Bangladesh J. Bot. **54**(2): 369-376, 2025 (June) DOI: https://doi.org/10.3329/bjb.v54i2.82324

EFFECTS OF GRAPE (*VITIS VINIFERA* L.) R2R3-MYB TRANSCRIPTION FACTOR, MYB6 ON DROUGHT STRESS TOLERANCE IN TRANSGENIC TOBACCO

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Keywords: Grape, Drought tolerance, *VvMYB6*, Physiological and biochemical indicators, Transgenic tobacco, Gene function, Gene functional verification

Abstract

The identification of stress-associated genes in grape are essential for improving its drought resistance. In this study, *MYB6*, a R2R3-MYB in grape 'Yatomi Rose' (*Vitis vinifera* L.) was identified, and the stress response phenotypes of tobacco lines overexpressing this gene were investigated. The results revealed that root lengths, fresh weights, and heights of transgenic tobacco with overexpression of *VvMYB6* were significantly different from empty vector-transformed (EV) tobacco under optimum stress conditions. Under drought stress, the leaves of transgenic tobacco with overexpression of *VvMYB6* had less blue-brown patches and lighter color compared with the EV-transformed tobacco, which indicates a reduction in the content of reactive oxygen species (ROS). *VvMYB6*-overexpressing tobacco plants showed less electrolyte leakage, but increased contents of chlorophyll and proline. Additionally, the contents of malondialdehyde (MDA) and H_2O_2 decreased in *VvMYB6*-overexpressing tobacco plants owing to increased activity of antioxidant enzymes. This study significantly deepens the understanding of the roles of R2R3-MYB TFs in drought tolerance of plants and facilitates the development of cultivars with enhanced stress tolerance.

Introduction

Grapes are vital economic fruits globally (Yu *et al.* 2020). Currently, European grape (*Vitis vinifera* L.) cultivars are the dominant ones, however, their yield and quality are threatened by various environmental factors (Li *et al*. 2021). Hence, identification of drought tolerance-related genes is of great significance as it may promote the development of cultivars with improved adversity resistance (Vannozzi *et al.* 2018).

Drought response pathways in plants are influenced by the R2R3-MYB transcription factors (TFs). Overexpression of the *MdSIMYB1* gene in apple is an indicator of improved tolerance to various stresses (Wang *et al*. 2013). Under drought conditions, *GmMYB76*, *GmMYB92*, and *GmMYB177* were up-regulated in soybean (Sun *et al.* 2014). The amount of mRNA transcribed from the *GmMYBJ6* rose dramatically under osmotic stress, demonstrating that the transition of the *GmMYBJ6* from DNA to mRNA and then to protein was directly related to abiotic stress (Tamura *et al.* 2011). Overall, MYB TFs are involved in plants environmental stress responses, especially under drought stress.

MYB TFs are DNA-binding proteins playing key roles in growth and development, organ formation, and abiotic stress responses of plants (Wang *et al.* 2013). Meanwhile, they regulate the expressions of some other genes, thereby affecting the morphology of plant tissues under Environmental stresses. MYB TF genes in various plants have been thoroughly investigated (Li *et al.* 2021). The results of this study revealed that the *VvMYB6* positively regulates anthocyanin synthesis. For clarification of the function of *VvMYB6* under drought stress, its gene expression

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was analyzed and the functional validation of its drought resistance in transgenic tobacco was conducted. This study will facilitates the understanding of the effect of the *VvMYB6* gene in drought tolerance of plants.

Materials and Methods

Four-year old 'Yatomi Rose' (*Vitis vinifera* L.) was grown in the Henan Institute of Science and Technology, Xinxiang, Henan, China. Tobacco seeds were germinated and grown on Murashige and Skoog (MS) medium under a 16 h/8 h light/dark photoperiod at 25°C. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* GV3101 strains (Shanghai Sangon Biotech, China) were used for molecular cloning and genetic transformation.

Total RNA from grape leaves was extracted using the SDS/phenol method (Oner *et al.* 2022), while genomic DNA was isolated via the CTAB protocol. The first-strand cDNA was synthesized using Promega's M-MLV reverse transcriptase according to the manufacturer's instructions. Primers for the *VvMYB6* gene were designed based on the *V. vinifera* cv. 'Pinot Noir' genome: ATGGGAAGAGCTCCCTGTTG (forward) and TTAAGCAGATAGCGATTCCACT (reverse).

The *VvMYB6* coding sequence was amplified by PCR, cloned into the pMD18-T vector, and transformed into *E. coli* DH5α. Positive clones were identified by colony PCR and double digestion with *SalI*/*BstEII*, followed by sequence verification (Shanghai Sangon Biotech.). The validated fragment was subcloned into the pCAMBIA1301 overexpression vector, generating pCAMBIA1301: *VvMYB6*. The recombinant plasmid was introduced into A. tumefaciens GV3101 via electroporation.

Tobacco transformation was performed using the leaf disc method. Briefly, tobacco leaves were infected with *A. tumefaciens* GV3101 carrying pCAMBIA1301: *VvMYB6* and cultured on selective MS medium containing kanamycin (50 mg/L). Positive T0 plants were confirmed by PCR and self-pollinated to generate T1 seeds. Homozygous T2 lines (100% kanamycin-resistant T3 progeny) were selected for subsequent experiments. Three independent transgenic lines (OE#1, OE#2, OE#3) and empty vector (EV)-transformed control plants were used for phenotypic and molecular analyses.

For seed germination assays, sterilized seeds were sown on MS medium supplemented with 0.15 or 0.25 mM mannitol. Germination rates were recorded after 7 days. For seedling growth analysis, seedlings were transferred to MS medium with mannitol (0.15 or 0.25 mM) for 7 days, followed by measurement of root length, plant height, and fresh weight.

ROS accumulation was visualized using 3,3'-diaminobenzidine (DAB, for H_2O_2) and nitroblue tetrazolium (NBT, for superoxide anion) staining. Five-centimeter leaf segments were vacuum-infiltrated with 1% DAB (pH 6.5) or 0.1% NBT (pH 7.8) solutions, incubated in the dark for 20 h (DAB) or overnight (NBT), and decolorized with boiling ethanol. Staining intensity was documented under a light microscope (Nikon, Japan).

Chlorophyll content was measured using a commercial spectrophotometric kit. Proline content was determined via the acid-ninhydrin method, while malondialdehyde (MDA) content and electrolyte leakage (EL) were assessed using established protocols. Activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were quantified with spectrophotometric kits, and H_2O_2 levels were measured using a colorimetric assay.

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical significance was evaluated using one-way analysis of variance (ANOVA) with SPSS 20.0, and differences were considered significant at $p < 0.05$ or $p < 0.01$.

Results and Discussion

To clarify the function of *VvMYB6* under drought stress, seed germination experiments were conducted. The germination rate of seeds from *VvMYB6*-OE lines was higher than that of EV-transformed tobacco lines under mannitol-induced osmotic stress (Fig. 1A). At a mannitol concentration of 0.15 mM, the germination rates of tobacco lines with overexpression of *VvMYB6* after the seven-day growth were 95.56, 97.78 and 100% for OE#1, OE#2, OE#3, respectively, which were significantly higher than that of EV-transformed tobacco (62.22%, $p<0.05$); at mannitol concentration of 0.25 mM, the germination rates for OE#1, OE#2, OE#3 were 97.78, 100 and 94.44%, respectively, which were significantly higher than that of EV-transformed tobacco seeds (71.11%, $p<0.05$) (Fig. 1B). These results indicated that overexpression of *VvMYB6* enhanced the germination rate of tobacco seeds under osmotic stress conditions.

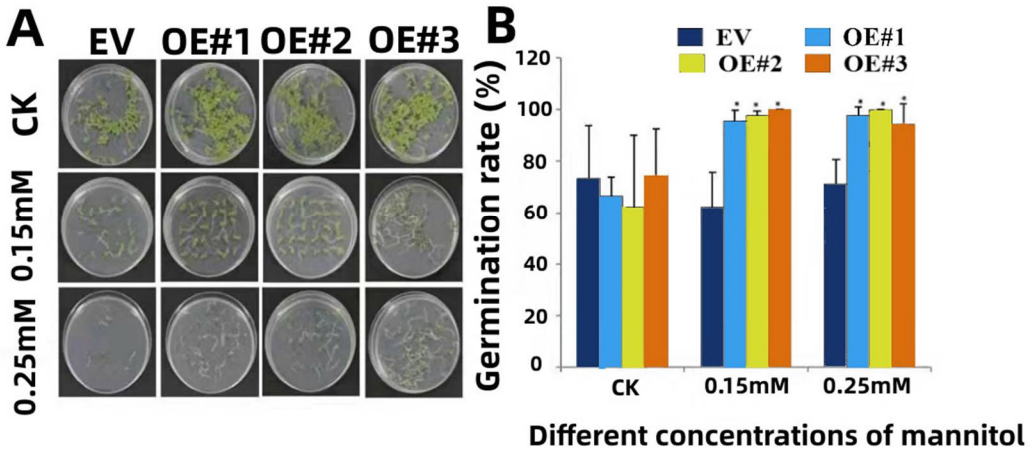

Fig. 1. Effects of different concentrations of mannitol on seed germination. A: Seed germination of EV-transformed and *VvMYB6*-OE lines and B: Germination rate of EV-transformed and *VvMYB6*-OE lines. Dat presented as the mean ± SD. Statistically significant differences are indicated by *$p<0.05$ and **$p<0.01$.

The *VvMYB6*-OE lines grew faster than the EV-transformed ones under mannitol-induced osmotic stress (Fig. 2A). The roots of *VvMYB6*-OE lines in case of 0.15 mM mannitol were 16.93, 16.11, and 15.07 mm, respectively, which were significantly longer than that of the EV-transformed tobacco ($p<0.01$); in the case of 0.25 mM mannitol, the root lengths of OE#1, OE#2, OE#3 were 9.34, 9.60, and 9.93 mm, respectively. The growth of EV-transformed and *VvMYB6*-OE lines was negatively related to the mannitol concentration (Fig. 2B), and the trends of plant height and fresh weight of *VvMYB6*-OE lines were consistent (Figs 2C, 2D). Under osmotic stress conditions, *VvMYB6*-OE lines exhibited robust growth compared to controls. Although the growth rates of both EV-transformed and *VvMYB6*-OE lines declined with increasing osmotic stress intensity, the OE lines maintained significantly better growth performance across all stress levels.

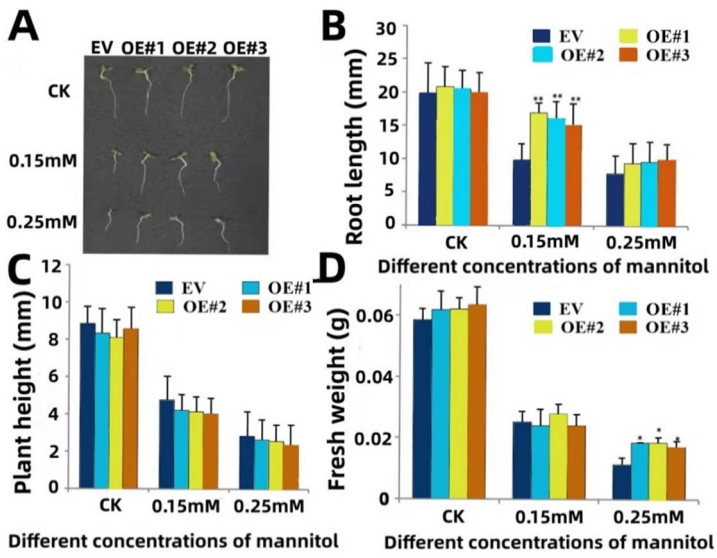

Fig. 2. Effects of different concentrations of mannitol on plantlet growth. A: Plantlet growth of EV-transformed and *VvMYB6*-OE lines. B: Root length, C: Plant height and D: Fresh weight of EV-transformed and *VvMYB6*-OE lines. Data presented as the mean ± SD. Statistically significant differences are indicated by *p<0.05 and **p<0.01.

To assess the oxidative stress response under mannitol treatment, NBT and DAB staining were performed on leaves of EV-transformed and *VvMYB6*-OE tobacco lines (Fig. 3). Under normal conditions, both genotypes displayed minimal staining, with no noticeable differences in the intensity of blue (NBT, indicative of superoxide anion) or brown (DAB, indicative of hydrogen peroxide) spots. However, under mannitol stress, the *VvMYB6*-OE lines exhibited significantly fewer blue and brown deposits compared to EV-transformed plants (Fig. 3). This reduction in staining intensity suggests that *VvMYB6* overexpression effectively mitigates the accumulation of reactive oxygen species (ROS) in transgenic tobacco under osmotic stress conditions. These results implied that *VvMYB6* enhanced ROS scavenging capacity, thereby contributing to improved stress tolerance in tobacco.

The EL and the contents of chlorophyll, proline, and MDA under osmotic stress were assessed to clarify the mechanisms of increased tolerance of the *VvMYB6*-OE lines towards osmotic stress. Under normal conditions, the chlorophyll contents of the two groups were highly consistent. The chlorophyll contents in leaves of the *VvMYB6*-OE lines were considerably higher than that in leaves of the EV-transformed lines in the case of 0.15 mM mannitol ($p<0.01$) (Fig. 4A). Additionally, the proline contents of the two groups were highly consistent under normal conditions; the proline content was higher in the *VvMYB6*-OE lines than in the EV-transformed ones under osmotic stress (Fig. 4B). Overall, increased contents of chlorophyll and proline were correlated with improved osmotic stress in the *VvMYB6*-OE lines.

EL is a method for determining plant osmotic tolerance. The water contents of plant samples were assessed after osmotic treatment with 0.15 mM mannitol. The results indicated that the EL of treated plants were higher than that of the control (0 d). However, ELs of the *VvMYB6*-OE lines were drastically lower than those of the EV-transformed ones ($p<0.01$) (Fig. 4C). The MDA levels of the *VvMYB6*-OE lines were similar to that of the EV-transformed lines under normal conditions, but considerably lower after osmotic treatment with (Fig. 4D). Under osmotic stress,

high EL and MDA levels indicated severe cell membrane damage and stress severity. Compared to EV-transformed lines, *VvMYB6*-OE lines showed lower EL and MDA levels, confirming reduced membrane damage under stress. Overall, *VvMYB6* overexpression enhances osmotic stress tolerance.

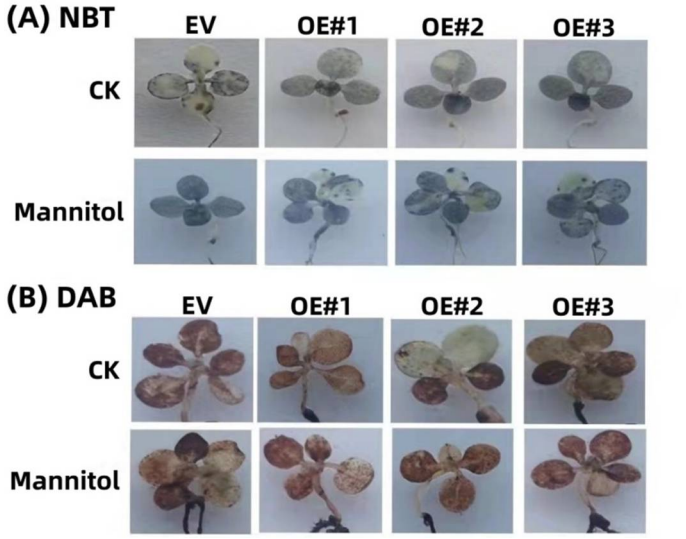

Fig. 3. Changes in reactive oxygen in leaves of EV-transformed and *VvMYB6*-OE lines. A: NBT staining for superoxide anion detection and B: DAB staining for hydrogen peroxide (H_2O_2) detection.

Enzymatic antioxidants are crucial for ROS homeostasis. To determine their influences on the stress tolerance of *VvMYB6*-OE lines, the activities of antioxidant enzymes in the *VvMYB6*-OE lines and the EV-transformed tobacco plants were investigated. As indicated, the activities of SOD in the *VvMYB6*-OE lines were substantially higher than that in EV-transformed plants in the case of 0.15 mM mannitol ($p<0.01$). Similar results were found for POD and CAT activities (Fig. 5A-C). Overall, overexpression of *VvMYB6* positively affected the osmotic tolerance of plants by tuning the activity of key antioxidant enzymes. H_2O_2 is essential for signal transduction under abiotic stress. After osmotic treatment with 0.15 mM mannitol, the H_2O_2 contents in both *VvMYB6*-OE lines and the EV-transformed lines decreased (Fig. 5D). After 10 days of osmotic stress, the H_2O_2 content in *VvMYB6*-OE lines was significantly lower ($p<0.01$) than that in EV-transformed plants. Collectively, *VvMYB6* overexpression enhances stress tolerance in transgenic tobacco under osmotic stress conditions.

Drought stress causes various changes in the morphological structure and physiological activities of plants, thereby affecting seed germination, plant growth, blooming, and fruiting (Wang and Cheng 2017), as well as diminishing yield and quality (Lu *et a*l. 2017). Plants have evolved multiple metabolic pathways with sophisticated networks that control gene expression under drought stresses. Indeed, MYB TFs play a prominent role in morphological alteration, differentiation, and functional maturity of plant tissues and organs under adverse environmental conditions (Yang *et al.* 2012). The response pathways of drought stress are heavily dependent on the R2R3-MYB TFs, and abundant MYB TFs linked to stress tolerance have been cloned from various plants to date. For wheat, high osmotic conditions lead to up-regulations of *TaMYB30*, *TaMYB73*, *TaMYB32*, *TaMYB56-B*, *TaMYBsdu1*, and *TaMYB33*, resulting in enhanced stress tolerance (Rahaie *et al*. 2010, Zhang *et al.* 2011). In addition, overexpression of *OsMYB2*,

OsMYB59, *OsMYB3R*-2, and OsMYB3R-2 in rice lead to increased resistance to environmental stresses (Quan *et al*. 2010, Yang *et al*. 2012, Nakabayashi *et al*. 2014). The sensitivity of sugarcane to osmotic stress is influenced by the gene expressions of the *ScMYBS1* (Prabu and Prasad 2012) and the *VvMYB60* participates the osmotic stress response of grape (Galbiati *et al*. 2011).

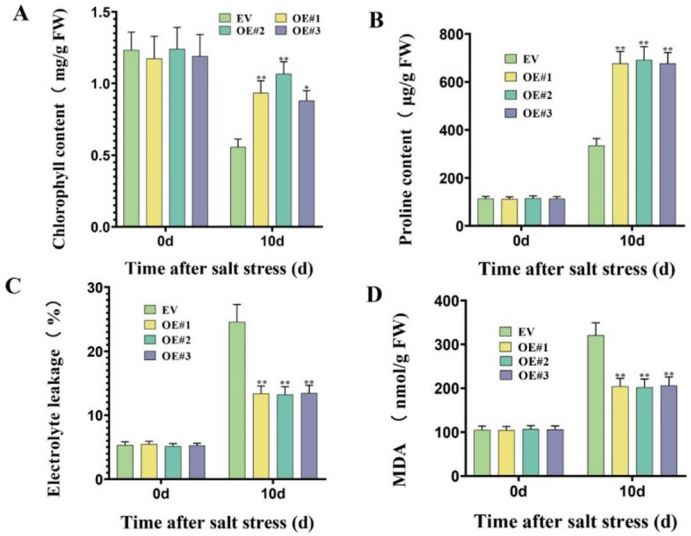

Fig. 4. Chlorophyll content, proline content, EL, and MDA content of the three *VvMYB6*-OE tobacco lines and EV plants under 0.15 mM mannitol osmotic treatment. A: Chlorophyll content, B: Proline content, C: Electrolyte leakage and D: MDA content in leaves. Data presented as the mean ± SD. Significant differences are indicated by *p<0.05 and **p<0.01.

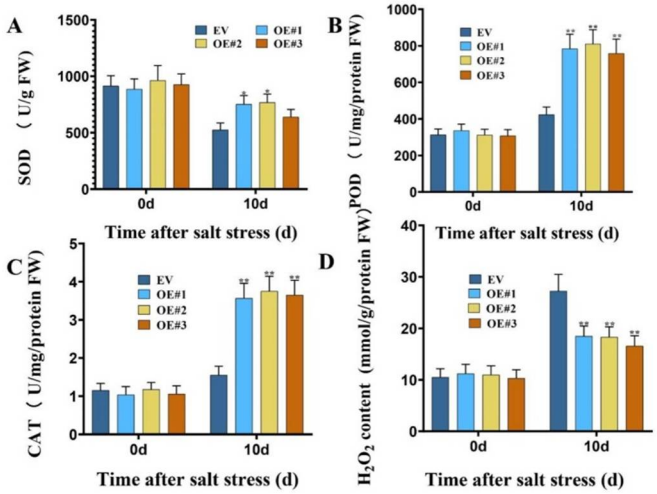

Fig. 5. Oxidase enzyme activities and H_2O_2 content of *VvMYB6*-OE tobacco lines and EV plants under 0.15 mM mannitol osmotic treatment. A: SOD, B: POD, C: CAT and D: H_2O_2. Data presented as the mean ± SD. Significant differences are indicated by *p<0.05 and **p<0.01.

The root system plays a vital role in stress reactions of plants. Dalal *et al.* (2018) reported that root growth under drought stresses is an adaptive plant trait, especially under extreme conditions such as restricted irrigation regime and rain fed. Additionally, plant growth is inversely proportional to the severity of drought stress (Yang *et al.* 2020). Drought stress led to decreased growth indexes of tobacco, while such influences on transgenic tobacco with overexpression of *VvMYB6* were significantly relieved.

Furthermore, the content of ROS in plants was proportional to the visibility of cell damages. However, excess oxygen-free radicals can be eliminated by plants in order to counteract the adverse conditions. The results of DAB and NBT staining showed reduced staining in transgenic tobacco with overexpression of *VvMYB6* compared to the EV-transformed lines under abiotic stress, indicating that the ROS content in transgenic tobacco was lower, resulting in reduced damage.

SOD, POD, and CAT are essential for the removal of ROS. Specifically, SOD catalyzes generation of O_2 and H_2O_2, while CAT and POD trigger the metabolization of H_2O_2 into H_2O. In this study, numerous physiological and biochemical parameters in both plants were investigated after osmotic treatment to clarify the mechanisms by which *VvMYB6* overexpression causes an increase of plants' tolerance of osmotic stress. Compared with the EV-transformed plants, the *VvMYB6*-OE lines exhibited higher activities of the three enzymes under osmotic conditions, implying that these enzymes contributed to the increased osmotic tolerance of the *VvMYB6*-OE lines. After osmotic treatment, the contents of both chlorophyll and proline were higher in the *VvMYB6*-OE lines than in the EV-transformed plants, while the EL and MDA levels were higher in the EV-transformed plants. These findings are consistent with the work of Feng *et al.* (2015), reinforcing the role of *VvMYB6* in enhancing ROS scavenging capacity and cellular membrane stability under osmotic stress.

VvMYB6 enhances drought tolerance by regulating ROS homeostasis, osmolyte biosynthesis, and membrane integrity. This discovery advances our understanding of MYB transcription factors in stress regulation and offers a promising target for breeding drought-resistant crops.

Acknowledgements

This project was supported by Henan Provincial Key Research and Development Special Project (241111113200).

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(Manuscript received on 05 December 2024; revised on 04 June, 2025)