

Article

VvMYBPA2 Regulated the Accumulation of Flavan-3-ols though Forming a Trimeric Complex in ‘Zaoheibao’ Grape

Changmei Liang ^{1,†}, Jianyong Guo ^{2,†}, Mingxiang Chen ², Xuehui Zhang ², Guorong Zhang ², Pengfei Zhang ², Jinjun Liang ² and Pengfei Wen ^{2,*}

¹ College of Information Science and Engineering, Shanxi Agricultural University, Taigu, Jinzhong 030801, China

² College of Horticulture, Shanxi Agricultural University, Taigu, Jinzhong 030801, China

* Correspondence: wenpengfei@126.com; Tel.: +86-136-3354-8791

† These authors contributed equally to this work.

Abstract: Flavan-3-ols are monomers of Proanthocyanidins (PAs), which are important polyphenolic compounds in grapes. Previous studies had shown that *VvMYBPA2* was closely related to grape flavan-3-ol monomers biosynthesis, but its regulatory network is still unclear. Here, we found that the contents of (+)-catechin and (–)-epicatechin, the enzyme activities of anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR) and the expression of *VvANR* and *VvLAR1* were increased in the *VvMYBPA2* overexpression grape leaves compared to the control. It was proved that *VvMYBPA2* protein interacted with *VvWDR1* and *VvWDR1* protein interacted with *VvMYC2* by yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC). The promoters of *VvANR* and *VvLAR1* were bound by *VvMYBPA2* using yeast one-hybrid (Y1H) assay. These results suggested that *VvMYBPA2* could form a trimeric complex with *VvWDR1* and *VvMYC2* and jointly regulated the expression of flavan-3-ol monomers related genes *VvANR* and *VvLAR1*, thereby affecting the enzyme activities of ANR and LAR and ultimately regulating the contents of flavan-3-ols.

Keywords: grape; flavan-3-ols; proanthocyanidins; *VvMYBPA2*; MBW complex



Citation: Liang, C.; Guo, J.; Chen, M.; Zhang, X.; Zhang, G.; Zhang, P.; Liang, J.; Wen, P. *VvMYBPA2* Regulated the Accumulation of Flavan-3-ols though Forming a Trimeric Complex in ‘Zaoheibao’ Grape. *Agriculture* **2022**, *12*, 1414. <https://doi.org/10.3390/agriculture12091414>

Academic Editor: Vitale Nuzzo

Received: 17 August 2022

Accepted: 2 September 2022

Published: 7 September 2022

Publisher’s Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Proanthocyanidins (PAs), namely condensed tannins (CTs), are formed from flavan-3-ol monomers and their polymers, which could be decomposed to produce anthocyanins when heated in an acidic environment [1]. It plays an important role in grape stress resistance and human health. PAs are commonly found in many different tissues of plants and play important roles in plant defense against pathogens and other diseases, regulation of seed dormancy, pigmentation and stomatal opening. Numerous studies had shown that dietary PAs had the potential to prevent various diseases in the human body [2]. The contents of PAs affected the taste, flavor and organoleptic properties of grapes [3–6]. It is closely related to the shelf life and color stability of wine [7] and is also an important health care component in wine [8]. Therefore, elucidating the molecular mechanism of PA biosynthesis in grapes is of great significance to improve its stress resistance and fruit quality.

PAs take the dihydroquercetin produced by the phenylalanine metabolic pathway as the substrate, under the catalysis of dihydroflavonol reductase (DFR), to generate leucoanthocyanidins. Then catechin is produced under the action of leucoanthocyanidin reductase (LAR). Epicatechin is generated under the action of anthocyanin synthase (ANS) and anthocyanidin reductase (ANR). They form dimers, trimers and multimers with leucoanthocyanidins under the action of condensed enzyme (CON), namely Pas [9,10]. Two key structural genes, *ANR* and *LAR*, unique to the PA synthesis pathway [11,12], and the *LAR* and *ANR* enzymes encoded by them catalyze the formation of flavan-3-ol monomers (+)-catechin and (–)-epicatechin, respectively. One *ANR* gene (*VvANR*) and two *LAR* genes (*VvLAR1*, *VvLAR2*) were identified in grapes, and related studies confirmed

the specificity of *VvANR*, *VvLAR1* and *VvLAR2* for grape PA synthesis [3,13]. In the previous research in our laboratory, silencing *VvANR* in grape leaves reduced the content of (–)-epicatechin and (–)-epigallocatechin [14].

The most important transcription factors regulating PA biosynthesis are the MYB family genes. For example, *AtTT2* [15], *MdMYB9/11* [16], *GsMYB60* [17], *DkMYB19/20* [18], *PuMYB134* [19], *GmTT2A/B* [20], etc. Previous studies had shown that the MYB protein was usually involved in the formation of the MYB-bHLH-WD40 (MBW) complex, which regulated flavonoid biosynthesis in turn [21]. *AtTT2* (MYB) interacted with the transcription factors *AtTT8* (bHLH) and *AtTTG1* (WD40) to form the MBW complex, which induced the accumulation of PAs in *Arabidopsis* seed coat by directly regulating the transcription level of PAs synthesis structural gene *AtBANYULS* (*AtBAN*) [22]. MBW complexes also existed in other horticultural plants involved in their PA biosynthesis, such as *FaMYB9/FaMYB11-FaTTG1-FabHHLH3* [23]; *CsMYB60-CsbHLH42/MYC1-CsWD40* [17]; *DkMYB2/DkMYB4-DkMYC1-DkWDR1* [24], etc.

VvMYBPA1 was the first factor found to regulate PA synthesis in grape [25], and ectopic expression of *VvMYBPA1* resulted in increased PA synthesis in *Arabidopsis* and complemented the phenotype of the *Arabidopsis* mutant *tt2*, although there was no homologous gene of *VvMYBPA1* in *Arabidopsis*. While *VvMYBPA2* was mainly expressed in young fruits and peels [26], its expression pattern was consistent with the accumulation of PA during grapevine development. *VvMYBPA2* was highly homologous to *AtTT2*, and heterologous overexpression also increases PA biosynthesis of *Arabidopsis*. Compared with *VvMYBPA1*, *VvMYBPA2* had a preferential role in the synthesis and accumulation of PAs in berry pericarp [26]. *VvWDR1* (WD40 protein) [27] and *VvMYC2* (bHLH protein) [27,28], which had high similarity to *AtTTG1* [22] and *ZmMYC2* [29], respectively, had been proved to be involved in the biosynthesis of PAs or anthocyanidins in grapes. MBW or MBWW complexes in grapes had been shown to be involved in their flavonoid biosynthesis, for example, *VvMYBA1-VvWDR1-VvMYC1* regulated grape anthocyanin synthesis [30]; the *VvMYB5-VvMYC1-WDR1-WRKY26* complex was associated with accumulation of flavonoids in grapes [31,32]. However, there were few studies on the regulatory mechanism of MBW complex for grape flavan-3-ols biosynthesis.

In this study, the positive regulation of *VvMYBPA2* on grape flavan-3-ols biosynthesis was further demonstrated by transient overexpression experiments. The *VvMYBPA2* protein had been proved that it interacted with *VvWDR1* and *VvMYC2* to form a complex by Y2H and BiFC assays, and the promoters of *VvANR* and *VvLAR1* were bound by *VvMYBPA2* through Y1H assay. These results confirmed the role of the MBW complex in grape flavan-3-ols biosynthesis and enriched our understanding of the regulatory network of flavan-3-ols biosynthesis.

2. Materials and Methods

2.1. Test Materials

Leaves were collected from *Vitis vinifera* L. cv. ‘Zaoheibao’ grown at the horticultural station of Shanxi Agricultural University, with plant spacing and row spacing of 1.0 m and 2.5 m, respectively; vines were planted in hedgerows and managed routinely. New shoots with stems were collected as infection materials at 3 pm on 23 September 2021 year.

Seeds of *Nicotiana benthamiana* were planted in an incubator with a 16 h/8 h photoperiod and 25 °C temperatures.

The vectors pCAMBIA1300, pCAMBIA1300-35S-YFPc and pCAMBIA1300-35S-YFPn were donated by Professor Xu Jin of Shanxi Agricultural University. Yeast strains AH109, Y1Hgold, vector pCAMBIA1300-35S-GFP, pGBKT7, pGADT7 and pAbAi were maintained in our laboratory. The primer sequences used are listed in Appendix A.

2.2. Subcellular Localization

The full-length CDS of *VvMYBPA2* without the termination codon was fused to the pCAMBIA-1300-GFP plant expression vector that was driven by the CaMV35S promoter

containing the green fluorescent protein (GFP) reporter gene to produce the fusion vector 35S::VvMYBPA2-GFP. The recombinant vector and control vector 35S::GFP were then introduced into tobacco (*Nicotiana benthamiana*) leaves by agroinfiltration. The cells of transformed tobacco leaves were observed via a laser scanning confocal microscope (Leica TCS SP8, Wetzlar, Germany).

2.3. Transient Expression of Grape Leaves

The full-length CDS sequence of VvMYBPA2 was inserted into pCambia1300 overexpression vector to construct 35S::VvMYBPA2 and transformed into *Agrobacterium tumefaciens* GV3101 competent cells. A total of 120 healthy grape shoots were collected, and 60 shoots were set as a group. The grape leaves were infected by vacuum for 10 min, the infection solution was gently wiped from the leaves and leaves were placed in an LED light incubator with light intensity of 2000 lx, relative humidity of 60–70% and temperature of 25 °C for light and dark alternate culture of 16 h and 8 h.

2.4. DMACA Staining

According to the method of Li et al. [33], the infected grape leaves were soaked in ethanol–glacial acetic acid (GAA) solution (3:1, v/v) for discoloration, then rinsed with 75% ethanol for 12 h and washed with distilled water. Finally, the leaves were dyed in 0.6% DMACA solution (6N HCl:methanol = 1:1) for 2 min, observed and photographed. The darker staining indicated the higher content of PAs.

2.5. Epicatechin and Catechin Content

According to the method of Liang et al. [14], we accurately weighed grape leaves (0.3 g) into a centrifuge tube, added 3 mL of 70% methanol and extracted by ultrasonic for 25 min. Centrifuged at 12,000 rpm for 10 min, the supernatant was collected and centrifuged repeatedly and filtered through a 0.45 µm organic microporous membrane. After rotary evaporation, 1 mL of pure water was added for extraction, and 2 mL of ethyl acetate was added for extraction, and the upper layer was taken and extracted with 1 mL pure methanol. Then, the epicatechin and catechin contents were determined by HPLC (main instruments include autosampler, chromatographic column and detector, etc.) (Thermo Fisher, Waltham, MA, USA) after filtration through a 0.22 µm organic microporous (Shengze Technology Co., Ltd., Tianjin, China) membrane.

Mobile phase B was 1.3% glacial acetic acid; the detection wavelength was 280 nm; the injection volume was 5 µL; the flow rate was 1.0 mL·L^{−1}; and the column temperature was 30 °C.

2.6. Determination of ANR and LAR Enzyme Activity

The determination of ANR enzyme activity was performed according to Liang et al. [14]: we accurately weighed grape leaves (0.4 g) into a centrifuge tube and added 2 mL phosphate-buffered saline (PBS) (pH = 7.4), mixed and let stand for 10 min. Then, the mixture was centrifuged at 1500 rpm at 4 °C for 15 min, and the supernatant was taken as the crude enzyme solution. An accurately weighed total of 1.0 mL of crude enzyme solution, Tris-HCl (pH = 7.7), cyanidin chloride (CYA) and reduced coenzyme II tetrasodium salt (NADPH·Na₄) was added, and the mixture was reacted at 45 °C for 20 min. HCl–methanol and vanillic aldehyde–methanol were added. The ANR enzyme activity was mainly determined by measuring the content of epicatechin as follows: $U = \text{mg EC} \cdot \text{mg}^{-1} \cdot \text{prot}^{-1} \cdot \text{min}^{-1}$.

The LAR enzyme activity was determined according to Wen et al. [34] with slight modifications: We put accurately weighed grape leaves (0.4 g) into a centrifuge tube, and 2 mL boric acid buffer solution at pH = 8.8 was added. After mixing via vortex and centrifugation for 15 min at 1500 rpm, the supernatant was taken as the crude enzyme solution. A total of 1.0 mL of crude enzyme solution was accurately weighed, reduced coenzyme II (NADPH), Dihydroquercetin (DHQ) and Tris-HCl (pH = 7.7) were added in turn and then the mixture was reacted at 37 °C for 1 h. Then HCl–methanol and vanillic aldehyde–methanol were added and further reacted at 37 °C for 1 h. The LAR

enzyme activity was mainly determined by measuring the content of catechin as follows:
 $U = \text{mg CAT} \cdot \text{mg}^{-1} \cdot \text{prot}^{-1} \cdot \text{h}^{-1}$.

2.7. Gene Expression Analysis

Total RNA was extracted by the cetyltriethylammonium bromide (CTAB) method with slight modifications, and the cDNA was obtained by reverse transcription. *VvActin* was used as the internal reference gene. According to the instructions of the $2 \times$ Realtime PCR Supper mix (SYBR green, with anti-Taq), we performed predenaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing temperature at 55 °C for 15 s; fluorescence was measured at 72 °C for 30 s, and the relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

2.8. Gene Isolation and Sequence Analysis

The ORFs of grape PA biosynthesis regulators (*VvMYBPA2*, *VvWDR1*, *VvMYC2*) were cloned by PCR. Homologous search was performed on the NCBI BLAST server. Molecular evolutionary genetic analysis (MEGA) software version 7 was used to construct phylogenetic tree by adjacency method, and sequence alignment was performed by CLUSTAL X program. Bootstrap analysis is implemented by 500 repetitions. Protein ID: *VvMYBPA1*, CAJ90831; *VvMYBPA2*, EU919682; *VvMYBPA2*, BAP39802; *VvMYBA1*, BAD18977; *VvMYBA2*, BAD18978; *VvMYB5b*, AAX51291; *AtTT2*, Q9FJA2; *MdMYB9*, ABB84757; *MdMYB11*, AAZ20431.1; *FaMYB9*, AFL02460; *ZmC1*, 1613412E; *DkMYB2*, AB503699; *PtMYB134*, ACR83705; *DkMYB4*, AB503671; *NtMYB2*, BAA88222; *AtMYB114*, AEE34502; *AtMYB90*, NP_176813; *AtMYB75*, NP_176057; *MrMYB1*, ADG21957; *AtMYB113*, NP_176811; *PhAN2*, AAF66727; *VvMYC1*, ACC68685; *VvMYCA1*, NP_001267954; *VvMYC2*, NP_001267974; *AtTT8*, OAO98324; *PhAN1*, AF260919_1; *MtTT8*, AKN79606; *MdbHLH33*, ABB84474; *MdbHLH3*, ADL36597; *ZmMYC2*, QDM55339; *AtMYC2*, NP_174541; *PhAN11*, AAC18941; *AtTTG1*, NM122360; *NtTTG1*, ACJ06978; *VvWDR2*, ABF66626; *VvWDR1*, ABF66625; *MdTTG1*, AAF27919; *VcTTG1*, MH717246; *ZmPAC1*, AAM76724; *FaTTG1*, JQ989287; *InWDR1*, AB232779; *PfWD40*, AB059642.

2.9. Y2H Assays

The full-length CDS sequences of *VvMYBPA2*, *VvWDR1* and *VvMYC2* were cloned and connected to pGADT7 and pGBKT7 vectors, respectively. The recombinant pGADT7 and pGBKT7 plasmids were co-transformed into yeast AH109 competent cells and then coated on SD/-Trp-Leu defect medium for screening. The positive colonies were inoculated on SD/-Trp-Leu-His-Ade, SD/-Trp-Leu-His-Ade-X- α -gal medium for interaction screening, and the colony growth was observed and recorded. pGADT7-T and pGBKT7-53 were used as positive controls, and pGADT7-T and pGBKT7-Lam were used as negative controls.

2.10. BiFC Assays

The CDS sequences of *VvMYBPA2*, *VvWDR1* and *VvMYC2* were cloned and ligated into pCAMBIA1300-35S-YFPc and pCAMBIA1300-35S-YFPn vectors, respectively. The recombinant plasmids were introduced into the competent cells of *A. tumefaciens* GV3101. The cYFP-WDR1/nYFP-MYBPA2, cYFP-MYC2/nYFP-MYBPA2, cYFP-WDR1/nYFP-MYC2 and the negative control cYFP/nYFP in the experimental group were injected into the tobacco leaves of the four-week-old tobacco plants, respectively. Two to three days after injection, the laser confocal microscope (Leica) was used to observe and record. DAPI was used for nuclear staining.

2.11. Y1H Assays

The cis-acting elements of promoter *VvANR* and *VvLAR1* were predicted according to the PlantCARE online website. The fragments containing MBS elements (100–200 bp) were inserted into a pAbAi vector to construct the pAbAi-ANR and pAbAi-LAR1 vectors. The mutation-related vectors pAbAi-anr and pAbAi-lar1 were constructed by Shang-

hai Biological Engineering Co., Ltd. They were transformed into Y1Hgold competent cells and coated on SD/-Ura medium to screen positive colonies and prepare bait vector competent cells. The pGADT7 empty vector was transformed into pAbAi-ANR, pAbAi-LAR1, pAbAi-anr and pAbAi-lar1 competent cells, respectively, and coated on SD/-Leu, SD/-Leu/AbA⁵⁰ ng/mL, SD/-Leu/AbA⁷⁵ ng/mL, SD/-Leu/AbA¹⁰⁰ ng/mL and SD/-Leu/AbA²⁰⁰ ng/mL. The concentration of AbA was screened. Finally, the experimental group pGADT7-MYBPA2/pAbAi-ANR, pGADT7-MYBPA2/pAbAi-LAR1 and negative control pGADT7-MYBPA2/pAbAi-anr, pGADT7-MYBPA2/pAbAi-lar1 co-transformed yeast Y1Hgold competent state and coated with the corresponding concentration of AbA SD/-Leu screening medium was observed and recorded.

2.12. Data Processing

GraphPad Prism 9.0 and TBtools were used for data processing, and SAS 8.0 software was used for significant difference analysis. Photoshop CS6 was used for image processing.

3. Results

3.1. Subcellular Localization of VvMYBPA2

In order to explore the subcellular localization of grape VvMYBPA2 protein, the subcellular localization vector 35S::VvMYBPA2-GFP was constructed and transiently expressed in tobacco leaves, and 35S::GFP was used as a control. The results of laser confocal microscopy showed that the fluorescent green signal in the experimental group was concentrated in the nucleus, while it was dispersed throughout the cells in the empty vector group (Figure 1).

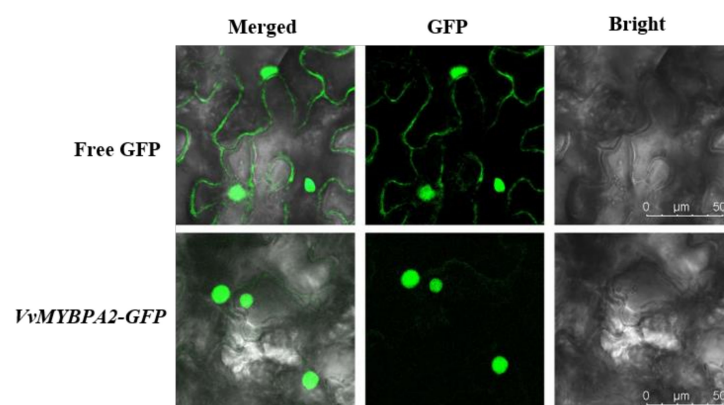


Figure 1. Subcellular localization of VvMYBPA2 in tobacco leaf cells. The empty GFP vector was used as a control. The images were taken in the dark field for green fluorescence (middle), bright light to demonstrate the morphology of the cells (right) and in overlay (left).

3.2. VvMYBPA2 Promoted the Accumulation of PAs in Grape Leaves

In order to explore whether VvMYBPA2 could promote the accumulation of PAs in grape leaves, we constructed an overexpression vector 35S::VvMYBPA2 and used the empty vector pCambia1300 as a control to infect grape leaves by vacuum infiltration. We found significant differences in DMACA staining, which indicated the higher content of PAs, in grape leaves after transient expression (Figure 2a), which indicated that overexpression of VvMYBPA2 increased the content of PAs in grape leaves. Fluorescence quantitative results showed that the expression of VvANR in grape leaves was significantly increased after transient overexpression, the expression of VvLAR1 was extremely significantly increased and there was no significant difference between VvLAR2 and the control (Figure 2b). The epicatechin content of the control group was 2.34 μg/g FW, while the content of the overexpression was 5.82 μg/g FW; the catechin content in the control group was 13.27 μg/g FW, while the content after overexpression was 51.06 μg/g FW. It was found that the epicatechin and catechin content were significantly higher (Figure 2c). By measuring the ANR and LAR enzyme activities of the samples, it was found that the ANR enzyme activity

of the grape leaves overexpressing *VvMYBPA2* was significantly increased (Figure 2d), and the LAR enzyme activity was extremely significantly increased (Figure 2e).

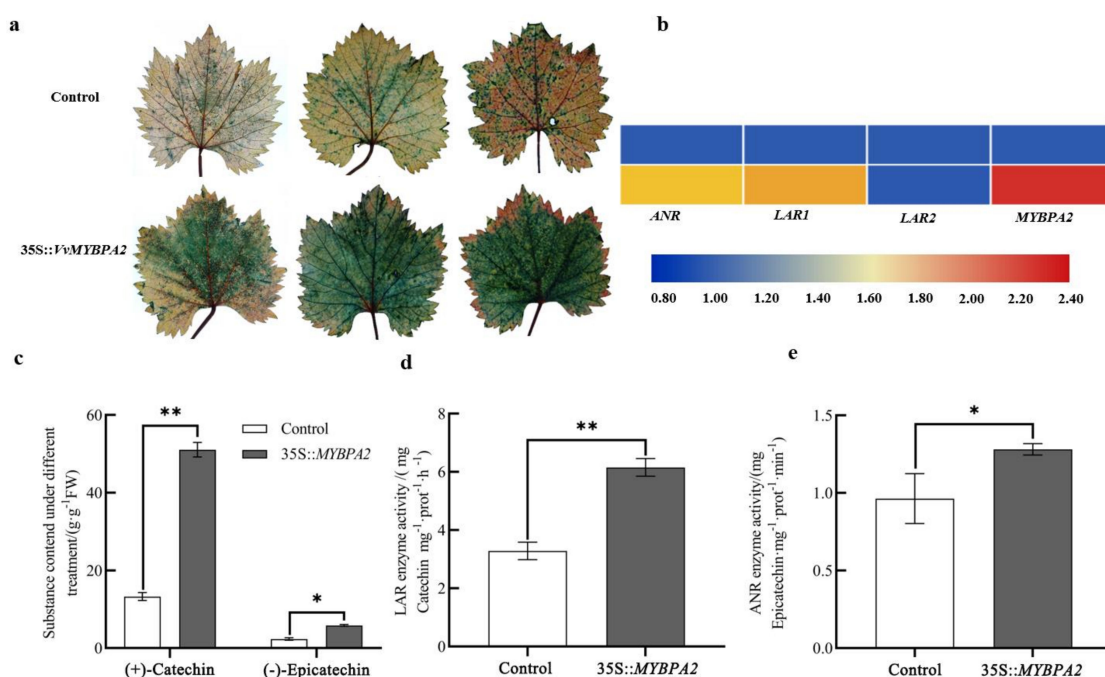


Figure 2. Effect of transient overexpression of *VvMYBPA2* in grape leaves. (a) DMACA staining results under different treatments. (b) Effects of different treatments on gene expression related to proanthocyanidin biosynthesis. (c) Effects of different treatments on catechin and epicatechin content. (d,e) Effects of different treatments on activities of LAR and ANR enzymes. Note: ** indicates extremely significant differences at the level of $p < 0.01$; * indicates significant differences at the level of $p < 0.05$, while ns indicates no significant difference.

3.3. Phylogenetic Analysis of Genes Related to Grape PAs Synthesis

Phylogenetic analysis of the deduced amino acid sequences was performed using the neighbor-joining method. Phylogenetic analysis showed that the amino acid sequence of *VvMYBPA2*, *VvMYC2* and *VvWDR1* was the most similar to *AtTT2*, *ZmMYC2* and *AtMYC2*, *AtTTG1* and *PhAN11*, respectively (Figure 3a–c).

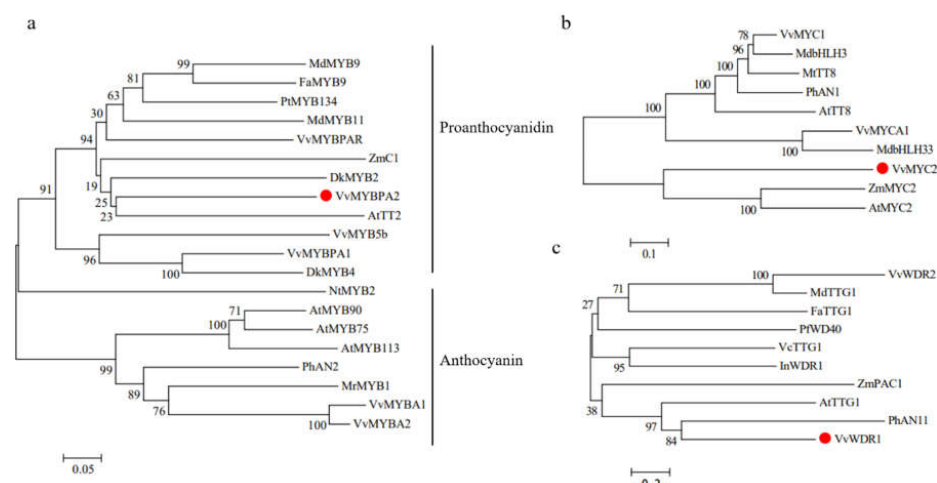


Figure 3. Phylogenetic analysis of transcription factors related to grape proanthocyanidin synthesis. (a–c) means the phylogenetic relationships between MYB, bHLH, WD40 transcription factors with their closest homologues, respectively.

3.4. *VvMYBPA2* Formed a Trimeric Complex with *VvWDR1* and *VvMYC2*

In order to clarify the interaction between *VvMYBPA2* protein, bHLH and WD40, we performed Y2H and BiFC assay. The Y2H results showed that both the positive control and the experimental groups could grow on the four-deficient plate, but only positive control and *VvWDR1/VvMYC2* turned blue on SD/-Trp-Leu-His-Ade/*X-α-Gal* plates (Figure 4a). It indicated that the *VvMYBPA2* protein interacted with the *VvWDR1* and *VvMYC2* proteins at the yeast level. We further verified using a BiFC assay, which showed that in tobacco leaves co-transformed with nYFP-*VvMYBPA2* and cYFP-*VvWDR1*, nYFP-*VvMYC2* and cYFP-*VvWDR1*, yellow fluorescence was detected in the nucleus, but no fluorescence was observed in tobacco leaves co-transformed with nYFP-*VvMYBPA2* and cYFP-*VvMYC2* (Figure 4b). It indicated that *VvMYBPA2* could interact with *VvWDR1* protein, but not *VvMYC2*, while *VvWDR1* could interact with *VvMYC2*.

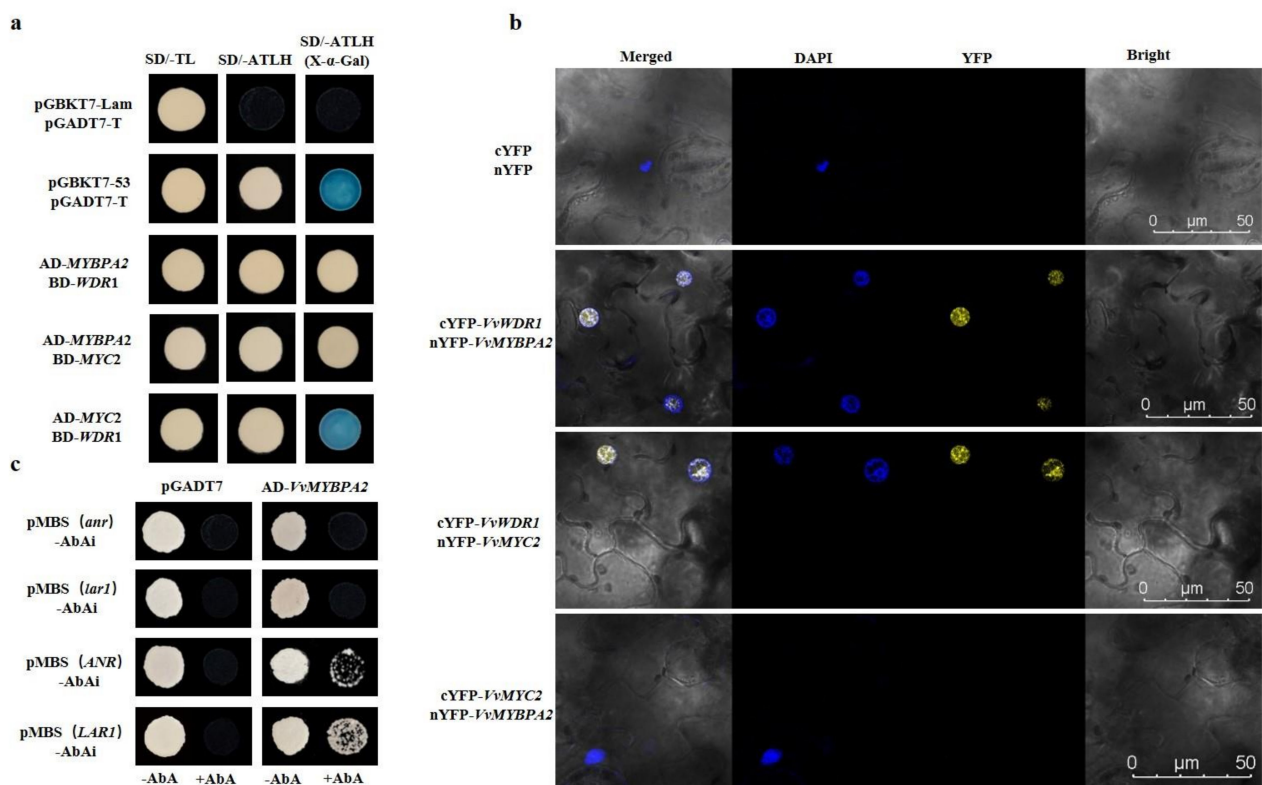


Figure 4. Interaction between *VvMYBPA2* and PAs biosynthesis-related genes. (a) Y2H assay analyzed the interaction among MYBPA2, WDR1 and MYC2 in vitro. (b) The interaction of *VvMYBPA2*, *VvWDR1* and *VvMYC2* in tobacco cells was analyzed by BiFC assay. (c) The interaction between *VvMYBPA2* and the promoters of *VvANR* and *VvLAR1* was analyzed by Y1H assay.

3.5. *VvMYBPA2* Directly Activated *VvANR* and *VvLAR1* Promoters

To clarify the binding between *VvMYBPA2* and the promoters of proanthocyanidin biosynthesis-related genes *VvANR* and *VvLAR1*, we carried out Y1H assay. The results showed that both the empty vector and mutant control successfully inhibited the growth on the SD/leu+75 ng AbA plate (Figure 4c), while the experimental group grew on the SD/leu+75 ng AbA plate, indicating that *VvMYBPA2* can directly bind to the MBS acting elements of *VvANR* and *VvLAR1* promoters. Similarly, the expression of *VvANR* and *VvLAR1* genes and related enzyme activities were significantly increased in the transient over-expression test of grape leaves. Therefore, *VvMYBPA2* can directly activate *VvANR* and *VvLAR1* promoters and then affect the accumulation of grape proanthocyanidins.

4. Discussion

Research had shown that plant proanthocyanidin biosynthesis was regulated by the TT2 type genes, a large branch of R2R3 MYBs, which belongs to the fifth subgroup [35], including *AtTT2* [15], *MdMYB9/11* [16], etc. Previous localization studies on *VvMYBPA2* homologous proteins *AtTT2* [15] and *MdMYB9/11* [16] showed that they were all localized in the nucleus. In this study, the TT2 transcription factor *VvMYBPA2* in ‘Zaoheibao’ grape was cloned, and the subcellular localization-related vector 35S::*VvMYBPA2*-GFP was successfully constructed and injected into tobacco to observe its localization in the nucleus (Figure 1).

Previous studies had found that *VvMYBPA2*, which is highly homologous to *Arabidopsis* TT2, was mainly expressed in the pericarp and leaves of young grape fruit, and its expression pattern was consistent with the accumulation of PAs during grape fruit development [25]. In addition to grapes, poplar [19], alfalfa [36], narcissus [37] and cocoa beans [36] were also found in MYB transcription factors homologous to *Arabidopsis* TT2, and the overexpression of these transcription factors all up-regulated the gene expression levels of LAR and ANR in transgenic plants, eventually leading to the accumulation of PAs. Overexpression of *VvMYBPA2* in grape hairy roots induced its accumulation of PAs and significantly activated *VvANR*, *VvLAR1* and *VvMYBPA1* [25]. Preliminary research in our laboratory found that the content of PAs in silencing *VvMYBPA2* grape leaves was significantly lower than that of the control, the activities of ANR and LAR enzymes were significantly reduced and the content of flavan-3-ol monomers was reduced. In this study, we found that the contents of (+)-catechin and (–)-epicatechin in grape leaves were significantly increased, ANR and LAR enzyme activities were significantly increased and levels of *VvANR* and *VvLAR1* gene expression were increased after transient overexpression of *VvMYBPA2* (Figure 2).

The R2R3-MYB transcription factor was involved in regulating different branches of flavonoid biosynthesis. To regulate PAs and anthocyanin biosynthesis, MYB transcription factors interacted with basic helix-loop-helix (bHLH) and WDR-repeat (WDR) factors to form ternary complexes to control related structural gene expression [38]. *Arabidopsis* AtTT2-AtTT8-AtTTG1 [22], kiwifruit AcMYBF110-AcbHLH1-AcWDR1 [39], poplar PuMYB134-PubHLH131 [19] and others had been shown to form MBW complexes that were involved in the regulation of anthocyanin and proanthocyanidin biosynthesis. Instead, there were exceptions to the MBW complex model. For example, the Apple WD40 protein MdTTG1 interacted with bHLH but did not interact with the MYB protein to regulate the accumulation of anthocyanins [40]. In grapes, studies had found that MYBA1 interacted with WDR1, and WDR1 interacted with MYC1 to form a complex to regulate the synthesis of grape anthocyanins ‘Yan’ 73 in tobacco [30]. Studies had shown that WD40 provided a docking platform for MYB-bHLH interaction so that MYB-WD40-bHLH could form a ternary complex to regulate the biosynthesis of anthocyanins [41]. In this study, we found that *VvMYBPA2* could interact with *VvWDR1* through Y2H and BiFC experiments, but there was no in vivo interaction with *VvMYC2*. We also observed that *VvWDR1* interacted with *VvMYC2*, which is similar to previous studies. Taken together, *VvMYBPA2* and *VvMYC2* form a trimeric complex by cooperating with *VvWDR1*, and affect proanthocyanidin. Previous studies had found that *VvWDR1* protein could bind to *VvWRKY26* protein [32]. It was speculated that the MBWW complex (*VvMYBPA2*-*VvMYC2*-*VvWDR1*-*WRKY26*) might be involved in regulating the synthesis of grape proanthocyanidins.

Genes related to proanthocyanidin biosynthesis are divided into two categories: structural genes and transcription factors. Among them, the structural genes directly encode the biosynthetic enzymes in the process of proanthocyanidin synthesis, namely anthocyanin reductase (ANR) and leucoanthocyanidin reductase (LAR), which catalyze the production of epicatechin and catechin monomers, respectively. Generally, transcription factors enhance or inhibit gene expression by binding to specific DNA sequences within the promoter region of the target gene [42]. For example, *GsMYB60* [17], *DkMYB2/4* [24], *MdMYB9/11* [16], *PtBBX23* [43], etc. could activate the expression of proanthocyanidin

biosynthesis-related outcome genes ANR or LAR directly. Grape MYBPA2 [44] could also activate PA synthesis-specific genes *VvCHS3*, *VvF3'5'H*, *VvLAR2* individually. However, some MYB transcription factors must rely on the interaction of bHLH or WD40 proteins to activate the promoters of structural genes, such as *Arabidopsis* AtTT2-AtTT8-AtTTG1 [22] and *strawberry* FaMYB9/FaMYB11-FabHLH3-FaTTG1 [23]. In this study, the Y1H assay showed that *VvMYBPA2* could directly bind to the promoters of *VvANR* and *VvLAR1*, thereby regulating the biosynthesis of (+)-catechin and (−)-epicatechin.

5. Conclusions

In short, it was found that *VvMYBPA2* could form a trimeric complex with *VvMYC2* and *VvWDR1* in grape and jointly regulated the expression of structural genes *VvANR* and *VvLAR1*, thereby promoting the ANR and LAR enzymatic activities, which ultimately positive regulated (+)-catechin and (−)-epicatechin contents (Figure 5).

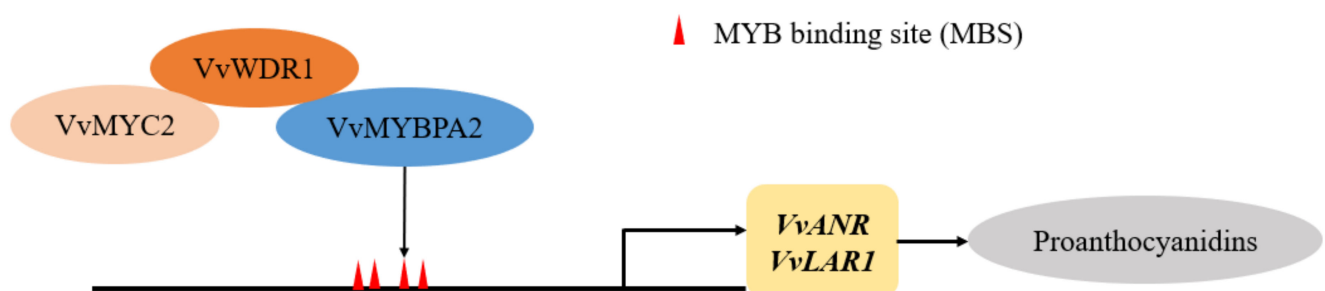


Figure 5. Prediction of the biosynthesis pattern of grape PAs regulated by MBW complex.

Author Contributions: Conceptualization, J.L. and P.W.; methodology, J.L.; validation, P.Z.; formal analysis, X.Z. and G.Z.; investigation, C.L.; resources, J.G., M.C., J.L. and C.L.; data curation, X.Z.; writing—original draft preparation, J.G.; writing—review and editing, C.L. and J.G.; visualization, C.L.; supervision, P.W.; project administration, J.L.; funding acquisition, J.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Biological Breeding Engineering Project of Shanxi Agricultural University (Grant Nos: YZGC113), Youth Scientific Research Project of Shanxi Province (Grant Nos: 20210302124067), Shanxi Province doctoral graduates and postdoctoral researchers come to work in Shanxi to reward scientific research projects (Grant Nos: SXBYKY2021075), and Shanxi Fruit Tree Industry Technology System Post Expert Special Project.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: Thanks to Xu Jin for donating tobacco seeds and related vectors.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A. Information of primers used in PCR

Primers Name	Primers Sequence	Accession Number
GFP-MYBPA2-F	GGGGTACCATGGGAAGAAGACCTT	EU919682.1
GFP-MYBPA2-R	CGGGATCCTGGGACTTGATTATTTTC	
OX-MYBPA2-F	GGGGTACCATGGGAAGAAGACCTT	EU919682.1
OX-MYBPA2-R	CGGGATCCCTATGGGACTTGATTATTT	
RT-VvActin-F	CCCCATGCTATCCTTCG	AF369524.1
RT-VvActin-R	AGGCAGCTCATAGTTC	
RT-VvANR-F	AGAACTACAGGAGTTGGGTGAC	DQ129684.1
RT-VvANR-R	CCTTGAATTGCTGGCTTG	
RT-VvLAR1-F	ACGATGTCCGAACACTGAAC	AJ865336
RT-VvLAR1-R	TGAACGCCGCTACTACACTC	
RT-VvLAR2-F	TCTCGACATACATGATGATGTG	AJ8653
RT-VvLAR2-R	TGCAGTTTCTTTGATTGAGTTC	
RT-VvMYBPA2-F	GTGCGATGCTCCAAGGTTAT	EU919682.1
RT-VvMYBPA2-R	CAGTGCTGAACCTGAGGGC	
BD-VvWDR1-F	CGGAATTCATGGAGAGATCAAGCC	DQ517913.2
BD-VvWDR1-R	CGGGATCCCTAAACTTTAAGAAGC	
AD-VvMYC2-F	GGAATTCCATATGATGAAAAGTAAATGGG	EF636725.2
AD-VvMYC2-R	TCCCCCGGGTTACCCAAGTATGATGAC	
BD-VvMYC2-F	CGCCCGGGATGAAAAGTAAATGGGTATG	EF636725.2
BD-VvMYC2-R	GGCGTCGACTTACCCAAGTATGATGAC	
BiFC-MYBPA2-F	CGGGATCCATGGGAAGAAGACCTT	EU919682.1
BiFC-MYBPA2-R	GCGTCGACTGGGACTTGATTATTTTCAG	
BiFC-WDR1-F	CGGATCCATGGAGAGATCAAGCCT	DQ517913.2
BiFC-WDR1-R	GGCGAATTCAACTTTAAGAAGCTGCAGTTT	
BiFC-MYC2-F	GGCGTCGACATGAAAAGTAAATGGGTATG	EF636725.2
BiFC-MYC2-R	GGACTAGTCCCAACTGATGATGACAAAG	
Y1H-ANR-F	CGAGCTCGTTAGTTGGGAACCATC	DQ129684.1
Y1H-ANR-R	GCGTCGACGCATATCTCAACAGCAG	
Y1H-LAR1-F	CGAGCTCACATAAATCCGGCCTAG	AJ865336
Y1H-LAR1-R	GCGTCGACTGACTCACCATTTCATGA	

Note. The underlined part is the enzyme cleavage site.

References

- Wen, P.F. *Studies on Flavanols in Wine and Grape Berry and Expression of Genes Involved in Proanthocyanidins Biosynthesis during Berry Development*; China Agricultural University: Beijing, China, 2005.
- Zeng, Y.X.; Wang, S.; Wei, L.; Cui, Y.Y.; Chen, Y.H. Proanthocyanidins: Components, pharmacokinetics and biomedical properties. *Am. J. Chin. Med.* **2020**, *48*, 813–869. [[CrossRef](#)]
- Bogs, J.; Downey, M.O.; Harvey, J.S.; Ashton, A.R.; Tanner, G.J.; Robinson, S.P. Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. *Plant Physiol.* **2005**, *139*, 652–663. [[CrossRef](#)] [[PubMed](#)]
- Ma, W.; Guo, A.; Zhang, Y.; Wang, H.; Liu, Y.; Li, H. A review on astringency and bitterness perception of tannins in wine. *Trends in Food Sci. Technol.* **2014**, *40*, 6–19. [[CrossRef](#)]
- Ding, Y.; Han, X.M.; Wang, C.P.; Wang, Q.C. Effects of different fermentative maceration methods on the flavor components and sensory characteristics of red wine. *Liquor-Mak. Sci. Technol.* **2021**, *323*, 39–45.
- Bertelli, A.A.; Das, D.K. Grapes, wines, resveratrol, and heart health. *J. Cardiovasc. Pharmacol.* **2009**, *54*, 468–476. [[CrossRef](#)] [[PubMed](#)]
- Dixon, R.A.; Xie, D.; Sharma, S.B. Proanthocyanidins—A final frontier in flavonoid research? *New Phytol.* **2005**, *165*, 9–28. [[CrossRef](#)] [[PubMed](#)]
- Wilska-Jeszka, J. Proanthocyanidins: Content in fruits and influence on health. *Food Chem.* **1996**, *57*, 57. [[CrossRef](#)]
- Xing, Y.F. *The Accumulation of Flavanols, Expression of Leucoanthocyanidin Reductase Induced by Uv-C Irradiation in Grape Breezy*; Shanxi Agricultural University: Taiyuan, China, 2013.
- Dixon, R.A.; Sarnala, S. Proanthocyanidin biosynthesis—A matter of protection. *Plant Physiol.* **2020**, *184*, 579–591. [[CrossRef](#)] [[PubMed](#)]
- Xie, D.Y.; Sharma, S.B.; Paiva, N.L.; Ferreira, D.; Dixon, R.A. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. *Science* **2003**, *299*, 396–399. [[CrossRef](#)]

12. Tanner, G.J.; Francki, K.T.; Abrahams, S.; Watson, J.M.; Larkin, P.J.; Ashton, A.R. Proanthocyanidin biosynthesis in plants. Purification of legume leucoanthocyanidin reductase and molecular cloning of its cDNA. *J. Biol. Chem.* **2003**, *278*, 31647–31656. [\[CrossRef\]](#)
13. Pfeiffer, J.; Kühnel, C.; Brandt, J.; Duy, D.; Punyasiri, P.A.; Forkmann, G.; Fischer, T.C. Biosynthesis of flavan 3-ols by leucoanthocyanidin 4-reductases and anthocyanidin reductases in leaves of grape (*Vitis vinifera* L.), apple (*Malus × domestica* Borkh.) and other crops. *Plant Physiol. Biochem.* **2006**, *44*, 323–334. [\[CrossRef\]](#)
14. Liang, C.M.; Yang, B.; Wei, Y.; Zhang, P.F.; Wen, P.F. SA incubation induced accumulation of flavan-3-ols through activated VvANR expression in grape leaves. *Sci. Hort.* **2021**, *287*, 110269. [\[CrossRef\]](#)
15. Debeaujon, I.; Nesi, N.; Perez, P.; Devic, M.; Grandjean, O.; Caboche, M.; Lepiniec, L. Proanthocyanidin-accumulating cells in *Arabidopsis* testa: Regulation of differentiation and role in seed development. *Plant Cell.* **2003**, *15*, 2514–2531. [\[CrossRef\]](#) [\[PubMed\]](#)
16. An, X.H.; Tian, Y.; Chen, K.Q.; Liu, X.J.; Liu, D.D.; Xie, X.B.; Cheng, C.G.; Cong, P.H.; Hao, Y.J. MdMYB9 and MdMYB11 are Involved in the Regulation of the JA-Induced Biosynthesis of Anthocyanin and Proanthocyanidin in Apples. *Plant Cell Physiol.* **2015**, *56*, 650–662. [\[CrossRef\]](#)
17. Li, J.; Luan, Q.; Han, J.; Zhang, C.; Liu, M.; Ren, Z. CsMYB60 directly and indirectly activates structural genes to promote the biosynthesis of flavonols and proanthocyanidins in cucumber. *Hort. Res.* **2020**, *7*, 103–118. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Yang, S.; Zhang, M.; Xu, L.; Luo, Z.; Zhang, Q. MiR858b inhibits proanthocyanidin accumulation by the repression of DkMYB19 and DkMYB20 in persimmon. *Front. Plant Sci.* **2020**, *1*, 576378. [\[CrossRef\]](#)
19. James, A.M.; Ma, D.; Mellway, R.; Gesell, A.; Yoshida, K.; Walker, V.; Tran, L.; Stewart, D.; Reichelt, M.; Suvanto, J.; et al. Poplar MYB115 and MYB134 transcription factors regulate proanthocyanidin synthesis and structure. *Plant Physiol.* **2017**, *174*, 154–171. [\[CrossRef\]](#)
20. Lu, N.; Rao, X.; Li, Y.; Jun, J.H.; Dixon, R.A. Dissecting the transcriptional regulation of proanthocyanidin and anthocyanin biosynthesis in soybean (*Glycine max*). *Plant Biotechnol. J.* **2021**, *19*, 1429–1442. [\[CrossRef\]](#)
21. Broun, P. Transcriptional control of flavonoid biosynthesis: A complex network of conserved regulators involved in multiple aspects of differentiation in *Arabidopsis*. *Curr. Opin. Plant Biol.* **2005**, *8*, 272–279. [\[CrossRef\]](#)
22. Baudry, A.; Heim, M.A.; Dubreucq, B.; Caboche, M.; Weisshaar, B.; Lepiniec, L. TT2, TT8, and TTG1 synergistically specify the expression of banyuls and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. *Plant J.* **2010**, *39*, 366–380. [\[CrossRef\]](#)
23. Schaart, J.G.; Dubos, C.; Romero De La Fuente, I.; van Houwelingen, A.; de Vos, R.; Jonker, H.H.; Xu, W.; Routaboul, J.M.; Lepiniec, L.; Bovy, A.G. Identification and characterization of MYB-bHLH-WD40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (*Fragaria × ananassa*) fruits. *New Phytol.* **2013**, *197*, 454–467. [\[CrossRef\]](#)
24. Gil-Muoz, F.; Sánchez-Navarro, J.A.; Besada, C.; Salvador, A.; Badenes, M.L.; Naval, M.D.M.; Rios, G. MBW complexes impinge on anthocyanidin reductase gene regulation for proanthocyanidin biosynthesis in persimmon fruit. *Sci. Rpt.* **2020**, *10*, 3543–3554. [\[CrossRef\]](#)
25. Bogs, J.; Jaffé, F.W.; Takos, A.M.; Walker, A.R.; Robinson, S.P. The grapevine transcription factor VvMYBPA1 regulates proanthocyanidin synthesis during fruit development. *Plant Physiol.* **2007**, *143*, 1347–1361. [\[CrossRef\]](#)
26. Terrier, N.; Torregrosa, L.; Ageorges, A.; Vialet, S.; Verriès, C.; Cheynier, V.; Romieu, C. Ectopic expression of VvMYBPA2 promotes proanthocyanidin biosynthesis in grapevine and suggests additional targets in the pathway. *Plant Physiol.* **2009**, *149*, 1028–1041. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Matus, J.T.; Poupin, M.J.; Cañón, P.; Bordeu, E.; Alcalde, J.A.; Arce-Johnson, P. Isolation of WDR and bHLH genes related to flavonoid synthesis in grapevine (*Vitis vinifera* L.). *Plant Mol. Biol.* **2010**, *72*, 607–620. [\[CrossRef\]](#)
28. Wang, P.F.; Su, L.; Gao, H.H.; Jiang, X.L.; Wu, X.Y.; Li, Y.; Zhang, Q.Q.; Wang, Y.M.; Ren, F.S. Genome-wide characterization of bHLH genes in grape and analysis of their potential relevance to abiotic stress tolerance and secondary metabolite biosynthesis. *Front. Plant Sci.* **2018**, *1*, 64–78. [\[CrossRef\]](#)
29. Fu, J.Y.; Liu, L.J.; Liu, Q.; Shen, Q.Q.; Wang, C.; Yang, P.P.; Zhu, C.Y.; Wang, Q. ZmMYC2 exhibits diverse functions and enhances JA signaling in transgenic *Arabidopsis*. *Plant Cell Rep.* **2020**, *39*, 273–288. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Xie, S.; Qiao, X.L.; Chen, H.W.; Nan, H.; Zhang, Z.W. Coordinated regulation of grape berry flesh color by transcriptional activators and repressors. *J. Agr. Food Chem.* **2019**, *67*, 11815–11824. [\[CrossRef\]](#)
31. Hichri, I.; Heppel, S.C.; Pillet, J.; Léon, C.; Czemmel, S.; Delrot, S.; Lauvergeat, V.; Bogs, J. The basic helix-loop-helix transcription factor MYC1 is involved in the regulation of the flavonoid biosynthesis pathway in grapevine. *Mol. Plant.* **2010**, *3*, 509–523. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Amato, A.; Cavallini, E.; Walker, A.R.; Pezzotti, M.; Bliet, M.; Quattrocchio, F.; Koes, R.; Ruperti, B.; Bertini, E.; Zenoni, S.; et al. The MYB5-driven MBW complex recruits a WRKY factor to enhance the expression of targets involved in vacuolar hyper-acidification and trafficking in grapevine. *Plant J.* **2019**, *99*, 1220–1241. [\[CrossRef\]](#)
33. Li, Y.G.; Tanner, G.; Larkin, P. The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in Forage Legumes. *J. Sci. Food Agri.* **1996**, *70*, 89–101. [\[CrossRef\]](#)
34. Wen, P.F.; Ji, W.; Gao, M.Y.; Niu, T.Q.; Xing, Y.F.; Niu, X.Y. Accumulation of flavanols and expression of leucoanthocyanidin reductase induced by postharvest UV-C irradiation in grape berry. *Genet. Mol. Res.* **2015**, *14*, 7687–7695. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Nesi, N.; Jond, C.; Debeaujon, I.; Caboche, M.; Lepiniec, L. The *Arabidopsis* TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. *Plant Cell* **2001**, *13*, 2099–2114. [\[CrossRef\]](#) [\[PubMed\]](#)

36. Liu, C.; Jun, J.H.; Dixon, R.A. MYB5 and MYB14 play pivotal roles in seed coat polymer biosynthesis in *Medicago truncatula*. *Plant Physiol.* **2014**, *165*, 1424–1439. [[CrossRef](#)]
37. Fan, Y.; Peng, J.; Wu, J.; Zhou, P.; He, R.; Allan, A.C.; Zeng, L. NtHLH1, a JAF13-like bHLH, interacts with NtMYB6 to enhance proanthocyanidin accumulation in Chinese Narcissus. *BMC Plant Biol.* **2021**, *21*, 275–289. [[CrossRef](#)] [[PubMed](#)]
38. Gonzalez, A.; Brown, M.; Hatlestad, G.; Akhavan, N.; Smith, T.; Hembd, A.; Moore, J.; Montes, D.; Mosley, T.; Resendez, J.; et al. TTG2 controls the developmental regulation of seed coat tannins in Arabidopsis by regulating vacuolar transport steps in the proanthocyanidin pathway. *Dev. Biol.* **2016**, *419*, 54–63. [[CrossRef](#)]
39. Liu, Y.; Ma, K.; Qi, Y.; Lv, G.; Ren, X.; Liu, Z.; Ma, F. Transcriptional regulation of anthocyanin synthesis by MYB-bHLH-WDR complexes in Kiwifruit (*Actinidia chinensis*). *J. Agr. Food Chem.* **2021**, *69*, 3677–3691. [[CrossRef](#)]
40. An, X.H.; Tian, Y.; Chen, K.Q.; Wang, X.F.; Hao, Y.J. The apple WD40 protein MdTTG1 interacts with bHLH but not MYB proteins to regulate anthocyanin accumulation. *J. Plant Physiol.* **2012**, *169*, 710–717. [[CrossRef](#)] [[PubMed](#)]
41. Zhang, B. *Evolutionary Analysis of Mybs-Bhlh-Wd40 Protein Complexes Formation and Their Functional Relationship in Planta*; University of Cologne: Cologne, Germany, 2018.
42. Fickett, J.W.; Wasserman, W.W. Discovery and modeling of transcriptional regulatory regions. *Curr. Opin. Biotech.* **2000**, *11*, 19–24. [[CrossRef](#)]
43. Li, C.; Pei, J.; Yan, X.; Tsuruta, M.; Liu, Y.; Lian, C. A poplar B-box protein PtrBBX23 modulates the accumulation of anthocyanins and proanthocyanidins in response to high light. *Plant Cell Environ.* **2021**, *44*, 3015–3033. [[CrossRef](#)] [[PubMed](#)]
44. Kazuya, K.; Mineyo, N.; Ikuko, N.; Goto-Yamamoto, N.; Matsumura, H.; Tanaka, N. Functional characterization of a new grapevine MYB transcription factor and regulation of proanthocyanidin biosynthesis in grapes. *J. Expt. Bot.* **2014**, *65*, 4433–4449.