A specific allele of MYB14 in grapevine correlates with high stilbene inducibility triggered by Al\(^{3+}\) and UV-C radiation

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Abstract
Key message The structural differences of MYB14 promoter in two grapevine genotypes affect the expression of MYB14 and stilbene synthesis in response to Al\(^{3+}\) and UV-C radiation.

Abstract Grapevines provide an important fruit crop worldwide, but production is often limited by pathogen infection. Stilbenes, a class of secondary metabolite, represent phytoalexins that contribute to defence against pathogens in many plants, including grapevine. It is known that the transcription factors MYB14 and MYB15 are required for the activation of the promoters of resveratrol synthase to regulate stilbene biosynthesis. In the current study, we observed that stilbene levels were more highly induced by Al\(^{3+}\) and UV-C radiation treatments in the cultivar Vitis labrusca ‘Concord’ than in the cultivar V. vinifera ‘Cabernet Sauvignon’. We investigated whether genetic/structural variations in the MYB14 and MYB15 promoters between these two representative genotypes are responsible for the differences in stilbene accumulation. Significant differences in the structure and activity of the promoter of MYB14, but not MYB15 were identified between the two genotypes, following heterologous expression in Nicotiana benthamiana system and treatments with Al\(^{3+}\) and UV-C. Hydrogen peroxide (H\(_2\)O\(_2\)) was detected in Concord soon after the stress treatments, but after diphenyleneiodonium chloride pre-treatment, the expressing level of VlMYB14, the promoter activity of VlMYB14 and the accumulation of stilbenes was significantly reduced.

A model is presented where the induction of MYB14 contributes to stilbene accumulation in Concord following Al\(^{3+}\) and UV-C treatments involving reactive oxygen species (ROS) production as an early signal.

Keywords Defence · Grapevines · MYB14 · Stilbene accumulation · Al\(^{3+}\) · UV-C

Introduction
Grapevine (Vitis spp.) provides an economically important fruit crop and cultivars are grown in temperate areas worldwide. However, production can be severely restricted by environmental factors and so a range of distinctive defence responses to various biotic and abiotic stresses, especially to pathogen attack, has been studied in order to enhance yield. American grapevines are generally more resistant to pathogens than are the genotypes from Europe. For instance, V. labrusca ‘Concord’ is characterized as a pathogen-resistant genotype (Pearson and Gadoury 1992; Pearson and Goheen 1988), while V. vinifera ‘Cabernet Sauvignon’ is relatively pathogen-sensitive (Boso and Kassemeyer 2008; Marsh et al. 2010). However, due to the extended coevolution of grapevines and pathogens, some American grapevines, such as the V. labrusca ‘Concord’, which is known for its ability to resist the destructive disease ‘powdery mildew’, now suffer from infection by pathogens, such as Plasmopara viticola strains.
et al. 1996). However, even though a number of transcription 
application of chemicals, including aluminium ions (Adrian 
mulation of stilbenes, the underlying molecular mechanisms 
Factors have been demonstrated to play key roles in the accu-
berry, including the relationship between enzyme coding 
Specific differences in the expression of stilbene synthase 
of the promoter that could account for the stress-
R2R3-MYB-type TFs have been 
Promoters of stilbene synthase and be involved in stilbene 
MYB13, an uncharacterized close homologue of 
MYB15, may also be involved in the accumulation of 
stilbene in different organs and in response to biotic and 
abiotic stresses through co-operation with other TF families 
(Wong et al. 2016). Recently, WRKY transcription factors 
were also reported to regulate stilbene synthesis pathway: 
VviWRKY24 was shown to enhance the promoter activity of 
VviSTS29 to regulate the stilbene synthesis (Vannozzi et al. 
Stilbene synthesis is thus controlled by a complex 
regulatory system that likely involves multiple TFs and regu-
laratory conditions that have yet to be characterized. Here, we 
investigated the regulation of stilbene biosynthesis by MYB 
TFs, based on the results of previous studies (Duan et al. 
2015, 2016). We identified differences in the inducibility of the 
MYB14 promoter that could account for the stress-
specific differences in the expression of stilbene synthase 
among V. vinifera ssp. Sylvestris, which is the ancestor of 
cultivated grapevine in European. We speculate that structural 
variations between the MYB14 and MYB15 promoters 
may contribute to the differences in stilbene inducibility, as 
well in other grapevines, including genotypes from North 
America and cultivars in Europe.

Reactive oxygen species (ROS) are produced as a result of a perturbed redox balance in response to various biotic 
and abiotic stresses (Apel and Hirt 2004; Boscolo et al. 
2003; Wojtaszek 1997; Yamamoto et al. 2003; Ghanati et al. 
2005; Gao et al. 2008; Ke et al. 2010). Recent research has 
indicated that ROS not only function as toxic compounds 
that damage cells (Buchanan et al. 2000), but also act impor-
tant early signal molecules (Fath et al. 2002; Vranová et al. 
2002; Wang and Nick 2017). Importantly, ROS have been 
proposed to be involved in the production of phytoalexins 
and are necessary for the induction of stilbene synthase 
(Rustérucci et al. 1996; Mithöfer et al. 1997; Chang et al. 
2011; Duan et al. 2016).

In this current study, we choose two representative gen-
types, V. labrusca ‘Concord’, which is native to North 
America and widely planted in the United States, and V. 
vitis vinifera ‘Cabernet Sauvignon’, which is a common culti-
vated in Europe. Both cultivars are well known sources of 
grape juice and wine. We used two stress treatments, UV-C 
radiation and Al3+, which are potent elicitors (Adrian et al. 
1996; Ahad and Nick 2007; Duan et al. 2015), to induce the 
stilbene synthesis in grapevines tested the hypothesis that 
genetic diversity between the MYB14 and MYB15 promoters 
is responsible for the differential accumulation of stilbenes 
between the grapevine genotypes. The overarching longer-
term objective is to develop methods to increase resistance of 
grapevines to fungal pathogens through environmentally 
benign approaches, avoiding the use of fungicides. Our results 
reveal a specific allele of MYB14 in Concord that is a 
potential target for improving resistance to pathogens.

Materials and methods

Plant materials

Two grapevine genotypes, V. labrusca ‘Concord’ and V. 
vitis vinifera ‘Cabernet Sauvignon’, were cultivated in the Life 
Science Experimental Park of Northwest University, Xi’an, 
Shaanxi, China. Nicotiana benthamiana plants were grown 
in growth cabinets with controlled 16 h light/8 h dark period 
at 23 °C. The third to fifth fully-expanded leaves from the 
plant apex of Concord and Cabernet Sauvignon were ran-
domly selected for treatment with UV-C and aluminium 
chloride. For the UV-C treatment, leaves were placed upside 
down on moist filter paper in petri dishes and the abaxial 
surface of an entire leaf was exposed to UV-C light (254 nm, 
15 W, FSL, China) for 10 min at a distance of 12.5 cm from 
the light source. For treatment with aluminium chloride, 
leaves were separately placed in petri dishes on filter paper 
and soaked in 1.5 mL freshly prepared 1.0% AlCl3 solution 
(Tianli Chemical Reagent Co., Ltd, China). The same exper-
imental treatment, but with sterile water rather than AlCl3, 
was performed as a negative control. After that, the leaves of 
Concord and Cabernet Sauvignon were harvested at different
time points, immediately frozen with liquid nitrogen and then stored at −80 °C for RNA extraction or stilbene, H₂O₂ and malondialdehyde (MDA) analysis.

**Stilbene analysis and quantification**

For the UV-C treatment, leaves of Concord and Cabernet Sauvignon were collected at the following time points: C (control fresh leaf, without UV-C treatment), 6 h and 24 h (from the end of the 10 min UV-C pulse), respectively. For the aluminium chloride treatment, leaves of two genotypes were collected at the following time points: C (control fresh leaf, without aluminium chloride treatment), 6, 12, 24, and 48 h. All of the sterile water treated samples were collected as a negative control at the corresponding time points. Stilbenes were extracted as described by Duan et al. (2015) and analysed using high-performance liquid chromatography (HPLC, Waters 2696, America). Chromatographic separations were performed on an Agilent ZORBAX SB-C18 column maintained at room temperature. The mobile phase consisted of acetonitrile (eluent A) and phosphoric acid (0.1%, v/v) (eluent B) at a flow rate of 0.8 mL min⁻¹. The gradient elution program was as follows: 0–20 min, 15–30% A; 20–35 min, 30–50% A; 35–45 min, 50% A. The injecting volume was 20 µL. The peak areas were recorded and calculated using an external standard method.

**Determination of H₂O₂ and MDA in grapevine leaves**

The leaves of Concord and Cabernet Sauvignon were harvested at different time points after the UV-C irradiation: C (control fresh leaf, without UV-C treatment), 0, 10, 15, 30 and 60 min after the 10 min UV-C pulse. The time points for the aluminium chloride treatment as follows: C (control fresh leaf, without 1.0% AlCl₃ solution), 15, 30, 60, 90 and 120 min. All of the sterile water treated samples were used as a negative control at the corresponding time points. Levels of H₂O₂ in leaves were determined as described by Wang et al. (2017), by using a peroxide assay kit (Comin Biotechnology Co., Ltd. Suzhou, China). Hydrogen peroxide (H₂O₂) is one of the most important ROS. Lipid peroxidation as readout for oxidative burst was determined by measuring the production of MDA as previously described (Hodgson and Raison 1991), but with minor changes: the plant leaves (100 mg) were ground in liquid nitrogen using a pestle and mortar, vortexed for 45 s in 1 mL 0.1 M phosphate buffer (pH 7.4) in a 2.0 mL Eppendorf (EP) tube, centrifuged for 4 min at 8000g and the pellet discarded. The remaining 200 µL of supernatant were added to a reaction mixture containing 750 µL acetic acid (20% v/v), 750 µL thiobarbituric acid (TBA) (aqueous solution, 0.8% w/v), 200 µL Milli-Q water and 100 µL sodium dodecyl sulphate (8.1% w/v). An identical reaction mixture, where the supernatant from the sample was replaced by an equal volume of phosphate buffer, was used as a control. The reaction mixture was then incubated for 1 h at 98 °C and cooled to room temperature. The absorbance of the solution at 535 nm (specific signal) and 600 nm (background) were measured using a microplate reader (Synergy 2, BioTek, America). Lipid peroxidation was calculated as µM MDA from A535 to A600 using an extinction coefficient of 155 mM⁻¹ cm⁻¹.

To further investigate potential ROS signalling, leaves of Concord were treated with 10 min UV-C, 1% AlCl₃, or 1% H₂O₂ (w/v) (FuYu Fine Chemical Co., Ltd., China). The leaves were harvested at 24 h post-treatment. For the diphenyleneiodonium chloride (DPI) (Sigma-Aldrich, shanghai) treatment, the leaves were pretreated with 100 µM DPI by soaking for 1 h before the UV-C, Al³⁺ or H₂O₂ treatments. DPI was also added without a subsequent treatment to evaluate the effect of the inhibitor alone. Leaves treated with sterile water in which the AlCl₃ and H₂O₂ were dissolved served as negative controls. After the treatments, the leaves were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction and stilbene analysis. Three samples were treated in each treatment, and each treatment was repeated three times.

**Isolation of MYB14/15 promoter fragments and sequence analysis**

Genomic DNA was extracted from the leaves of both grapevine genotypes using a new rapid plant genomic DNA extraction kit (BioTeke, Beijing, China) according to the manufacturer’s protocol. MYB14-F: (5′-CTACTGACGTGACACTAGCCT-3′), MYB14-R: (5′-GCAGAGTGAAGTGCAACACG-3′) and MYB15-F: (5′-GCCAAGGACTTGAACCTTTGAAGA-3′) and MYB15-R: (5′-CTTCTGATGACCAAATCTTTGGA-3′). PCR primers were used to amplify the full length MYB14 and MYB15 genes respectively, using genomic DNA as a template. Fragments were amplified using LA Taq DNA Polymerase (TaKaRa) following the manufacturer’s recommended reaction conditions. The PCR products were cloned into T-Vector pMD™19 (Simple) (TaKaRa, Dalian, China) and the resulting constructs were transformed into Escherichia coli DH5α competent cells to produce pMD-VvMYB14/15 and pMD-VvMYB14/15, which were then sequenced (Sangon Biotech, Shanghai, China). PCR primers were designed to amplify the promoters with restriction enzyme sites HindIII and BglII (Table S1). The PCR products were respectively linked to T-Vector pMD™19 to produce pMD-pVvMYB14/15 and pMD-pVvMYB14/15, which were then sequenced. Potential cis-elements in the promoter sequences were predicted with the online software packages Plant CARE (http://bioinforma
by treatments with Al3+ and UV-C for 60 min and 30 min DPI for 30 min as described in Duan et al. (2016), followed the negative control. For the DPI pre-treatment experiments, C, 6, 12 and 24 h with sterile water treatment used as a parallel to the aluminium chloride treatment, leaves were collected at the negative control at the corresponding time points. For UV-C treatment, leaves were collected at C and 24 h with to the various treatments as described as above. For the needle-free syringe. After 48 h, the leaves were subjected benthamiana leaves that had grown for 6–8 weeks with a 0.6 with permeation buffer.

spectrophotometer (JINGHUA, Shanghai), and adjusted to OD600, measured using a Nicolet Evolution 754 UV–Vis

depend on a shaker (ZHCHENG, Shanghai) at 200 rpm for 24 h at 28 °C then centrifuged at 2773g for 10 min at room temperature. The pellets were re-suspended in 10 mL of permeation buffer (250 mg n-glucose, 5 mL 500 mM MES, 5 mL 20 mM Na2PO4·12H2O, 5 µL 1 M acetosyringone adjusted to a final volume of 50 mL with ddH2O). The bacterial suspensions were incubated at 28 °C for 3 h and the absorbance at OD600, measured using a Nicolet Evolution 754 UV–Vis spectrophotometer (JINGHUA, Shanghai), and adjusted to 0.6 with permeation buffer.

The bacterial suspensions were then injected into N. benthamiana leaves that had grown for 6–8 weeks with a needle-free syringe. After 48 h, the leaves were subjected to the various treatments as described as above. For the UV-C treatment, leaves were collected at C and 24 h with the negative control at the corresponding time points. For the aluminium chloride treatment, leaves were collected at C, 6, 12 and 24 h with sterile water treatment used as a parallel negative control. For the DPI pre-treatment experiments, the N. benthamiana leaves were pre-treated with 100 µM DPI for 30 min as described in Duan et al. (2016), followed by treatments with Al3+ and UV-C for 60 min and 30 min respectively. X-Gluc staining, GUS expression and GUS enzymatic activity were analysed as described by Xu et al. (2010) and Jiao et al. (2016).

cDNA synthesis and quantitative real-time PCR

Total RNA from both grapevine genotypes and N. benthamiana leaves was extracted using an EZNA® Total RNA kit (Omega Bio-tech) according to the manufacturer’s instructions. mRNA was transcribed into cDNA using Prime Script Reverse Transcriptase (TaKaRa). Quantitative real-time PCR was conducted as described in Xu et al. (2011). NbEF1-a (for N. benthamiana leaves) (Zhang et al. 2015) and EF1-a (for grapevine leaves) (Duan et al. 2015) were used for gene expression normalization and marker genes were used for comparison (Belhadj et al. 2008; Xu et al. 2010; Höll et al. 2013; Duan et al. 2015, 2016). Primers used in the experiment are shown in Table S2. Each experiment was carried out in three biological repetitions.

Results

Stilbenes are highly induced in Concord compared to Cabernet Sauvignon following UV-C or Al3+ treatments

Stilbene biosynthesis is known to be induced by different stresses, including UV-C (Duan et al. 2015) and AlCl3 (Adrian et al. 1996), and in this study we examined their effects on stilbene accumulation in Concord and Cabernet Sauvignon. As shown in Fig. 1, following either treatment trans-resveratrol and viniferins were the main stilbenes in leaves and we noted especially high levels of trans-resveratrol. Stilbene accumulation was rapidly induced to higher levels in Concord than in Cabernet Sauvignon. For example, 24 h following UV-C treatment, we detected 860 µg g−1 fresh weight (FW) of trans-resveratrol and 200 µg g−1 FW of viniferins in Concord, compared with 230 µg g−1 FW and 135 µg g−1 FW, respectively, in Cabernet Sauvignon (Fig. 1b, d). Similarly, an increase in stilbene levels was observed following AlCl3 treatment from 6 to 48 h in Concord (Fig. 1a), whereas no obvious changes were observed over time in Cabernet Sauvignon (Fig. 1c). UV-C treatment induced greater stilbene accumulation than did the Al3+ treatment at same time points in both genotypes. For example, total stilbenes levels reached 1200 µg g−1 FW 24 h after UV-C treatment in Concord, (Fig. 1b), while the Al3+ treatment resulted in 100 µg g−1 FW (Fig. 1a). The accumulation of trans-resveratrol was detected at early time points (e.g. 6 h) in both genotypes and treatments, while the viniferins accumulated later and were most abundant at 24 h.

The expression levels of key stilbene-related genes are higher in Concord than in Cabernet Sauvignon following Al3+ or UV-C treatments

We investigated whether the different accumulation of stilbenes correlated with the expression of key genes involved in stilbene synthesis pathway, including the upstream enzyme in the phenylpropanoid pathway phenylalanine ammonium lyase (PAL), trans-resveratrol synthase (RS), as well as MYB14 and MYB 15, which are known to regulate the expression of resveratrol synthase (Höll et al. 2013; Duan et al. 2016). The transcript levels of PAL, RS, MYB14 and MYB15 were investigated by real-time quantitative PCR (RT-qPCR) at the early time points in leaves of Cabernet Sauvignon and Concord following Al3+ and UV-C treatments. Following the Al3+ treatment, the expression of PAL...
and RS in Concord increased from 30 min (five- and twofold, respectively) and peaked at 60 min (Fig. 2a). The expression of MYB14 showed the same pattern and had maximal levels at 60 min (almost threefold compared with control). However, these responses were less pronounced in Cabernet Sauvignon. Transcript levels of PAL, RS and MYB14 were rapidly induced after the 10 min UV-C pulse in Concord (Fig. 2b), whereas the induction of these transcripts in Cabernet Sauvignon was relatively slower and peaked at lower levels. Regardless of the expression levels, the induction levels of MYB15 showed no significant difference between Cabernet Sauvignon and Concord for both treatments.

Differences in the MYB14 promoter sequences between Concord and Cabernet Sauvignon

We next investigated whether differences in the MYB14 and MYB15 promoter sequences from the two genotypes might be responsible for the differential induction of RS, thereby influencing stilbene diversity. As indicated in Fig. 3, we identified a large deletion (300 bp) in pVvMYB14 compared to pVlMYB14; however, the sequences shared 99% nucleic acid identity and showed no other differences between pVvMYB15 and pVlMYB15 (Fig. S1).

To identify possible regulatory cis-acting elements in the promoters, both pVvMYB14 and pVlMYB14 were analysed using the PlantCARE algorithm (Lescot et al. 2002). As shown in Fig. 3 and Fig. S2, the promoter region of VlMYB14 was predicted to have more cis-elements than VvMYB14. For example, the 5'-UTR Py-rich stretch has been reported to confer high transcriptional levels (Daraselia et al. 1996; Wang et al. 2013); the TATA-box is a sequence of DNA found in the core promoter region of genes (Bae et al. 2015; Whittington et al. 2008); the AT-rich element acts as an enhancer (Bustos et al. 1989; Sandhu et al. 1998); Box III is predicted to be a protein binding site involved in salt stress responses (Sun et al. 2010) and several cis-elements (e.g. a GATA-motif, GT1-motif and MRE) are linked to light responsiveness. In addition, the transcription factor binding sites (TFBSs) in the promoter sequences of pVlMYB14 and pVlMYB14 were analysed using PlantPAN 2.0 (Chow et al. 2016). The transcription factors that were specific to Vitis were selected. In general, more TFBSs were identified in pVlMYB14 than in pVvMYB14 (Table S3). Several sequence motifs that are present in the binding sites of some key
transcription factors, such as WRKY, bHLH, bZIP, NAC, AP2-ERF, Alpha-amylase, AT-Hook, C2H2, Dof, GATA, MADF/Trihelix, and that might play roles in growth and development, hormone regulation and stress response were identified.

The promoter induction is stronger in \textit{VlMYB14} than in \textit{VvMYB14} induced by Al\textsuperscript{3+} and UV-C

To investigate the functional significance of the structural differences between \textit{pVlMYB14} and \textit{pVvMYB14}, the 1625 bp
(VlMYB14) and 1346 bp (VvMYB14) promoter fragments were inserted into the vector pCAMBIA1301, replacing the Cauliflower mosaic virus (CaMV) 35S promoter region, upstream of a GUS reporter (Xu et al. 2010), and transiently expressed in N. benthamiana. GUS expression was monitored by X-Gluc staining (Fig. 4a), the RT-qPCR analysis of GUS transcript levels (Fig. 4b) and the quantification of GUS enzymatic activity (Fig. 4c). As shown in Fig. 4a, pVlMYB14 was more strongly induced than pVvMYB14 following Al³⁺ or UV-C treatments. Quantification of GUS transcript abundance (Fig. 4b) and enzymatic activity (Fig. 4c) in pVlMYB14 revealed a strong induction at 6 h after incubation with Al³⁺, which continuously increased until 24 h. In contrast, the induction of pVvMYB14::GUS increased slightly but did not show a statistically significant difference during the same time period. Similar response patterns were observed for pVlMYB14 and pVvMYB14 when exposed to UV-C irradiation (Fig. 4b and c). pVlMYB14 was highly induced in the 24 h treatment (~ ninefold), while the induction of pVvMYB14 remained below the significance threshold. These results suggest that the promoter induction following Al³⁺ or UV-C treatments is correlated with the promoter structural differences in the heterologous N. benthamiana system, and that the specific allele of pVlMYB14 may contribute to the high stilbene inducibility.

**ROS levels are induced in Concord by Al³⁺ or UV-C**

ROS are known to act as signals that orchestrate cellular adaptations to stresses. To investigate whether ROS might contribute to the activation of VIMYB14, we examined the effects of the Al³⁺ and UV-C treatments on ROS production. Specifically, we measured the production of hydrogen peroxide (H₂O₂), one of the major ROS species, and the production of MDA, one of the main products of stress-induced membrane lipid peroxidation (Draper and Hadley 1990; Janero 1990).

As shown in Fig. 5 and S4, both the Al³⁺ and UV-C treatments induced a rapid production of H₂O₂ and MDA in Concord, but not in Cabernet Sauvignon. Following the Al³⁺ treatment (Fig. 5a and Fig. S4a), levels of H₂O₂/MDA increased from around 15 min and peaked at 60 min in Concord, then sharply declined from 60 to 120 min and approached control levels at 120 min. A similar pattern was observed after UV-C irradiation in Concord, but the maximal levels of H₂O₂ and MDA were significantly higher and the induction was more rapid than following the Al³⁺ treatment (Fig. 5b and Fig. S4b).

**ROS are necessary for the activation of pVIMYB14, induction of VIMYB14 and accumulation of stilbenes**

To investigate whether the induction of ROS triggered by the Al³⁺ or UV-C are necessary for the activation of pVIMYB14 in Concord, we measured the activity of pVIMYB14 in the heterologous N. benthamiana system following treatment with DPI, a specific inhibitor of NADPH oxidase. As shown in Fig. 6, we observed that pVIMYB14 was strongly induced after treatment with Al³⁺ or UV-C. However, after a DPI pretreatment, the induction of pVIMYB14 was substantially decreased, whereas treatment with DPI alone did not affect the modulation of pVIMYB14 expression.

In addition to Al³⁺/UV-C, we introduced exogenous H₂O₂ as a ROS-donor to investigate the effects on MYB14 expression. As shown in Fig. 7a–c, compared to the solvent control, the accumulation of VIMYB14 transcripts significantly increased after the treatments with Al³⁺/UV-C/H₂O₂. However, when the leaves of Concord were pre-treated with DPI for 1 h before the Al³⁺/UV-C/H₂O₂ treatment, the induction of VIMYB14 was abolished.
**Fig. 4** Time course of the heterologous expression of the GUS reporter following introduction of pVvMYB14::GUS or pVlMYB14::GUS in transgenic N. benthamiana leaves in response to Al<sup>3+</sup> and UV-C treatments. a Histochemical assay of GUS expression in the transiently transformed N. benthamiana leaves. b GUS transcript abundance in response to 1% Al<sup>3+</sup> treatment at different time points and at 24 h after UV-C irradiation for 10 min, measured by RT-qPCR. c GUS enzymatic activity. Values represent mean values and standard errors from three independent experimental series. *P < 0.05 and **P < 0.01 indicates statistical significant differences between Cabernet Sauvignon and Concord at the same time points (n = 3).

**Fig. 5** Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels in Cabernet Sauvignon and Concord leaves following 1% Al<sup>3+</sup> (a) and 10 min UV-C (b) treatments. As negative controls, leaves were incubated with sterile water, or not exposed to UV-C. Values represent means and standard errors from nine independent biological replicates.
treatment, *VlMYB14* transcript levels were higher than those in the solvent control, but significantly lower than in groups that were treated with Al³⁺/UV-C/H₂O₂ alone. DPI itself did not affect the expression of *VlMYB14*. These results indicate that *VlMYB14* acts downstream of ROS signalling in Concord.

We also measured stilbene accumulation following with DPI pretreatment prior to the Al³⁺/UV-C/H₂O₂ treatments.
We found that stilbene accumulation increased significantly after the treatments with Al\textsuperscript{3+}/UV-C/H\textsubscript{2}O\textsubscript{2}, but that this effect was strongly suppressed by DPI (Fig. 7d–f). Taken together, the data indicate that ROS enhance the activation of pV\textsubscript{l}MYB\textsubscript{14} by the Al\textsuperscript{3+} or UV-C treatments and are necessary for stilbene synthesis in Concord.

**Discussion**

In the current study, we examined the functional significance of the MYB\textsubscript{14} and MYB\textsubscript{15} in the context of stilbene accumulation in two grapevine genotypes. We observed that the activation of pV\textsubscript{l}MYB\textsubscript{14}, but not pV\textsubscript{v}MYB\textsubscript{14}, was strongly induced by Al\textsuperscript{3+} and UV-C treatments, and that these inductions were associated with ROS accumulation. We detected no differences in the sequences or activities of the MYB\textsubscript{15} promoters; however, we identified genetic variations in the MYB\textsubscript{14} promoters between Concord and Cabernet Sauvignon that correlated with stilbene levels. Higher stilbene levels were present in Concord associated with a specific MYB\textsubscript{14} promoter structure and corresponding promoter induction.

Most stilbenes are derivatives of the basic unit trans-resveratrol (3,5,4′-trihydroxy-trans-stilbene), with variants resulting from differences in the glycosylation pattern, trans-piceid, oxidation pattern viniferins and the methylated form pterostilbene. In general, resveratrol and viniferins are the main active and antimicrobial stilbenes (Jeanedt et al. 2002). In this study, we observed that trans-resveratrol and viniferins represent the primary stilbenes associated with both treatments in the two genotypes. In addition, UV-C triggered more rapid accumulation and higher overall levels of stilbenes than did the Al\textsuperscript{3+} treatment. Additionally, the pattern of stilbene accumulation was time dependent: trans-resveratrol accumulated earlier, whereas the viniferins were found later, which is consistent with our previous studies of *V. sylvestris* (Duan et al. 2015).

MYB\textsubscript{14} and MYB\textsubscript{15} are known to activate stilbene/resveratrol synthase and to contribute to stilbene accumulation (Höll et al. 2013); however, other factors are likely involved, such as members of the WRKY TF family. A close functional association between MYB and WRKY TFs has been demonstrated, and they have been reported to act combinatorially and synergistically to regulate grapevine STS genes (Vannozzi et al. 2018). For instance, *VviWRKY03* itself does not activate STS29, but in combination with *VviMYB14* significantly increases STS promoter activity. In addition, the APETALA2/ERF (AP2/ERF) and a bHLH TF are involved in the regulation of stilbene synthesis (Wong and Matus 2017; Vannozzi et al. 2018). Here we found structural differences between the MYB\textsubscript{14} promoters of Cabernet Sauvignon and Concord that may explain differences in the accumulation of stilbenes following Al\textsuperscript{3+} and UV-C treatments. We hypothesize that this may reflect the fact that the MYB\textsubscript{14} promoter from Concord has more regulatory cis-elements (Fig. 3) and TFBSs (Table S3) than the equivalent region from Cabernet Sauvignon. Moreover, the protective cis-element, ARE, which plays a role in the ROS signalling pathway, is absent from the promoter of pV\textsubscript{v}MYB\textsubscript{14} (Rushmore et al. 1991; Nguyen et al. 2009). Accordingly, this may explain why we observed earlier increases in the levels of ROS in Concord, which may in turn have contributed to the earlier activation of MYB\textsubscript{14}. Similarly, AT-Hook, WRKY, C2H2, Dof, GATA and MADF/Trihelix TFBSs (Boccacci et al. 2017) were identified in the promoter of pV\textsubscript{l}MYB\textsubscript{14}, which we propose may interact with MYB\textsubscript{14} to co-regulate the STS genes.

A specific group of NADP(H) oxidases located in the plasma membrane contribute to ROS release in plants (for review see Marino et al. 2012) and NADP(H) oxidase can be activated by different stress conditions. ROS play an important role in the early signalling transduction in response to biotic and abiotic stresses (Neill et al. 2002). Our data support a model in which ROS act as an early signal to activate downstream genes, such as MYB\textsubscript{14}, thereby promoting stilbene biosynthesis. In addition, the production of ROS is more pronounced and rapid in response to UV-C irradiation than following Al\textsuperscript{3+} treatment (Fig. 5 and Fig. S4). We hypothesize that Al\textsuperscript{3+} and UV-C may act through a similar defense pathway to activate stilbene synthesis in Concord, and that ROS may represent a common link, whereas the difference between types of treatment may reflect the timing of the ROS signal.

We propose a model (Fig. 8) where ROS function as an early signal to induce pV\textsubscript{l}MYB\textsubscript{14} and consequently stilbene synthesis in Concord. The early synthesis of ROS triggered by Al\textsuperscript{3+} is relatively slower than that induced by the UV-C treatment. The mechanism by which the ROS signal activates pV\textsubscript{l}MYB\textsubscript{14} has not yet been elucidated. Duan et al. (2016) demonstrated that flg22 can activate the ROS, MAPK cascade and jasmonate signalling, which then converge on the *V. sylvestris* MYB\textsubscript{14} promoter in the cultivar Hoe29. In addition, it has been reported that the ROS, superoxide, enters the cell through aquaporins and causes actin bundling and detachment from the membrane, thereby triggering defence signalling and the activation of phytoalexin and stilbene synthesis genes (Chang et al. 2015; Eggenberger et al. 2016). The regulatory pathway involving ROS and MYB\textsubscript{14} is clearly complex and may involve other molecular signals (Zhang et al. 2007; Duan et al. 2016; Qiao et al. 2010; Chang et al. 2015; Eggenberger et al. 2016).

**Author contribution statement** DD conceived and designed the work. RB, YY-L, JL and KR-W performed the experiments. RB and DD analysed the data. DD wrote the
Fig. 8 Model of defence responses triggered by Al3+ and UV-C in grapevine. Both treatments induce the accumulation of stilbenes, such as resveratrol and viniferins, to enhance the defence response in Concord by recruiting the activation of the transcription factor MYB14, but not in Cabernet Sauvignon. ROS plays an important role in the early signalling transduction to activate pVIMYB14. The production of ROS is induced more rapidly by UV-C than by the Al3+ treatment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. All forms of financial support are acknowledged in the contribution.

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