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Expression of the *VvMYB60* Transcription Factor Is Restricted to Guard Cells and Correlates with the Stomatal Conductance of the Grape Leaf

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Abstract: The modulation of stomatal activity is a relevant trait in grapes, as it defines the isohydric/anysohydric behavior of different cultivars and directly affects water-use efficiency and drought resistance of vineyards. The grape transcription factor *VvMYB60* has been proposed as a transcriptional regulator of stomatal responses based on its ectopic expression in heterologous systems. Here, we directly addressed the cellular specificity of *VvMYB60* expression in grape leaves by integrating independent approaches, including the qPCR analysis of purified stomata and the transient expression of a *VvMYB60* promoter: GFP fusion. We also investigated changes in the *VvMYB60* expression in different rootstocks in response to declining water availability. Our results indicate that *VvMYB60* is specifically expressed in guard cells and that its expression tightly correlates with the level of stomatal conductance (g_s) of the grape leaf. As a whole, these findings highlight the relevance of the *VvMYB60* regulatory network in mediating stomatal activity in grapes.

Keywords: guard cell; laser microdissection; MYB transcription factor; gene expression; stomatal conductance

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1. Introduction

Grapevine (*Vitis vinifera* L.) is traditionally grown under non-irrigated field conditions in many cropping environments, including drylands and semiarid regions [1]. Good osmotic adjustment, the architecture of the root system, xylem embolism, and efficient stomatal control of water loss account for the drought resistance traits broadly found within the *Vitis* genus [2].

Among these features, the regulation of stomatal activity is of particular relevance as it directly shapes the isohydric versus anysohydric behavior of different grape species and cultivars [3]. After experiencing soil water deficit, isohydric genotypes rapidly reduce stomatal conductance to prevent excessive transpiration and to limit a decline in leaf water potential. Conversely, under stress, anysohydric genotypes maintain elevated g_s levels to maximize photosynthesis, despite the significant drop in water potential [4]. In addition, the closure of the stomatal pore contributes to avoiding xylem tensions, which, in turn, could lead to cavitation and to decreased water conductance within the plant [5]. Stomata also play a central role in response to major pathogens, including downy mildew (*Plasmopora viticola*), as they represent primary ports of penetration for the germinating zoospores and preferential sites of exit for sporangiophores during sporulation [6]. Despite the prominent

role of stomata in the interaction between the plant and the surrounding environment, the molecular mechanisms underlying their activity have been poorly investigated in grapes.

Opening and closing of the pore are modulated by turgor-driven changes in the volume of the two surrounding guard cells [7]. Studies from model species, mainly Arabidopsis, have unraveled many components of the complex regulatory networks controlling stomatal movements [8]. Increasing evidence indicates a role for the transcriptional regulation of gene expression in modulating stomatal responses to both biotic and abiotic stimuli. Several guard-cell-related transcription factors have been identified as key regulators of stomatal development and guard cell activity. Among them, members of the R2R3 MYB subfamily of transcriptional regulators appear to play a central role in the control of stomatal opening and transpirational water loss under stress [9].

The grapevine genome contains nearly 280 MYB genes, of which 125 encode R2R3 MYB-type proteins [10]. This large gene family has been extensively investigated in grapes, as several R2R3 MYBs are directly involved in the regulation of anthocyanin and pro-anthocyanidin biosynthesis, two major determinants of the quality of berries and wine [11]. Very few studies have addressed the role of R2R3 genes beyond their function in flavonoid biosynthesis. In a previous work, we identified the grape gene *VvMYB60* (VIT_08s0056g00800) as the functional ortholog of *AtMYB60* (At1g08810), which is involved in the regulation of stomatal activity in Arabidopsis [12]. Expression of the Arabidopsis *AtMYB60* gene is restricted to guard cells and positively correlates with the opening of the stomatal pore. Genetic analyses indicate that *AtMYB60* is a positive regulator of light-induced stomatal opening [13]. Recently, it has been shown that *AtMYB60* negatively regulates the expression of guard-cell-related *LYPOXIGENASEs*, involved in the synthesis of oxylipins in stomata. The increased accumulation of active oxylipins, in the *atmyb60-1* mutant, results in reduced stomatal opening, decreased transpirational water loss, and enhanced drought resistance [14].

Histochemical and quantitative Real-Time PCR (qPCR) analyses of Arabidopsis transgenic lines harboring the GUS marker gene under the control of the *VvMYB60* regulatory region highlight the guard-cell-specific activity of the grape promoter in a heterologous system [12]. Most importantly, expression of the VvMYB60 protein in the *atmyb60-1* background fully rescues the stomatal defect depicted by the mutant, thus indicating the conservation of the MYB60 function between grapes and Arabidopsis [12].

Here, we report results from the analysis of *VvMYB60* expression in the grape leaf, which provide new insights into its cellular specificity. qPCR analyses of stomata-enriched epidermal fragments or pure preparation of guard cells microdissected from the grape leaf indicated that *VvMYB60* is preferentially expressed in stomata. Confocal analysis of grape leaves Agro-infiltrated with a transcriptional fusion between the *VvMYB60* promoter and the GFP reporter confirmed the guard-cell-specificity of *VvMYB60* expression. Analysis of changes in *VvMYB60* expression relative to variations in stomatal conductance in selected rootstock genotypes, grown under control or drought stress conditions, revealed a positive correlation between g_s and the accumulation of the *VvMYB60* transcript. As a whole, our data provide further support to the notion of *VvMYB60* being a transcriptional mediator of stomatal activity in grapes and highlight its potential as a target for modulating stomatal responses in this relevant crop.

2. Materials and Methods

2.1. Growth Condition, Leaf Sampling, and Stomatal Measurements

Two-year-old ungrafted *Vitis vinifera* L. cv. Cabernet Sauvignon were used in the ice-blinding and laser-microdissection experiments. Two-year-old ungrafted rootstocks (101.14 Millardet et de Grasset (101.14), Kober 5BB (K5BB), Milano4 (M4), and 1103Paulsen (1103P)) were used in the drought-stress experiment. All rootstocks were propagated from cuttings. Plants were grown in 3 L pots filled with sand–peat mixture (7:3 *v*/*v*), in a controlled greenhouse under a 16 h light and an 8 h dark photoperiod. Control plants were maintained at 80% of soil field capacity (FC), whereas the two water stress regimes were

imposed by reducing soil water content to 50% and 30% FC, respectively, as indicated in Meggio et al. [15].

2.2. Stomatal Conductivity (g_s) and Stomatal Density Measurements

The g_s value was measured in vivo using a portable AP4 leaf porometer (Delta-T Devices, Burwell, UK), following instructions from the manufacturer. The analysis was performed after three consecutive days at 50% or 30% FC on four leaves from five plants per genotype per treatment. Four adjacent g_s measurements were taken from the central part of each leaf. Stomatal density (SD) was manually measured counting the number of stomata per mm^2 of leaf surface in images of leaves cleared with 70% EtOH. Images were digitally recorded with a Leica DM2500 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany). Five leaves were analyzed for each genotype, for a total of 40 images per line, corresponding to an overall area of 10 mm^2. The g_s measurements and SD analyses were performed on leaves from the fourth to the seventh node of the primary shoot.

2.3. Ice Blending and Laser Microdissection of Grape Leaves

Four leaves of comparable size and developmental stage were sampled from five plants of *Vitis vinifera* L., cv Cabernet Sauvignon. Ice-blending purification of stomata was performed as previously described [16]. In brief, major veins were manually removed from grape leaves with a scalpel blade and tissues were mechanically fragmented by blending in ice-cold distilled water and crushed ice for 1 min and then filtered through a 210 µm nylon mesh. After three blending-filtration cycles, the resulting epidermal fraction was frozen in liquid nitrogen and stored at −80 °C. Samples of purified epidermal fraction were inspected by optical microscopy to ensure the absence of mesophyll cells and enrichment in intact stomatal guard cells.

For the laser microdissection (LM) experiment, major veins of the leaves were removed with a scalpel blade and samples were processed as previously described [17]. Ten micrometer sections were cut with a Leica RM2265 microtome (Leica Microsystems, GmbH, Wetzlar, Germany) from paraffin-embedded samples and mounted on PET membrane-coated glass slides (Leica Microsystems, GmbH, Wetzlar, Germany). Microdissection was performed using a Leica Laser Microdissection 6000 system (Leica Microsystems, GmbH, Wetzlar, Germany). Over 3000 stomata and mesophyll cells were dissected from each sample.

2.4. qPCR Analysis

RNA from intact leaves or from blended tissues was isolated using the Spectrum™ Plant Total RNA (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions. RNA from the LM sample was isolated using the PureLink® RNA Mini Kit (Life Technologies, Carlsbad, CA, USA). Complementary DNA synthesis and qPCR amplification were performed as described in [12], using the following primers: *VvMYB60* forw –TTGAGTACGAAAACCTGAATGAT-, *VvMYB60* rev–TTGAGTACGAAAACCTGAATGAT-; *VvSIRK* forw–AGTCCCCGTTACAGGGCTTGGG-, *VvSIRK* rev–AGTCCCCGTTACAGGGCT TGGG-; *VvG3PDH* forw–TTCTCGTTGAGGGCTATTCCA-, *VvG3PDH* rev–TTCTCGTTGAG GGCTATTCCA. qPCR amplification was performed using the Fast SYBR Green Master Mix (Applied Biosystem, Waltham, MA, USA), and real-time-monitored on a 7900 HT Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA). Relative gene expression was calculated using the ΔΔCt method according to Matus et al. [18].

2.5. Plasmid Constructs, Leaves Infiltration and Confocal Analysis

The 2239 bp *VvMYB60* promoter was excised from the *pVvMYB60:GUS* plasmid [12] with HindIII and XbaI and cloned into the pBINmGFP vector [19] to produce the *pVvMYB60:GFP* binary vector. Grape leaves from in vitro grown plants (cv Sugraone) were agro-infiltrated as described by Zottini et al. [20]. Inocula of Agrobacterium harboring the *pVvMYB60:GFP* plasmid were delivered to the lamina tissues of young grape leaves (before the full expansion, at about 2/3 of the full size) by gentle pressure to promote the infiltration through the stomata

of the lower epidermis using a 1 mL syringe without needle. Ten grapevine plants (5 leaves each) were Agro-infiltrated.

Confocal microscopy analyses were performed 10 days after the Agro-infiltration. Leaf samples were randomly cut from the infiltrated areas and mounted on slides for microscopic observations. Confocal microscope analyses were performed by using a Zeiss LSM 700 laser scanning confocal imaging system (Carl Zeiss Microscopy, Jena, Germany). For GFP fluorescence, excitation was at 488 nm and emission was between 515 and 530 nm. For the chlorophyll detection, excitation was at 488 nm and detection was over 650 nm.

3. Results and Discussion

3.1. Analysis of the Cellular Specificity of VvMYB60 Expression in the Grape Leaf

We sought to directly address the cellular domains of the *VvMYB60* expression in grape leaves (*Vitis vinifera* L., cv Cabernet Sauvignon) by employing two independent and complementary approaches: (i) the qPCR analysis of RNAs derived from epidermal fragments enriched in active stomata or purified from microdissected guard cells and mesophyll cells and (ii) the confocal microscopy analysis of the GFP expression in grape leaves transiently expressing the GFP reporter driven by the *VvMYB60* promoter (*VvMYB0$_{pro}$:GFP*).

Ice-blending of whole leaves has been proposed as a rapid and simple method to isolate epidermal fragments enriched in functional guard cells suitable for gene expression studies [16]. We tailored the blending technique to the grape leaf, obtaining epidermal pieces of approximately 0.5–1.0 mm^2 enclosing intact stomata and largely devoid of mesophyll tissue (Figure S1). Specificity of RNAs purified from the blended fragments was tested by qPCR analysis of the *STOMATAL INWARD RECTIFYING K$^+$ CHANNEL* (*VvSIRK*) gene. *VvSIRK* encodes a guard-cell-specific K$^+$ channel related to the *POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 2* (*KAT2*), which is involved in the regulation of the stomatal opening [21]. As expected, *VvSIRK* transcript was enriched in the blended-purified fraction compared with whole leaves, demonstrating efficacy of the purification method (Figure 1A). Expression of *VvMYB60* also showed significant over-representation in the stomata-enriched fraction, thus indicating its preferential expression in guard cells (Figure 1B).

The blended method implies the mechanical disruption of leaf tissues and possible contamination with other cell types, mainly from the vasculature [16]. We employed a laser-microdissection (LM) approach to accurately resolve the spatial distribution of *VvMYB60* expression in grape leaves. Since it is the opposite of blending methods, LM allows for the isolation of pure preparations of single cell types from intact complex tissues [22]. We assessed gene expression in LM-purified mesophyll and guard cells from Cabernet Sauvignon leaves (Figure S2). As shown in Figure 1C, the expression of *VvSIRK* was significantly enhanced in guard cells compared to mesophyll cells. Similarly, *VvMYB60* displayed a marked guard-cell-preferential expression, thus confirming its cellular specificity in the grape leaf (Figure 1D).

Finally, we employed a transient expression assay to further ascertain the spatial domains of *VvMYB60* expression. Leaves of the cultivar Sugraone were Agro-infiltrated with the control construct *35SCaMV$_{pro}$:GFP* or with the *VvMYB0$_{pro}$:GFP* construct expressing the reporter GFP under the control of the 2173 bp promoter region of *VvMYB60* [12]. As expected, GFP activity was detected throughout the leaves' tissues when expressed under the *35SCaMV* promoter (Figure 1E). Remarkably, in leaves infiltrated with the *VvMYB0$_{pro}$:GFP*, fusion GFP signals were specifically localized in stomata, unambiguously confirming the guard-cell-specific activity of the *VvMYB60* promoter in grape leaves (Figure 1F,G).

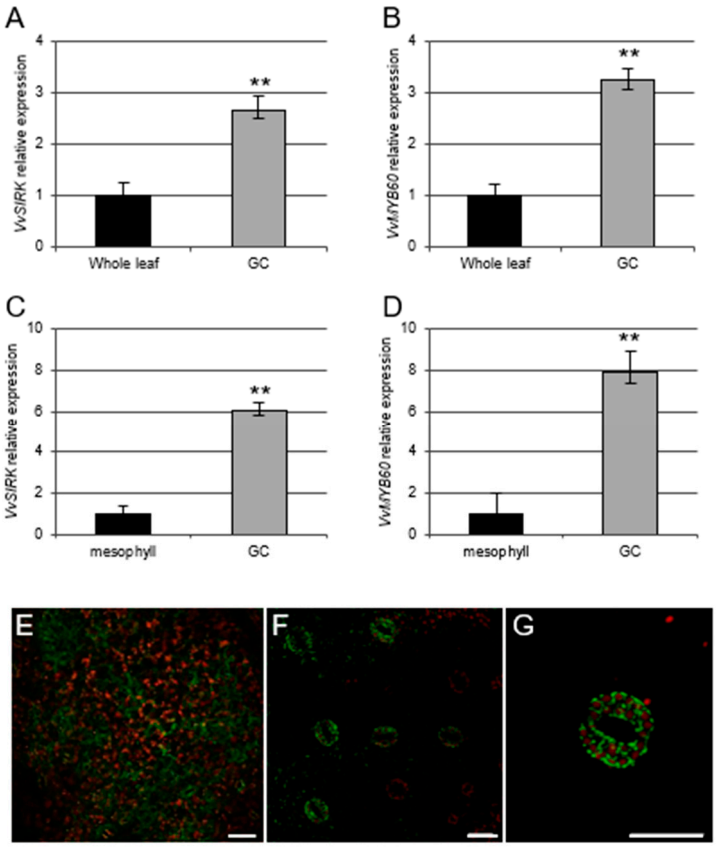

Figure 1. *VvMYB60* is preferentially expressed in guard cells. (**A**,**B**) qPCR analysis of *VvSIRK* (**A**) and *VvMYB60* (**B**) expression in epidermal fragments enriched in intact guard cells and in whole leaves. (**C**,**D**) Expression of *VvSIRK* (**C**) and *VvMYB60* (**D**) in LM-purified guard cells and mesophyll cells. Gene expression was normalized to the expression of the *GLYCERALDEHYDE-3-PHOSATE DEHYDROGENASE* (*G3PDH*) gene. Values represent means ± standard errors. Asterisks (**) indicate significant differences at $p < 0.01$ (ANOVA). (**E**–**G**) Confocal analysis of grape leaves agro-infiltrated with the $35SCaMV_{pro}$*:GFP* (**E**) or with the $VvMYB0_{pro}$*:GFP* construct (**F**,**G**). Chlorophyll autofluorescence is shown in red. Scale bars: (**E**), 50 μm; (**F**,**G**), 25 μm.

3.2. Different Rootstocks Disclose a Positive Correlation between VisMYB60 Expression and Stomatal Conductance

We investigated changes in the *VvMYB60* expression relative to variations in stomatal conductance to address its possible involvement in mediating stomatal opening in grapes. To this end, we selected two drought-susceptible rootstock genotypes, namely 101.14 and K5BB, and two drought tolerant genotypes, M4 and 1103P [15,23,24]. The short form *VvMYB60* refers to the *Vitis vinifera MYB60* gene, yet rootstocks are inter-specific hybrids of different Vitis species. We thus indicated the rootstocks *SIRK* and *MYB60* genes as *Vitis inter-specificSIRK* (*VisSIRK*) and *Vitis inter-specificMYB60* (*VisMYB60*), respectively.

Ungrafted plants were grown in pots under control or drought conditions. Control plants were maintained at a relative water soil content (RWSC) equivalent to 80% of the field capacity (FC), whereas the two drought stress regimes were imposed by reducing RWSC to 50% and 30% FC, respectively. First, we assessed stomatal density (SD) in leaves from plants grown under control conditions. Results revealed a comparable number of stomata per mm^2 of leaf area in 101.14, 1103P, and M4 leaves, while K5BB leaves disclosed a significantly increased number of stomata per unit area (Figure 2). Next, we performed g_s measurements in leaves from plants grown under control conditions or from plants maintained for three consecutive days at 50% or 30% FC. M4 leaves from untreated plants exhibited reduced g_s values compared with 101.14 and 1103P, despite possessing comparable SDs (Figure 3A).

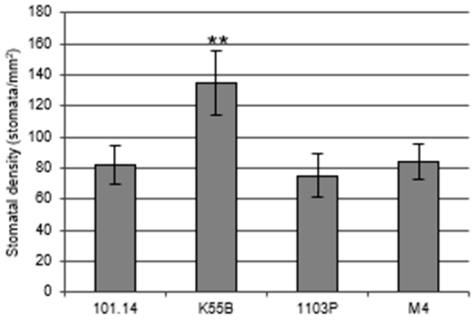

Figure 2. Analysis of stomatal density (SD) in the four rootstock genotypes. Values represent means ± standard errors. Asterisks (**) indicate significant differences among genotypes ($p < 0.01$, ANOVA).

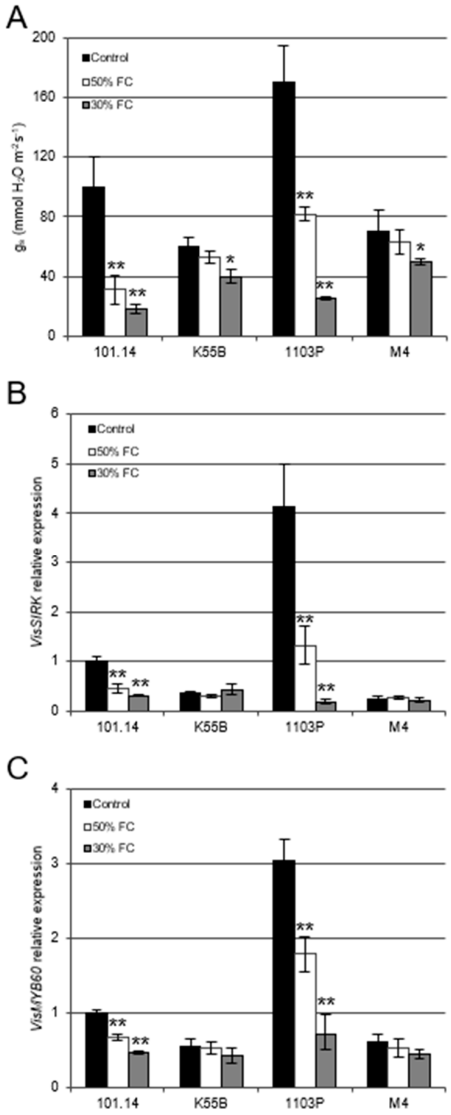

Figure 3. *VvMYB60* expression in the grape leaf correlates with stomatal conductance. (**A**) Comparison of g_s in leaves from control and stressed plants at 50% and 30% FC. (**B**,**C**) Comparison of *VisSIRK* (**B**) and *VisMYB60* (**C**) expression in leaves from plants grown under control conditions (80% FC) or maintained for three consecutive days at 50% or 30% FC. Values represent means ± standard errors. Gene expression was normalized to the expression of the *G3PDH* gene. Double (**) and single (*) asterisks indicate values significantly different from the untreated control at $p < 0.01$ and $p < 0.05$, respectively (ANOVA).

Interestingly, the higher number of stomata per mm^2 observed in K5BB leaves did not result in enhanced stomatal conductance, as K5BB plants disclosed g_s values comparable with those exhibited by M4 (Figure 3A). These findings suggest that differences in g_s likely reflected actual changes in stomatal opening rather than dissimilarities in the stomatal distribution across the four genotypes. From this perspective, 101.14 and 1103P leaves showed increased stomatal opening as compared with M4 and K5BB, when grown under optimal conditions.

After three consecutive days at 50% FC, stomatal conductance drastically declined in 101.14 and 1103P, whereas K5BB and M4 leaves sustained g_s values comparable with those of their respective unstressed controls. When plants reached 30% FC, we observed a further reduction in g_s in 101.14 and 1103P leaves, whereas K5BB and M4 leaves only revealed a moderate decrease (Figure 3A). Stomatal responses observed in 101.14 and M4 plants are consistent with results from a previous study, which investigated the different stress-adaptive strategies employed by these two genotypes [15]. Meggio and colleagues reported that under drought, 101.14 plants exhibited isohydric behavior, resulting in rapid stomatal closure and an almost complete inhibition of the net CO_2 assimilation rate (A_n). Conversely, under the same circumstances M4 plants were capable of maintaining relatively high levels of g_s and A_n, thus revealing a near-anysohydric response to water stress [15]. Adjustments in stomatal conductance to declining water availability have not been previously investigated in 1103P and K5BB plants. Our results indicate a strong stomatal control over the evaporative demand for 1103P plants, as opposed to K5BB, which showed a constitutive reduction in the stomatal opening under optimal conditions and limited adjustments in response to drought (Figure 3A).

Finally, we performed qPCR experiments to investigate changes in *VisSIRK* and *VisMYB60* expression in leaves from control and stress-treated plants. Under optimal conditions, the abundance of *VisSIRK* transcripts was augmented in 101.14 and 1103P leaves compared with M4 and K5BB (Figure 3B). This finding mirrors the enhanced g_s observed in 101.14 and 1103P and is consistent with the proposed role for *VisSIRK* in promoting stomatal opening [21]. Following an exposure to 50% and 30% FC, *VisSIRK* expression drastically declined in 101.14 and 1103P plants, but it did not show evident variations in M4 and K5BB plants (Figure 3B). Once more, changes in *VisSIRK* expression correlated with the different stomatal dynamics observed in 101.14 and 1103P compared with M4 and K5BB (R^2 = 0.87, Figure S3A). Under control conditions, *VisMYB60* disclosed the highest level of expression in 1103P leaves, followed by 101.14, M4, and K5BB, respectively (Figure 3C). Throughout the stress treatment, 1103P and 101.14 showed drastic reduction in *VisMYB60* expression as compared with the control plants. Conversely, M4 and K5BB did not reveal obvious changes in *VisMYB60* expression between control and stressed plants at either 50% or 30% FC (Figure 3C). As previously observed for the *VisSIRK* gene, changes in the *VisMYB60* expression closely reflected the stress-induced variations in g_s observed in the four rootstocks, thus suggesting a positive correlation between stomatal conductance and the level of *VisMYB60* expression (R^2 = 0.89, Figure S3B). This finding is in agreement with results from a previous comparative transcriptomic analysis of leaves from the two cultivars, Montepulciano (isohydric) and Sangiovese (anysohydric) [25]. Under water stress conditions, imposed by lowering the RWSC to 40% FC, variations in *VvMYB60* expression mirrored the different kinetic of stomatal closure observed in the two genotypes. Sangiovese leaves disclosed higher value of g_s throughout the duration of the stress treatment compared with Montepulciano. Consistently with our results, the anysohydric cultivar (Sangiovese) revealed enhanced expression of *VvMYB60* compared with the isohydric cultivar (Montepulciano) [25].

Recent evidence indicates that the 101.4 and M4 rootstocks can affect the stomatal behavior of the grafted scion [26]. Prinsi et al. reported that the Cabernet Sauvignon (Cab) scion displays enhanced stomatal conductance under drought conditions when grafted onto M4 compared with 101.4. Most interestingly, expression of *VvMYB60* is increased in

leaves from Cab/M4, relative to Cab/101.4, confirming the positive association between g_s and *VvMYB60* expression in these grafting combinations [26].

Our data provide novel evidence for the guard cell specificity of the *VvMYB60* gene and support its potential significance in mediating stomatal activity in grape leaves. It is important to emphasize that the link between *VvMYB60* expression and stomatal regulation does not support a causal role in determining the different levels of stress resistance depicted by the four rootstocks involved in this study. In our experimental setting, 1103P and 101.14 showed comparable responses in terms of stomatal behavior (i.e., elevated g_s under control conditions and drastic reduction under stress), despite 1103P being classically described as a drought-resistant rootstock, and 101.14 being known as a drought-susceptible genotype [23,27]. Likewise, the drought-resistant M4 rootstock [15] and the "less resistant" K5BB [23] displayed similar stomatal dynamics under both standard and stress conditions. These findings are not surprising considering that drought resistance in 1103P and M4 mainly relates to roots traits rather than to stomatal regulation [15,27]. Evidence indicates that 1103P plants sustain increased root growth and root conductance under drought stress compared with 101.14 [27]. Roots of M4 show enhanced biochemical and physiological responses to water stress, including increased accumulation of organic and inorganic osmolytes, as compared with 101.14 [15].

Nevertheless, the guard-cell specificity of *VvMYB60* and the correlation between its level of expression and stomatal activity underpin the extraordinary conservation of the *MYB60* function in distantly related species, such as Arabidopsis and grape. Considering that the same degree of conservation has been reported in tobacco and tomato [28,29], it is fascinating to speculate that the conservation of the *MYB60* stomatal regulatory network might extend across a vast array of plant species. Such conservation highlights the role of guard-cell-specific *MYB-like* genes as focal points in understanding stomatal regulation in plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12030694/s1, Figure S1: Ice-blending of grape leaves; Figure S2: Laser-microdissection of grape leaves; Figure S3: Linear regression analysis of *VvSIRK* and *VvMYB60* expression relative to changes in stomatal conductivity.

Author Contributions: Conceptualization, M.G. and C.T.; methodology, L.E. and L.C.; formal analysis, F.S., L.S., G.C. and M.Z.; resources, L.E.; writing—original draft preparation, M.G.; writing—review and editing, M.G., C.T., L.E., M.Z. and L.C. All authors have read and agreed to the published version of the manuscript.

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