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CLONING, SUBCELLULAR LOCATION AND EXPRESSION ANALYSIS OF GRAPE MYB GENE

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Abstract

MYB gene plays an important role in plant growth, development and response to abiotic stress. In the present study, 'Yatomi Rosa' grape (*Vitis vinifera* L.) was used as the test material. Two *MYB*, *VvMYBB1* and *VvMYBA3* gene were obtained by homologous cloning, and their subcellular location, different organs, and expression patterns under stress treatment and hormone induction were obtained. VvMYBB1 and VvMYBA3 proteins were located in the nucleus and belonged to nuclear proteins. They were highly expressed in roots and flowers. These genes might have a negative effect on the formation of peel color. *VvMYB1* gene and *VvMYBA3* gene might play an important role in drought resistance and salt stress resistance. Under the induction of exogenous hormones, relative expression of *VvMYBB1* gene was higher than that of *VvMYBA3* gene under IAA, ETH, SA and MeJA treatment. Relative expression of *VvMYBB1* gene was lower than that of *VvMYBA3* gene under 6-BA, GA_3 and ABA treatment. It showed that *MYB* gene played an important role in the development of different organs of grapes, fruit color, and response to abiotic stress and exogenous hormones induction in grape.

Introduction

Grapes are one of the most important fruit trees in the world. The widely cultivated grape cultivars are mainly Eurasian grapes (*Vitis Vinifera* L.). These grapes have excellent quality and processing properties, but their disease resistance and stress resistance are poor (Yu *et al*. 2020). In grapevine, 108 *MYB* genes have been identified from the genome of *Vitis vinifera* cv. Pinot Noir, which plays an important role in the grape growth and development (Zhu *et al*. 2019). This study is to explore the role of the new grape *MYB* gene in fruit color and response to abiotic stress and exogenous hormones induction. Two *MYB* genes, *VvMYBB1* gene and *VvMYBA3* gene, were cloned and their expression patterns under different organ development and peel color, abiotic stress and exogenous hormone induction were analyzed. These will help to understand the effect of grape *MYB* genes on grape fruit coloration, response to abiotic stress and exogenous hormones induction. This would provide a reference for the utilization of grape *MYB* gene resources.

Materials and Methods

Two-year-old cutting plants of 'Yatomi Rosa' grape (*Vitis Vinifera* L.) from the Grape Germplasm Resource Nursery of Henan Institute of Science and Technology were all planted in plastic pots (25 cm in diameter × 30 cm in height). Grape plants were grown in the cultivation substrate (garden soil: peat soil = 1 : 1) under normal conditions (25-28°C, 16/8 hrs light/dark photoperiod). New roots, tendrils, shoots and stems, mature leaves and flowers with caps were collected. The skins came from grapes at 2, 3, 5, 7 and 8 weeks after flowering.

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The control group was sprayed with sterile water, covered with white plastic bags, and the leaves were harvested at 0, 1, 2, 3, 4, 5, 7 and 9 days after treatment; Dryness treatment started when the water content of basin soil was 70%, and leaves were harvested 0, 1, 2, 3, 4, 5, 7 and 9 days after treatment; Under low temperature treatment, potted seedlings were placed in 4°C light incubator, and the leaves were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 hrs after treatment; High-salt treatment, with 0.1mol /L NaCl irrigation once, until the basin bottom solution outflow, leaves were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 hrs after treatment.

The control group was sprayed with sterile water and covered with a white plastic bag. Seven kinds of growth regulator (100 mmol/l IAA, 100 μmol/l GA_3, 100 μmol/l 6-BA, 3 mmol/l ETH, 100 μmol/l ABA, 100 μmol/l SA, 50 μmol/l MeJA solution) were used to sprayed grape leaves. Those leaves were all collected at 0, 3, 6, 9, 12, 24, 48 and 72 hrs after treatment (Zhu *et al.* 2019). All the treated materials were immediately frozen in liquid nitrogen and stored in -80°C refrigerator.

Specific primers of *VvMYBB1* and *VvMYBA3* gene were designed as the template. PCR amplification was performed by using the LA Taq® high-fidelity enzyme of TaKaRa. RNA was extracted from different tissues and organs of grapes by DS/phenol, its concentration and integrity were determined by Nanadrop2000C nucleic acid quantitative analyzer (Thermal USA) and 1% agarose gel electrophoresis. cDNA was performed using the a PrimeScript™ II 1st strand cDNA synthesis kit (TaKaRa, Beijing, China), which was template for quantitative real-time PCR (qRT-PCR) reaction with specific primers to detect the expression level of *VvMYBB1* and *VvMYBA3* gene in different tissues, organs and abiotic stresses of 'Yatomi Rose' grape. The relative mRNA abundance was normalized by the $2^{-\Delta\Delta CT}$ method. All experiments included three replicates (Li *et al.* 2021).

Subcellular localization is maintained using *VvMYBB1* and *VvMYBA3* gene to design specific primers containing restriction enzyme cutting sites, the CDs sequences of *VvMYBB1* and *VvMYBA3* gene were amplified, then inserted into the vector PBI221-GFP to construct a fluorescent fusion expression vector. The plasmids of the positive control of pBI221-GFP and pBI221-GFP/*VvMYBB1* and *VvMYBA3* were introduced into onion (*Allium cepa*) epidermal cells using a Bio-Rad He/1000 particle delivery system (Bio-Rad, Hercules, CA, USA) respectively. The inner epidermis of onion scales were exfoliated and placed on hypertonic solid MS medium for 4 hrs at 28°C. Live parasites could uptake of acridine orange (green fluorescence) in maximum emission of 488 nm as green color. Cell membrane was imaged by 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) staining. Dil fluorescence microscopy in Ex/Em, 510/530 nm emission. Cell nucleic DNA was imaged by 4′-6-diamidino-2-phenylindole (DAPI) staining. DAPI fluorescence microscopy in Ex/Em, 549/565 nm emission. A laser scanning confocal microscopy (LSCM) (LSM780, Carl Zeiss AG of Germany) at 40 × objective lens was used to observe subcellular localizationas (Zhang *et al.* 2018).

Results and Discussion

Using homologous cloning, two *MYB* gene fragments of 1680 bp and 1104 bp size were cloned using the cDNA of the leaves of 'Yatomi Rose' grape as the template (Fig. 1). Both contained a complete open reading frame with a sequence of 100% similarity to grape *VvMYBB1* gene and *VvMYBA3* gene (GenBank XM_002275810 and NP_001267927, respectively). ORF total length was 1350 bp and 834 bp. *VvMYBB1* gene total length was 762 bp, coding 254 amino acids, and the isoelectric point was 28.45 kD. The total length of the *VvMYBA3* gene was 477 bp, coding 159 amino acids, and the isoelectric point was 18.53 kD (Fig. 2).

pBI221-*VvMYBB1*-GFP and pBI221-*VvMYBA3*-GFP fusion proteins were localized in the nucleus, while the control GFP proteins covered the whole cell (Fig. 3), indicating that *VvMYBB1* gene and *VvMYBA3* gene proteins played a role in regulating the transcription level of other genes in the nucleus. It was consistent with those results of Xie *et al*. (2014), Liu *et al*. (2017) and Zhu *et al*. (2019). All these results showed that MYB gene were usually expressed in the nucleus and could specifically bind to cis-acting elements in the promoter region of eukaryotic genes to regulate gene expression.

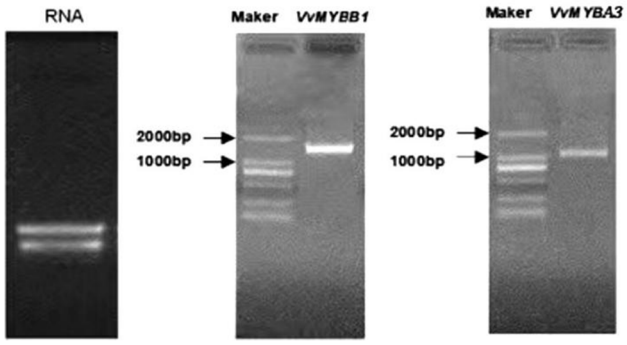

Fig. 1. Cloning of *two VvMYB* genes in 'Yatomi Rose' grape.

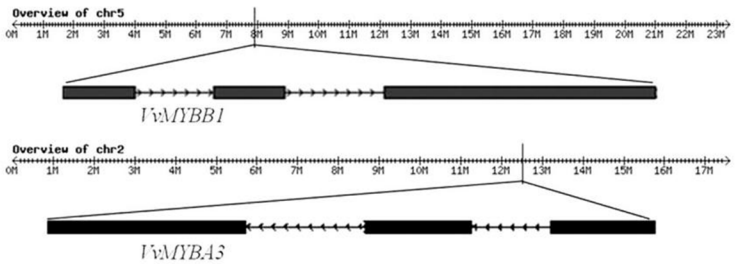

Fig. 2. Sequence analysis of two *MYB* genes in 'Yatomi Rose' grape.

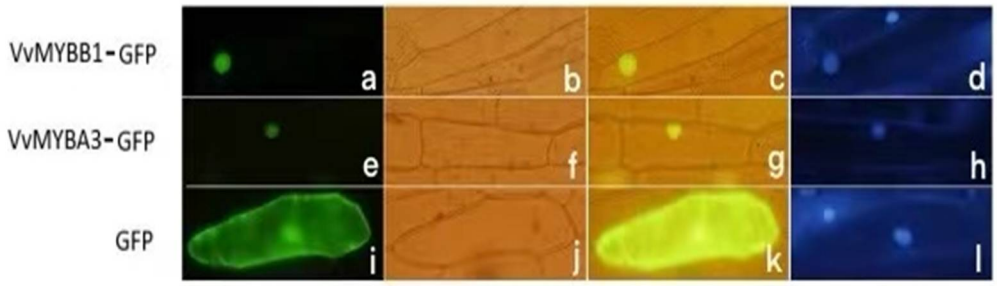

Fig. 3. Subcelluar localization of VvMYBB1 and VvMYBA3 protein.
a: VvMYBB1-GFP fluorescence; b: VvMYBB1-GFP bright-field; c: VvMYBB1-GFP with Dil staining; d: VvMYBB1-GFP with DAPI staining; e: VvMYBA3-GFP fluorescence; f: VvMYBA3-GFP bright-field; g: VvMYBA3-GFP with Dil staining; h: VvMYBA3-GFP with DAPI staining; i: GFP fluorescence; j: GFP bright-field; k: GFP with Dil staining; l: GFP with DAPI staining.

Plant MYB gene shows specific spatiotemporal expression characteristics in different organs and tissues (Jiang and Rao 2020). In this study, *r*elative expression of *VvMYBB1* gene was the highest in root, followed by tendrils, leaves and flowers, the lowest in stem. Relative expression of root was 50 times of that of stem. Relative expression of *VvMYBA3* gene was the highest in flowers, followed by roots, tendrils and leaves, and the lowest was in stems. Relative expression of flower was 100 times of that of stem (Fig. 4). Relative expression of *VvMYBB1* gene and *VvMYBA3* gene were specific in different tissues, with the highest expression of *VvMYBB1* gene in roots and the highest expression of *VvMYBA3* gene in flowers. Zhu *et al.* (2019) also found that *VvMYBC2L2* gene was strongly expressed in root, flower and seed tissue. Cavallini *et al.* (2015) found *MYBC2L2* showed very low relative expression in almost all organs including the berry and seed. These results indicated that MYB gene expression was different in different organs of grape, the present study indicated *VvMYBB1* gene and *VvMYBA3* gene played an important role in the development of grape roots and flower organs of 'Yatomi Rose' grape.

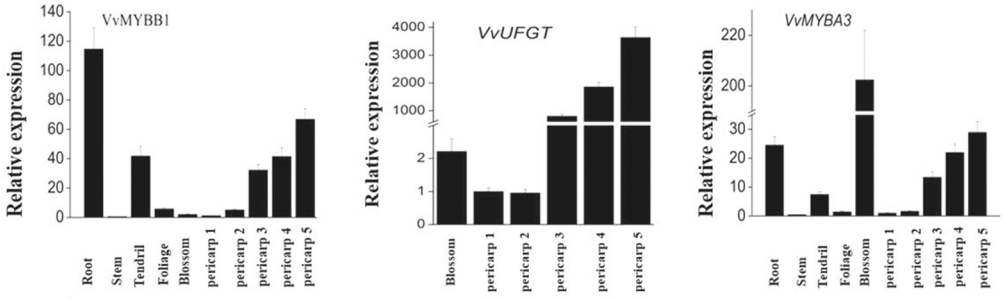

Fig. 4. Expression analysis of *VvMYBB1* gene and *VvMYBA3* gene in different tissues and organs of 'Yatomi Rose' grape.

There are many *MYB* genes involved in the regulation of anthocyanin synthesis in grape, such as *VvMYBA1* gene, *VvMYBA2* gene, *VvMYBA6* gene (Kobayashi *et al*. 2004, Cutanda-perez *et al*. 2009, Sun *et al*. 2016). In this study, relative expression of *VvMYBB1* gene and *VvMYBA3* gene were the lowest in the early stage of fruit development, and then increased gradually, and reached the highest value at 8 weeks after flower development. This showed that with the prolongation of fruit development period, the two genes played an important role in fruit maturation. Compared with the relative expression of control VvUFGT, the relative expression of *VvMYBB1* gene and *VvMYBA3* gene were 5 and 14 times higher than that of *VvMYBB1* gene and *VvMYBA3* gene at 8 weeks after flowering, respectively. So *VvMYBB1* gene and *VvMYBA3* gene could have a negative effect on the formation of grape epidermis color (Fig. 4). Zhu *et al*. (2019) found *VvMYBC2L2* gene weakly expressed during the fruit development in grape, and *VvMYBC2L2* gene played a role as a negative function of anthocyanin biosynthesis. Ni *et al.* (2020) studied that relative expression of *PpMYB140* to inhibit anthocyanin biosynthesis, and *PpMYB140* gene played a negative function in red pear fruit. These results are consistent with the present results, suggesting that *VvMYBB1* gene and *VvMYBA3* gene might have a negative effect on the formation of grape skin color of 'Yatomi Rose' grape.

MYB gene is involved in various aspects of plant response to abiotic stress. *AtMYB96* in *Arabidopsis thaliana* regulates drought stress responses by integrating ABA and IAA signals (Seo and Park 2010). In the present study, under drought treatment, relative expression of *VvMYBB1* gene increased gradually, reaching the highest value at 9 d, about 12 times of 0 hr. Relative

expression of *VvMYBA3* gene increased gradually, reaching the highest value at 9 days, about 6.5 times of 0 h. Under the treatment of low temperature (4°C), relative expression of *VvMYBB1* gene was higher than that of *VvMYBA3* gene. Relative expression of *VvMYBB1* gene increased gradually, reaching the highest value in 72 hrs, about 12 times of 0 hr, but relative expression of *VvMYBA3* gene changes were not obvious. Under salt treatment, relative expression of *VvMYBB1* gene increased first and then decreased, reaching the highest value in 12 hrs, about 8 times of 0 hr. Relative expression of *VvMYBA3* gene reached the highest value at 24 hrs, about 2 times of 0 hr (Fig. 5). These results suggest that *VvMYBB1* gene and *VvMYBA3* gene might play an important role in grape drought resistance and salt stress resistance. Prabu and Prasad (2012) found that relative expression of *SCMYBAS1* gene affects the response of sugarcane to drought and salt stress. Yang *et al*. (2012) found that relative expression of *OsMYB2* could be induced by salt stress, cold and drought in rice. Hao *et al*. (2020) found that *MdMYB308L* acted as a positive regulator in low temperature stress. The present results also indicated that *MYB* gene was involved in response to abiotic stress in grape.

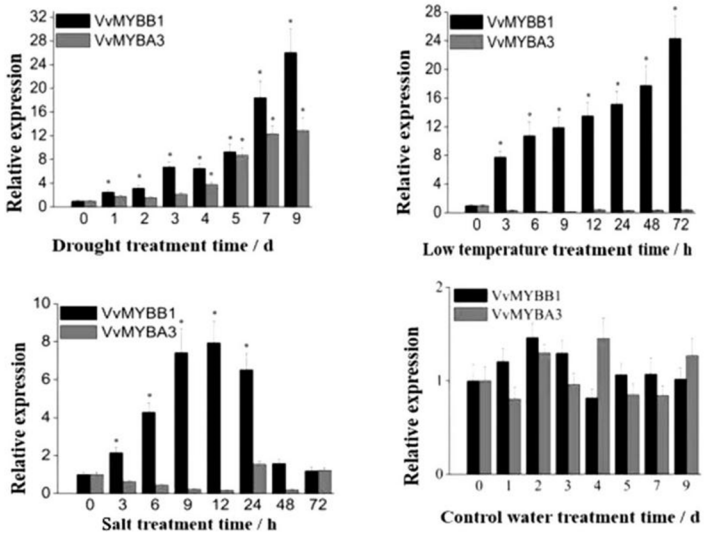

Fig. 5. Relative expression of *VvMYBB1* gene and *VvMYBA3* gene in 'Yatomi Rose' grape treated with abiotic stress. *: Significant differences of genes compared with 0 h ($P < 0.05$).

Jiang and Rao (2020) also found that *MYB* gene was involved in the plant hormone response process. For example, multiple *MYB* gene was found to be involved in the response of auxin, ethylene and cytokinin, and *AtMYB2* gene was induced by ABA, which was the first *MYB* gene induced by ABA in *Arabidopsis thaliana* (Abe *et al*. 1997). In this study, under IAA treatment, relative expression of *VvMYBB1* gene increased first and then decreased, at 12 hrs, its relative expression reached the highest, about 5 times of 0 hr. Relative expression of *VvMYBA3* gene showed a downward trend. Under GA_3 treatment, relative expression of *VyMYBB1* gene and *VyMYBA3* gene increased first and then decreased. Relative expression of *VyMYBB1* gene reached the maximum value in 12 hrs, about 10 times of 0 hr. Relative expression of *VyMYBA3* gene reached the maximum at 9 hrs, about 2.2 times of 0 hr. Under 6-BA treatment, relative expression of *VyMYBB1* gene and *VyMYBA3* gene increased first and then decreased. *VyMYBB1* gene relative expression reached the maximum at 9 hrs, about 6 times of 0 hr. Relative expression of *VyMYBA3*

gene reached the maximum in 3 hrs, about 1.2 times of 0 hr. Under ETH treatment, *VyMYBB1* gene relative expression increased first and then decreased, and its relative expression reached the maximum at 48 hrs, about 9 times of 0 hr. *VyMYBA3* gene relative expression showed a downward trend. Under ABA treatment, *VyMYBB1* gene relative expression increased first and then decreased, its relative expression reached the maximum value in 12 hrs, about 4 times of 0 hr. *VyMYBA3* gene relative expression showed an upward trend, its relative expression reached the

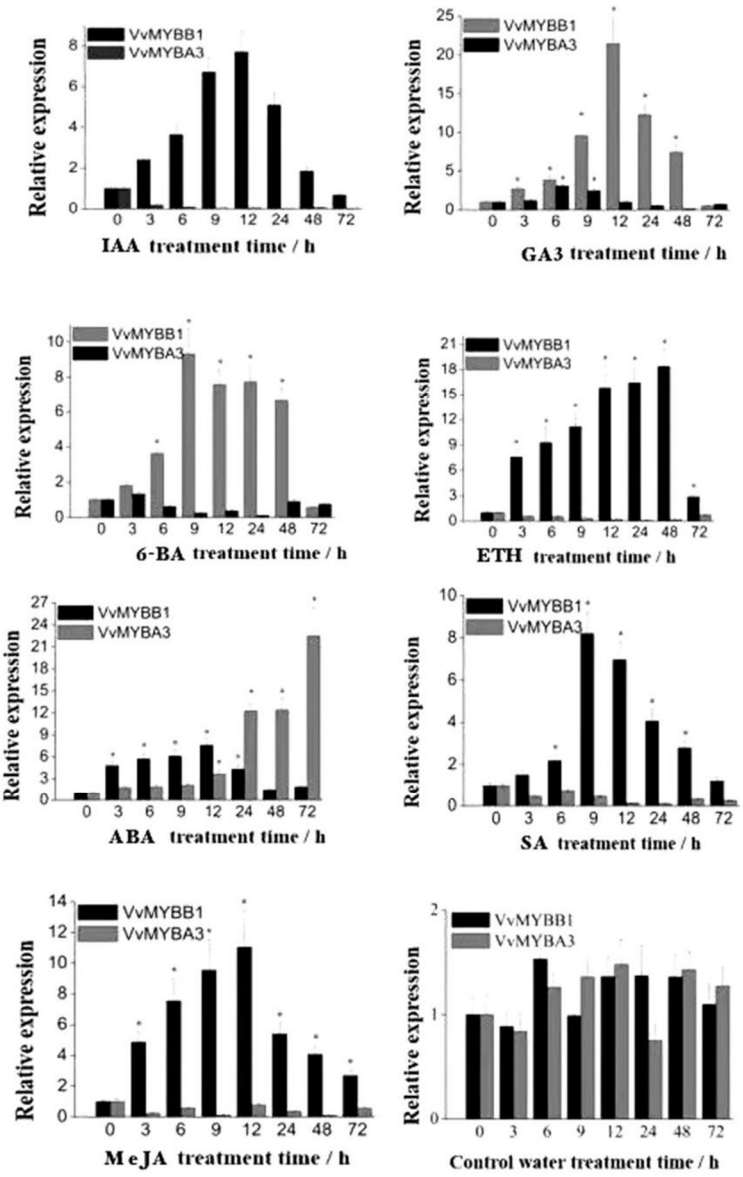

Fig. 6. Relative expression of *VvMYBB1* gene and *VvMYBA3* gene in 'Yatomi Rose' grape treated with exogenous hormone. *: Significant differences of genes compared with 0 h ($P < 0.05$).

maximum at 72 hrs, about 11 times of 0 hr. Under SA treatment, *VyMYBB1* gene relative expression increased first and then decreased, its relative expression reached the maximum at 9 hrs, about 8 times of 0 hr. *VyMYBA3* gene relative expression decreased. Under MeJA treatment, *VyMYBB1* gene relative expression increased first and then decreased, its relative expression reached the highest at 12 hrs, about 7.3 times of 0 hr. *VvMYBA3* gene relative expression showed a downward trend. The relative expressions of *VyMYBB1* gene and *VyMYBA3* gene were not obvious in the control water treatment at different times. Relative expression of *VvMYBB1* gene was higher than that of *VvMYBA3* gene under IAA, ETH, SA and MeJA treatment, and lower than that under 6-BA, GA_3, and ABA treatment (Fig. 6). These results indicated that *VyMYBB1* gene and *VyMYBA3* gene might play a very important role in physiological activities regulated by exogenous hormones in 'Yatomi Rose' grape. Chen *et al.* (2006) cloned 163 *MYB* genes in *Arabidopsis thaliana* and studied the treatment with exogenous hormones ABA, ETH, GA_3, IAA, JA and SA; and then found that relative expression of 50% of these *MYB* genes were changed when treated with SA. Nashaat *et al.* (2014) found that relative expression of *TAMYB4* gene in wheat was regulated by SA, ABA and MeJA. Shen *et al.* (2017) found *PacMYBA* gene relative expression in sweet cherry (*Prunus avium*) was induced by salt, MeJA and SA. The present study found that relative expression of *VvMYBB1* gene and *VvMYBA3* gene changed at different times which induced by 7 different exogenous hormones. These results indicate that plant *MYB* gene is widely involved in the regulation of exogenous hormones in plant growth.

The present results indicated that *VvMYBB1* gene and *VvMYBA3* gene played an important role in the development of different organs, fruit coloration, and response to abiotic stress and exogenous hormone in grape. This study provides reference for the utilization of *MYB* gene resources in grape.

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