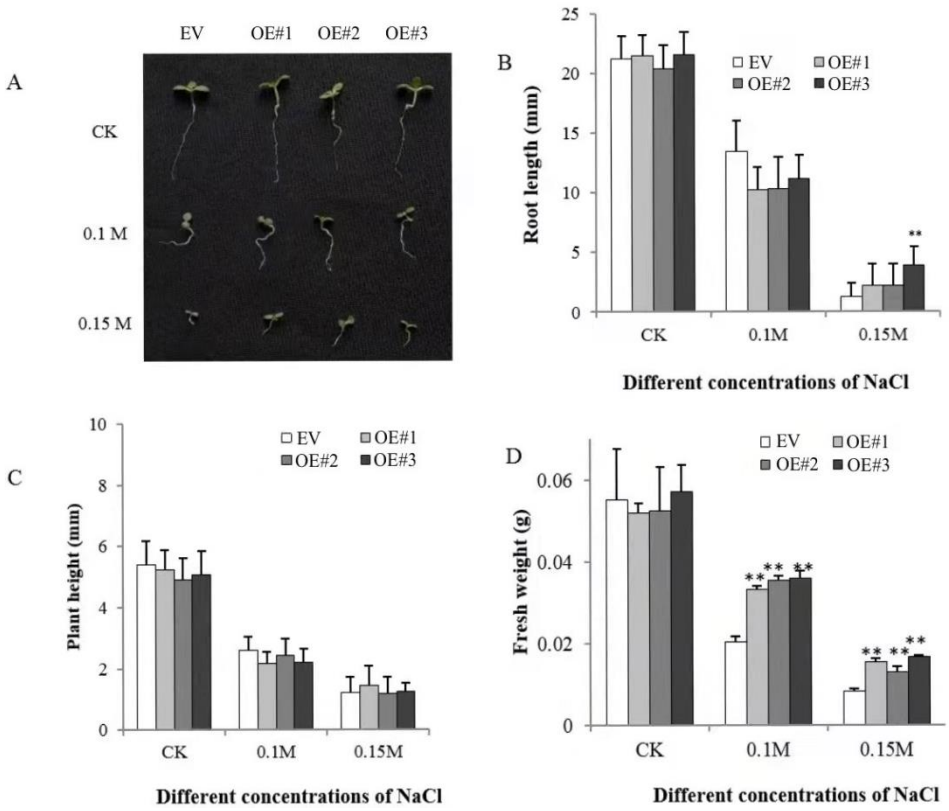

Figure 3. *Effects of different concentrations of NaCl on plantlet growth. (A) 7d plantlet growth of EV-transformed and VvMYB6-OE (OE#1, OE#2, OE#3) tobacco lines under NaCl salt stress, CK is EV-transformed; (B) Root length; (C) Plant height; (D) Fresh weight of EV-transformed and VvMYB6-OE lines (OE#1, OE#2, OE#3) under NaCl salt stress. CK is EV-transformed. Data presented as mean ± SD; level of significance indicated by **, P < 0.01*

Effect of VvMYB6-overexpression on ROS levels of tobacco plants under salt stress

Under NaCl stress, the NBT and DAB staining of EV-transformed and *VvMYB6*-OE lines (OE#1, OE#2, OE#3) were observed (*Fig. 4A, B*). There was no significant difference between the leaves of EV-transformed and *VvMYB6*-OE lines (OE#1, OE#2, OE#3) under normal conditions. There were fewer blue and brown spots on the leaves of *VvMYB6*-OE lines (OE#1, OE#2, OE#3) compared to the EV-transformed tobacco

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leaves under NaCl salt stresses, indicating that *VvMYB6*-OE reduced the accumulation of ROS in tobacco under NaCl salt stresses (*Fig. 4A, B*).

H_2O_2 is essential for signal transduction during abiotic stress reactions. At 10 d after 0.1 M NaCl treatment, H_2O_2 content was increased in both *VvMYB6*-OE lines (OE#1, OE#2, OE#3) and the EV-transformed plants compared to the control (0 d) (*Fig. 4C*). At 10 days after salt stress, the H_2O_2 level in *VvMYB6*-OE lines (OE#1, OE#2, OE#3) was considerably lower ($P < 0.01$) than that in the EV plants.

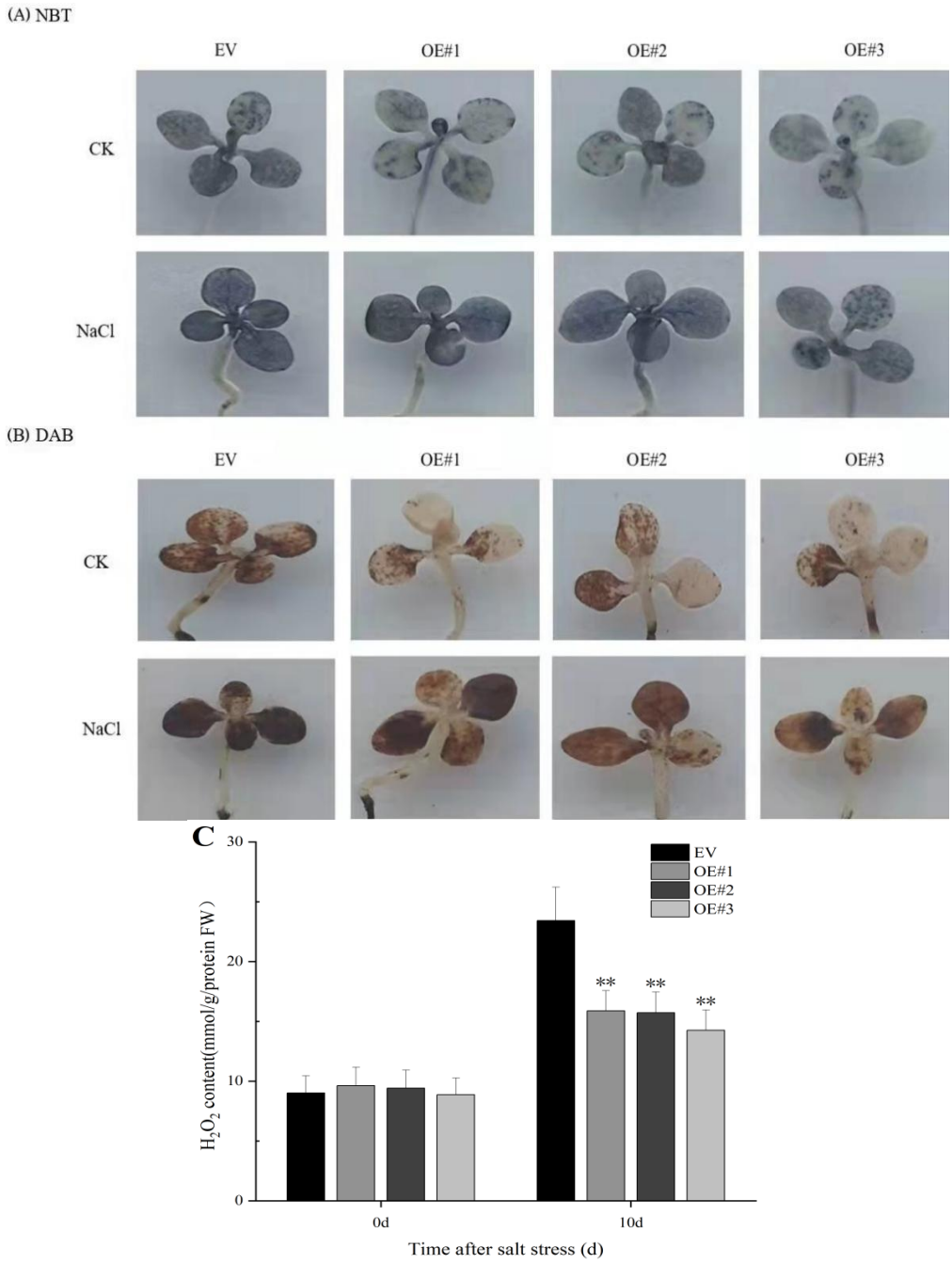

Figure 4. *Changes in ROS in leaves of EV-transformed and VvMYB6-OE (OE#1, OE#2, OE#3) tobacco lines under 0.1 M NaCl salt stress. Using 0 d as a control. (A) NBT staining of tobacco seedlings; (B) DAB staining of tobacco seedlings; (C) H_2O_2 content. Data presented as mean ± SD; level of significance indicated by **, P < 0.01*

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Effect of VvMYB6-overexpression on the physiological parameters of tobacco plants under salt stress

We assessed the chlorophyll, proline, EL, and MDA content under salt stress to determine the underlying mechanisms that caused the *VvMYB6*-OE lines (OE#1, OE#2, OE#3) to have increased salt stress tolerance. Under normal conditions, the chlorophyll content of *VvMYB6*-OE and EV-transformed leaves was identical. The chlorophyll content of *VvMYB6*-OE leaves was considerably higher than the EV-transformed leaves at 10 days after 0.1 M NaCl salt stress ($P < 0.01$) (*Fig. 5A*). Additionally, the proline content of *VvMYB6*-OE and EV-transformed plants was also identical under normal conditions, but was higher in the *VvMYB6*-OE plants under NaCl salt stress (*Fig. 5B*). These findings indicate that increased chlorophyll and proline accumulation was correlated with an improved salt stress in *VvMYB6*-OE lines (OE#1, OE#2, OE#3).

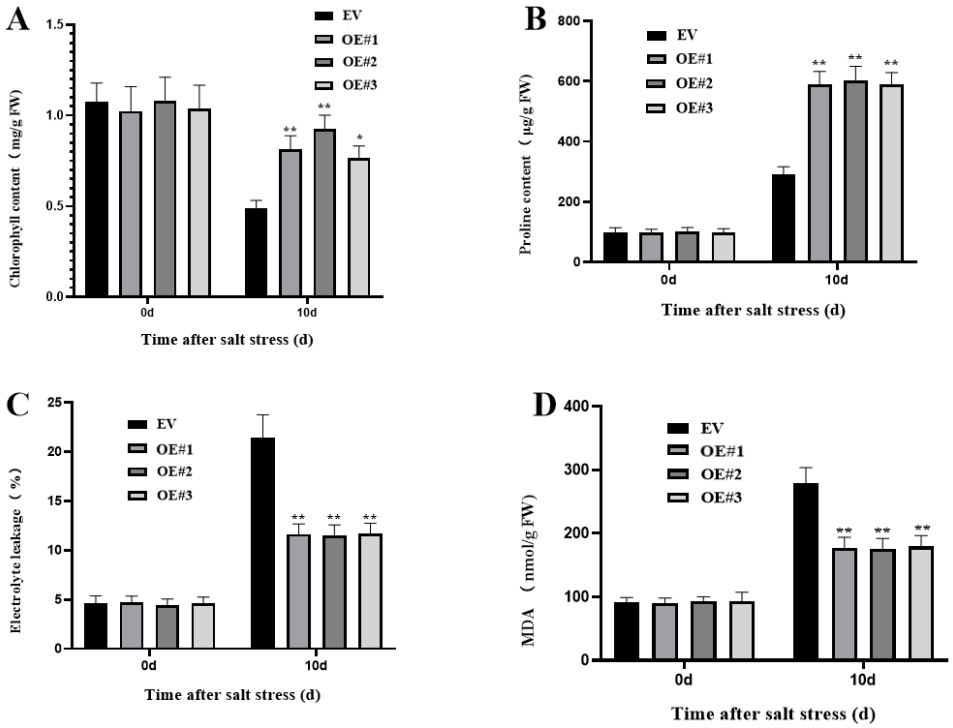

Figure 5. *Chlorophyll content, proline content, EL, and MDA content of the 3 VvMYB6-OE tobacco lines (OE#1, OE#2, OE#3) and EV plants under 0.1 M NaCl treatment. (A) Chlorophyll content; (B) Proline content; (C) Electrolyte leakage; (D) MDA content in leaves. CK is EV-transformed. Data presented as the mean ± SD, level of significance indicated by *, $P < 0.05$ and **, $P < 0.01$*

EL is a method for determining the osmotic tolerance of plants. The water status of plants was assessed after 0.1 M NaCl salt treatment, and the results indicated that the EL values of all plants were higher than the control (0 d). However, EL values in *VvMYB6*-OE lines (OE#1, OE#2, OE#3) were significantly lower than that in EV-transformed plants ($P < 0.01$) at 10 d post 0.15 M NaCl salt treatment (*Fig. 5C*). The MDA level of *VvMYB6*-OE lines (OE#1, OE#2, OE#3) was similar to that of EV-transformed under normal growth conditions, but 10 days after 0.1 M NaCl salt treatment, the MDA level of EV lines was considerably higher than *VvMYB6*-OE lines

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(OE#1, OE#2, OE#3) (*Fig. 5D*). Under salt stress conditions, a higher EL may cause more substantial damage to the cell membranes, whereas a higher MDA level suggests a severe salt stress. The differences in EL and MDA levels of the *VvMYB6*-OE lines (OE#1, OE#2, OE#3) indicated that they likely exhibited a reduced damage under salt stress compared to the EV-transformed seedlings. Taken together, these findings indicate that *VvMYB6*-OE results in increased tolerance to salt stress.

Effect of VvMYB6-overexpression on the biochemical parameters of tobacco plants under salt stress

Enzymatic antioxidants are crucial for maintaining ROS homeostasis. To determine their role in stress tolerance of *VvMYB6*-OE tobacco plants, we examined the oxidase enzyme activity in the 3 *VvMYB6*-OE tobacco lines (OE#1, OE#2, OE#3) and EV-transformed lines under 0.1 M NaCl treatment. SOD activity in *VvMYB6*-OE plants (OE#1, OE#2, OE#3) was substantially higher than that in the EV-transformed plants after 10 d NaCl treatment with 0.1 M NaCl ($P < 0.01$). Similar results were found for POD and CAT activities (*Fig. 6A-C*) after 10 days of 0.1 M NaCl treatment. These findings indicate that *VvMYB6*-OE tobacco plants exhibited an increased stress tolerance under salt stress conditions through higher levels of antioxidant enzyme activities.

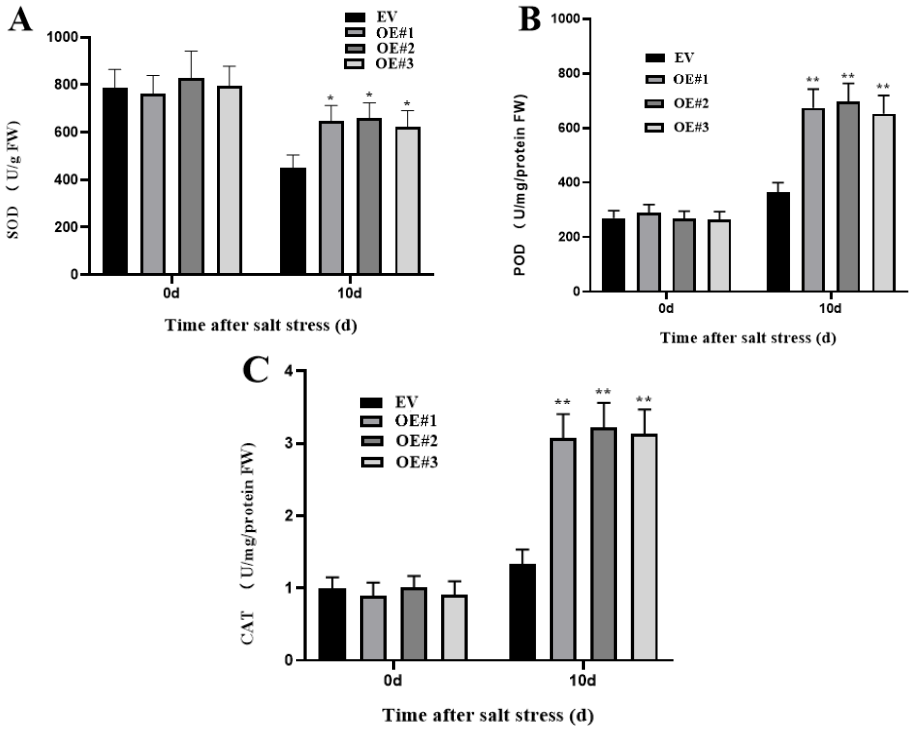

Figure 6. *Oxidase enzyme activities of VvMYB6-OE tobacco lines (OE#1, OE#2, OE#3) and EV plants under 0.1 M NaCl treatment. (A) SOD; (B) POD; (C) CAT. CK is EV-transformed. Data presented as mean ± SD; level of significance indicated by *, P < 0.05 and **, P < 0.01*

Discussion

Plants are frequently affected by abiotic stress throughout their growth and development, including drought, soil salinization, and other stresses (Wang and Cheng,

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2017). Abiotic stress causes a series of changes in the morphological structure and physiological activities of the plants, affecting seed germination, plant growth, blooming, and fruiting, as well as diminishing yield and quality (Guo et al., 2020; Urao et al., 1993). Plants have evolved multiple metabolic pathways with sophisticated networks that control gene expression in response to various abiotic stresses. MYB transcription factors have been shown to play a prominent role in the morphological alteration, differentiation, and functional maturity of plant tissues and organs in response to unfavorable environmental conditions (Mengiste et al., 2003). Abiotic stress response pathways have been found to be heavily reliant on the R2R3-MYB transcription factors, and many MYB transcription factors linked to stress tolerance have been cloned from various plants to date (Rahaie et al., 2010). High salt stress has been shown to alter the expression of many genes in *Arabidopsis*, including *AtMYB1*, *AtMYB2*, *AtMYB108*, and *AtMYB20*. Overexpression of these genes has also been shown to improve tolerance to salt stress (Zhang et al., 2011). In wheat, high salinity conditions have been shown to increase the expression levels of *TaMYB30*, *TaMYB73*, *TaMYB32*, *TaMYB56-B*, *TaMYBsdu1*, and *TaMYB33*, and overexpression of these genes results in more stress-tolerant wheat plants (Mao et al., 2011; Qin et al., 2012; Pasquali et al., 2008; Quan et al., 2010). *OsMYB91* and *OsMPS* genes in 2R-MYB class transcription factor members, improved the salt tolerance of rice in response to high salt signal (Yang et al., 2012; Zhu et al., 2015). Sugarcane sensitivity to salt stresses has been demonstrated to be influenced by the expression of the *ScMYBS1* gene (Prabu and Prasad, 2012), and the grape gene *VvMYB60* was found to be involved in osmotic stress response (Galbiati et al., 2011).

The root system is a vital part of a plant's reaction to stress, since it is responsible for taking in water and nutrients from the soil environment. Abiotic stress has been proven to significantly diminish the water content of plant roots in numerous studies. Plant growth reduction has been shown to become more evident with an increasing severity of the abiotic stress (Yang et al., 2020). Our results also indicated that abiotic stress reduced the growth index of tobacco; however, the *VvMYB6*-OE tobacco was impacted much less severely.

It is known that increasing levels of ROS present a visible damage to the plant cells. However, excess ROS can be eliminated by plants in order to compensate for stressful conditions (Luchi et al., 2001). In this study, DAB and NBT staining showed reduced staining in *VvMYB6*-OE tobacco compared to EV-transformed tobacco under salt stress, indicating that the transgenic tobacco eliminated more ROS, resulting in reduced damage. Taken together, our results demonstrate that *VvMYB6*-OE tobacco seedlings can effectively protect them from environmental stresses by increasing the activity of the antioxidant enzymes.

SOD, POD, and CAT all play essential roles in the scavenging of ROS. SOD first catalyzes O_2^- to produce oxygen and H_2O_2, whereas CAT and POD subsequently metabolize H_2O_2 to H_2O. We studied numerous physiological and biochemical parameters in both *VvMYB6*-OE and EV-transformed plants after salt treatment to better understand the mechanisms by which *VvMYB6*-OE results in increased salt stress tolerance. When compared to the EV-transformed lines, the SOD, POD, and CAT activities in *VvMYB6*-OE lines were higher under high salt conditions, implying that these enzymes likely contributed to the increased salt tolerance of the *VvMYB6*-OE lines. After salt treatment, both the chlorophyll and proline contents were found to be higher in the *VvMYB6*-OE lines than in the EV-transformed lines, although EL and

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MDA levels were higher in the EV-transformed lines. These findings are in line with a previous research (Feng et al., 2015), which suggested that MYB-mediated genes regulates the ROS buildup in plants, allowing them to withstand salt stress.

Our study demonstrated that *VvMYB6* gene from grapevine played an important role in the tolerance to salt stress. Under salt stress, the root length, fresh weight, and height of *VvMYB6*-OE tobacco was significantly different from the EV-transformed tobacco. The *VvMYB6*-OE tobacco showed fewer blue-brown patches on the leaves, and the color was lighter than that of EV-transformed tobacco, indicating a decrease in reactive oxygen species. The contents of chlorophyll and proline in *VvMYB6*-OE tobacco plants increased, while the contents of MDA and H_2O_2 decreased due to the increase of antioxidant enzyme activity. Furthermore, the *VvMYB6*-OE tobacco presented a significant salt stress tolerance than that of the EV-transformed tobacco. These findings indicate that the *VvMYB6*-OE gene significantly improved the salt stress tolerance of tobacco. Nevertheless, additional studies are needed in order to better understand the mechanisms by which the MYB transcription factor genes influences the grape salt stress tolerance.

Conclusions

In this paper, through the study of *VvMYB6* gene in plant salt tolerance, the following conclusions are drawn: (1) Through the comparative study on the phenotypic characteristics of *VvMYB6*-OE tobacco and EV-transformed tobacco, it was found that *VvMYB6*-OE tobacco showed strong salt tolerance. (2) Through the analysis of the results of physiological and biochemical indexes, it can be concluded that the increase and decrease of these indexes significantly improved the salt tolerance of the *VvMYB6* gene. This study shows that *VvMYB6* gene play an important role in plant salt tolerance, which provides an effective reference for the development of breeding in the future, and helps to cultivate more salt-tolerant cultivars, thus promoting the rapid development of grape industry.

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REFERENCES

[1] Chen, B. J., Wang, Y., Hu, Y. L., Wu, Q., Lin, Z. P. (2005): Cloning and characterization of a drought-inducible MYB gene from Boea crassifolia. – Plant Science 168(2): 493-500.

[2] Chen, Y. E., Cui, J. M., Su, Y. Q., Yuan, S., Yuan, M. (2015): Influence of stripe rust infection on the photosynthetic characteristics and antioxidant system of susceptible and resistant wheat cultivars at the adult plant stage. – Frontiers in Plant Science 6: 162219.

[3] Doyle, J. J., Doyle, J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. – Phytochemical Bulletin 19: 11-15.

[4] Estrada-Melo, A. C., Chao, C., Reid, M. S., Jiang, C. Z. (2015): Overexpression of an ABA biosynthesis gene using a stress-inducible promoter enhances drought resistance in petunia. – Horticulture Research 2: 15013.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_50175031

[5] Feng, J. Y. (2014): Effects of salt stress on seed germination and chlorophyll of transgenic tobacco with *ZMPP2C2* and wild tobacco. – Journal of Dezhou University 30: 79-81 + 92.
[6] Feng, S. Q., Xu, Y. C., Yang, L., Sun, S. S., Wang, D. Y., Chen, X. S. (2015): Genome-wide identification and characterization of R2R3-MYB transcription factors in pear. – Scientia Horticulturae 197: 176-182.
[7] Galbiati, M., Matus, J. T., Francia, P., Rusconi, F., Cañón, P., Medina, C., Conti, L., Cominelli, E., Tonelli, C., Arce-Johnson, P. (2011): The grapevine guard cell-related *VvMYB60* transcription factor is involved in the regulation of stomatal activity and is differentially expressed in response to ABA and osmotic stress. – BMC Plant Biology 11: 1-15.
[8] Ganesan, G., Sankararamasubramanian, H. M., Harikrishnan, M., Ashwin, G., Parida, A. (2012): A MYB transcription factor from the grey mangrove is induced by stress and confers NaCl tolerance in tobacco. – Journal of Experimental Botany 63(12): 4549-4561.
[9] Guo, Y., Ping, W. J., Huang, Y. Q., Chen, J. T., Zhu, Y. L., Zhao, Y. F., Guo, J. J. (2020): Integrative analysis of regulation of salt stress response by overexpression of MYB transcription factor in plants. – Journal of Shen Yang Agricultural University 51(1): 38-45.
[10] Horsch, M. A., Zhang, Z., Glotzer, S. C. (2005): Self-assembly of polymer-tethered nanorods. – Physical Review Letters 95(5): 056105.
[11] Jiang, Y., Qiu, Y., Yu, D. (2016): Heterologous expression of *AtWRKY57* confers drought tolerance in Oryza sativa. – Frontiers in Plant Science 7: 178425.
[12] Li, G. R., Xu, W. W., Jing, P. W., Hou, X. J., Fan, X. C. (2021): Overexpression of *VyDOF8*, a Chinese wild grapevine transcription factor gene, enhances drought tolerance in transgenic tobacco. – Environmental and Experimental Botany 190: 104592.
[13] Li, P., Ding, G., Deng, Y., Punyapitak, D., Li, D., Cao, Y. (2013): Determination of malondialdehyde in biological fluids by high-performance liquid chromatography using rhodamine B hydrazide as the derivatization reagent. – Free Radical Biology and Medicine 65(13): 224-231.
[14] Liao, Y., Zou, H. F., Wang, H. W., Zhang, W. K., Ma, B., Zhang, J. S., Chen, S. Y. (2008): Soybean *GmMYB76*, *GmMYB92*, and *GmMYB177* genes confer stress tolerance in transgenic Arabidopsis plants. – Cell Research 18(10): 1047-1060.
[15] Luchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K., Shinozaki, K. (2001): Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. – The Plant Journal 27(4): 325-333.
[16] Maghsoudi, K., Emam, Y., Niazi, A., Pessarakli, M., Arvin, M. J. (2018): P5CS expression level and proline accumulation in the sensitive and tolerant wheat cultivars under control and drought stress conditions in the presence/absence of silicon and salicylic acid. – Journal of Plant Interactions 13(1): 461-471.
[17] Mao, X. G., Jia, D. S., Li, A., Zhang, H. Y., Tian, S. J., Zhang, X. K., Jia, J. Z., Jing, R. L. (2011): Transgenic expression of *TaMYB2A* confers enhanced tolerance to multiple abiotic stresses in *Arabidopsis*. – Functional & Integrative Genomics 11: 445-465.
[18] Mengiste, T., Chen, X., Salmeron, J., Dietrich, R. (2003): The botrytis susceptible1 gene encodes an R2R3-MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. – Plant Cell 15: 2551-2565.
[19] Murashige, T., Skoog, F. (1962): A revised medium for rapid growth and bio assays with tobacco tissue cultures. – Physiologia Plantarum 15(3): 473-497.
[20] Pasquali, G., Biricolti, S., Locatelli, F., Baldoni, E., Mattana, M. (2008): *Osmyb4* expression improves adaptive responses to drought and cold stress in transgenic apples. – Plant Cell Reports 27: 1677-1686.
[21] Prabu, G., Prasad, D. T. (2012): Functional characterization of sugarcane MYB transcription factor gene promoter (*PScMYBAS1*) in response to abiotic stresses and hormones. – Plant Cell Reports 31: 661-669.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_50175031

[22] Qin, Y. X., Wang, M. C., Tian, Y. C., He, W. X., Han, L., Xia, G. M. (2012): Over-expression of *TaMYB33* encoding a novel wheat MYB transcription factor increases salt and drought tolerance in *Arabidopsis*. – Molecular Biology Reports 39: 7183-7192.
[23] Quan, R., Hu, S., Zhang, Z., Zhang, H., Zhang, Z., Huang, R. (2010): Overexpression of an ERF transcription factor *TSRF1* improves rice drought tolerance. – Plant Biotechnology Journal 8(4): 476-488.
[24] Rahaie, M., Xue, G. P., Naghavi, M. R., Alizadeh, H., Schenk, P. M. (2010): A MYB gene from wheat (*Triticum aestivum* L.) is up-regulated during salt and drought stresses and differentially regulated between salt-tolerant and sensitive genotypes. – Plant Cell Reports 29: 835-844.
[25] Sun, P., Zhu, X., Huang, X., Liu, J. H. (2014): Overexpression of a stress-responsive MYB transcription factor of Poncirus trifoliata confers enhanced dehydration tolerance and increases polyamine biosynthesis. – Plant Physiology and Biochemistry 78: 71-79.
[26] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011): MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. – Molecular Biology and Evolution 28(10): 2731-2739.
[27] The French-Italian Public Consortium for Grapevine Genome Characterization (2007): The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. – Nature 449: 463-468.
[28] Urao, T., Yamaguchi-Shinozaki, K., Urao, S., Shinozaki, K. (1993): An *Arabidopsis* MYB homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. – Plant Cell 5: 1529-1539.
[29] Vannozzi, A., Wong, D. C. J., Höll, J., Hmmam, I., Matus, J. T., Bogs, J., Ziegler, T., Dry, I., Barcaccia, G., Lucchin, M. (2018): Combinatorial regulation of stilbene synthase genes by WRKY and MYB transcription factors in grapevine (*Vitis vinifera* L.). – Plant and Cell Physiology 59(5): 1043-1059.
[30] Wang, B., Cheng, X. G. (2017): Physiological responses and regulatory pathways of transcription factors in plants under drought, high-salt, and low temperature stresses. – Journal of Plant Nutrition and Fertilizers 23(6): 1565-1574.
[31] Wang, R. K., Cao, Z. H., Hao, Y. J. (2014): Overexpression of a R2R3 MYB gene *MdSIMYB1* increases tolerance to multiple stresses in transgenic tobacco and apples. – Physiologia Plantarum 150(1): 76-87.
[32] Wang, Z., Qu, S., Zhang, Z., Zhang, J. (2004): A fast method for total RNA extraction from the tissue culture material of *malus sp*. – Journal of Fruit Science 4(26): 10-13925.
[33] Yang, A., Dai, X., Zhang, W. H. (2012): A R2R3-type MYB gene, *OsMYB2*, is involved in salt, cold, and dehydration tolerance in rice. – Journal of Experimental Botany 63(7): 2541-2556.
[34] Yang, W. J., Wu, Y. M., Tang, Y. X. (2009): Expressing and functional analysis of *GmMYBJ6* from soybean. – Yi Chuan = Hereditas 31(6): 645-653.
[35] Yang, Y. Y., Ren, Y. R., Zheng, P. F., You, C. X., Wang, X. F., Hao, Y. J. (2020): Investigation on *MdMYB2* from apple in response to abiotic stress. – Acta Horticulturae Sinica 47: 6-15.
[36] Yang, Y. Z., Wang, Y. J., Zhang, J. X., Zhang, C. H. (2007): Molecular marker linked to drought resistance gene in Chinese wild *Vitis* species and their genetic analysis. – Acta Horticulturae Sinica 34(5): 1087-1092.
[37] Yu, Y. H., Bian, L., Yu, K. K., Yang, S. D., Zhang, H. C., Wang, L. L., Zhang, G. H., Guo, D. L. (2020): *Vitis vinifera bZIP14* functions as a transcriptional activator and enhances drought stress resistance via suppression of reactive oxygen species. – Journal of Berry Research 10(4): 547-558.
[38] Zhang, X., Ju, H. W., Chung, M. S., Huang, P., Ahn, S. J., Kim, C. S. (2011): The RR-type MYB-like transcription factor, *AtMYBL*, is involved in promoting leaf senescence

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_50175031

and modulates an abiotic stress response in *Arabidopsis*. – Plant and Cell Physiology 52(1): 138-148.

[39] Zhu, N., Cheng, S. F., Liu, X. Y., Du, H., Dai, M. Q., Zhou, D. X., Yang, W. J., Zhao, Y. (2015): The R2R3-type MYB gene *OsMYB91* has a function in coordinating plant growth and salt stress tolerance in rice. – Plant Science 236: 146-156.

[40] Zhu, Z. G., Zhang, Q. T., Han, Z., Li, B., Li, G. D., Li, X. J. (2021): *VvMYB6*, an R2R3-MYB transcription factor, is involved in anthocyanin biosynthesis of grapevine. – Acta Horticulturae Sinica 48: 465-476.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_50175031