



ORIGINAL RESEARCH ARTICLE

Abscisic acid metabolism pathways differ between grapevine species, leaves, and roots during water deficit

Haley S. Toups^{1*}, Noé Cochetel^{1,2}, Laurent Deluc³ and Grant R. Cramer¹

¹ Department of Biochemistry and Molecular Biology, University of Nevada, Reno, Reno, NV 89557, USA

² Department of Viticulture and Enology, University of California, Davis, Davis, California, 95616 USA

³ Department of Horticulture, Oregon State University, Corvallis, OR, 97331, USA



*correspondence:
htoups@nevada.unr.edu

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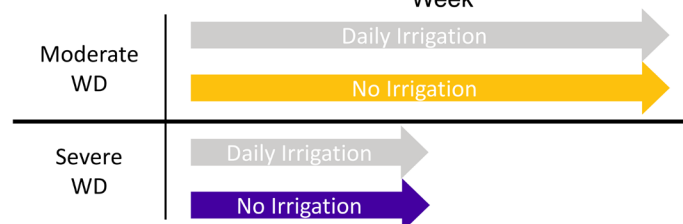
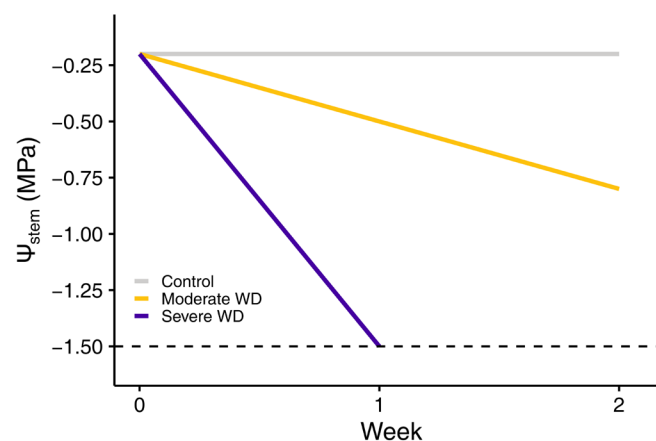
ABSTRACT

Abscisic acid (ABA) metabolism is complex involving biosynthesis, conjugation, and catabolism. Differences in ABA metabolism (ABA, ABA-related metabolites, and transcripts) in response to water deficit (WD) were detected in leaves and roots of four *Vitis* species differing in drought tolerance: *Vitis vinifera* (Cabernet Sauvignon), *Vitis champinii* (Ramsey), *Vitis riparia* (Riparia Gloire), and *Vitis vinifera* x *girdiana* (SC2). Concentrations of ABA and ABA-related metabolites increased after moderate or severe WD depending on species, organ, and time. Differences in ABA, glycosylated-ABA, and ABA catabolite concentrations, as well as previously investigated related transcript abundances, revealed differences in ABA metabolism pathways among the species and organs. NCED3 was a key gene in the WD response of leaves and roots of all species. NCED3 transcript abundance and ABA concentration in drought-tolerant Ramsey increased earlier and to a greater extent than other species. These species provide informative genetic resources to study ABA metabolism and drought tolerance further.



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KEYWORDS: abscisic acid, water deficit, nine-cis-epoxycarotenoid dioxygenase, grapevine

INTRODUCTION

Drought decreases global crop production and may become more frequent and severe in the near future (Gupta *et al.*, 2020). Abscisic acid (ABA) is an important phytohormone that increases in concentration during water deficit (WD), dormancy, and plant development (Chen *et al.*, 2020; Liu and Sherif, 2019). ABA metabolism and signalling is a complex pathway varying with plant species, organ, cell type, developmental stage, and the environment (Chen *et al.*, 2020).

ABA concentrations are regulated by biosynthesis, catabolism, conjugation, deconjugation, and transport. For example, genetic modifications of NINE-CIS-EPOXYCAROTENOID DIOXYGENASE (NCED), the enzyme responsible for the rate-limiting step of ABA biosynthesis, result in altered ABA concentrations, leading to improved or decreased drought tolerance (Huang *et al.*, 2018; Thompson *et al.*, 2007).

In addition to biosynthesis, ABA concentrations can be impacted by glycosylation to form ABA-glucose ester (ABA-GE) via UDP-glucose glucosyltransferase (UGT) and ABA-GE deglycosylation to reform ABA via β -d-glucosidases (BG or BGLU) (Chen *et al.*, 2020). ABA is catabolised to 7', 8', or 9'-hydroxy ABA via ABA hydroxylases (Chen *et al.*, 2020) that reduce ABA concentrations. ABA is also catabolised to neophaseic acid (NeOPA), phaseic acid (PA), and dihydrophaseic acid (DPA). Recently, PA was reported to have ABA-like physiological and signalling actions that contribute to long-term acclimation to WD (Weng *et al.*, 2016) in contrast to earlier work indicating that PA had minimal ABA-like activity (Kepka *et al.*, 2011).

ABA is transported throughout a plant via the phloem and xylem and distributed between tissues and organelles dependent on pH; alkaline conditions make ABA impermeable across plasma membranes (Finkelstein, 2013). Additionally, the ABA transporter, ABCG25, exports ABA from vascular tissue while ABCG40 imports ABA into guard cells to elicit stomatal closure during abiotic stress. Other ABA transporters include ABCG30, ABCG31, NPF4.5, and NPF4.6 (Chen *et al.*, 2020).

ABA metabolism and signalling pathways vary with plant species and are linked to the adaptation of plants to abiotic stress (Hanada *et al.*, 2011; Yadav *et al.*, 2020). ABA signalling genes are highly responsive to WD for four diverse *Vitis* species (*Vitis vinifera* cv. Cabernet Sauvignon clone-8 (CS), *Vitis champinii* cv. Ramsey (RM), *Vitis riparia* cv. Riparia Gloire (RG), and *Vitis vinifera* x *Vitis girdiana* hybrid SC2 (SC)) (Cochetel *et al.*, 2020). In previous experiments in which these vines experienced the same relative soil water content and stem water potentials during controlled natural dry-down water deficits, RM, the most drought-tolerant, and RG, the most drought-sensitive, had the highest and lowest stomatal conductance and photosynthesis, respectively, despite the different species experiencing the same level of stress. We hypothesised that the different transcriptional and

physiological responses among the species may have been reflective of differences in ABA metabolism.

In this study, this hypothesis was investigated further. ABA and ABA-related metabolites were quantified by LC-MS/MS along with comprehensive analyses of the related transcripts (RNA-Seq and weighted-gene co-expression network analysis (WGCNA)). ABA and ABA-related metabolites (including ABA-GE and four ABA catabolites) increased with WD and varied with species, organ, and time indicating that different ABA metabolism pathways were utilised among the roots and leaves of these species. This study provides insight into plant drought tolerance and ABA metabolism that may be valuable targets for breeding and genetic manipulation for improved water-use efficiency and drought tolerance.

MATERIALS AND METHODS

1. Plant material and growth conditions

Plant origins and growth conditions were previously described (Cochetel *et al.*, 2020). Briefly, *Vitis vinifera* (L.) cv. Cabernet Sauvignon clone 8 (CS), *Vitis champinii* (RM), *Vitis riparia* (RG), and a *Vitis vinifera* x *girdiana* hybrid (SC2) were grown in a greenhouse at 21–26 °C, 20–50 % relative humidity, and had average mid-day light intensities of 1200 $\mu\text{moles m}^{-2} \text{s}^{-1}$. Plants were grown in Stuewe and Son's Anderson AB39 pots (0.96 L) consisting of \sim 1.0 kg Quikrete medium grain sand and 80 g of fritted clay for plants used for one- and two-week moderate WD and one-week severe WD experiments. Plants were irrigated with Cramer's complete nutrient solution (1.5 mM $\text{Ca}(\text{NO}_3)_2$, 2 mM KNO_3 , 0.6 mM MgSO_4 , 1 mM KH_2PO_4 , 1.5 mM CaCl_2 , 36 μM Fe_2^+ Sprint 330, 1 μM MnSO_4 , 0.5 μM CuSO_4 , 20 μM ZnSO_4 , 20 μM H_3BO_3 , and 0.01 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$).

2. WD experiments

Moderate and severe WD experimental conditions performed in these small pots were previously described (Cochetel *et al.*, 2020). Briefly, the experiments performed were 1) WD and control treatments for one- and two-week moderate WD used for RNA-Seq, ABA, and ABA-related metabolite quantification 2) one-week severe WD and control treatments to support the original RNA-Seq experiment and quantify *NCED3* transcripts with RT-qPCR (see Cochetel *et al.*, 2020 Additional File 11) as well as ABA. Physiological measurements and a detailed report of related transcripts for both experiments were reported elsewhere (Cochetel *et al.*, 2020), and additional measurements not previously reported are in Supplementary Figure 3.

For the moderate WD RNA-Seq experiment previously described (Cochetel *et al.*, 2020), 3–5 individually potted CS, RM, RG, and SC vines were subjected to one or two weeks of moderate WD or well-watered daily with complete nutrient solution. The moderate WD experiment was comprised of vines with stem water potentials of -0.26 ± 0.02 to $-0.32 \text{ MPa} \pm 0.02$ after one- and two-weeks control treatment and $-0.49 \pm 0.04 \text{ MPa}$ to $-0.82 \pm 0.06 \text{ MPa}$ after one- and two-weeks WD, respectively.

Total plant leaves (excluding petiole) and roots were harvested after one and two weeks of treatment. Samples were immediately frozen in liquid nitrogen and stored at -80°C .

For the one-week severe WD experiment, 3–5 individually potted CS, RM, RG, and SC vines were subjected to one week of severe WD or watered daily to saturation with a complete nutrient solution. The severe WD resulted in stem water potentials of ~ -1.5 MPa, and the stem water potential for control-treated vines were similar to those of the moderate WD Controls (~ -0.2 to -0.3 MPa). At one week of treatment, various measurements (stem water potential, photosynthesis, stomatal conductance, relative soil water content, leaf and root fresh weight, and leaf surface area) were taken, and total plant leaves (excluding petiole) and roots were harvested. Samples were immediately frozen in liquid nitrogen and stored at -80°C . A visual representation of both the moderate and severe WD experiments is in the graphical abstract.

3. ABA quantification

ABA and ABA-related metabolites were quantified with LC-MS/MS using a methanol-based extraction as previously described (Gouthu *et al.*, 2013). Briefly, ABA derivative quantification from one- and two-week moderate WD and one-week severe WD samples was performed with methanol, formic acid, water (15:1:4) extraction, and deuterated standards. Samples were purified with Oasis® HLB 3.5 ml columns (Waters®) and eluted with methanol. Samples were reconstituted in acetonitrile, water, and formic acid (15:85:0.1) and separated with an Agilent™ Zorbax® Extend C₁₈ column (201 × 150 mm; 5 μm) with Opti-Solve® 2-micron guard column (Optimize Technologies™, USA). A binary gradient of LC-MS grade acetonitrile (ThermoFisher Scientific) acidified with 0.1 % formic acid, and HPLC grade water (ThermoFisher Scientific) also acidified with 0.1 % formic acid were used to resolve ABA derivatives. Mass spectrometry was carried out using multiple reaction monitoring (MRM) using a hybrid triple quadrupole/linear ion trap 4000 QTRAP LC-MS/MS instrument equipped with a Turbo V source (Applied Biosystems™, USA) with a 0.2 mL/min flow rate under the conditions previously described (Gouthu *et al.*, 2013). D6 deuterated ABA (Toronto Research Chemicals) was used as an internal standard for all samples, and D4 deuterated ABA (OIChemIm) was used as an extraction efficiency standard for one- and two-week moderate and one-week severe WD experiment samples.

4. CS ABA metabolism-related gene identification

ABA metabolism genes were identified in the CS genome (Chin *et al.*, 2016) with a protein basic local alignment search tool (BLASTP) using known ABA metabolism PN40024 protein sequences as a query. Orthologs were confirmed based on the highest total score, E-value, and length. When two BLAST hits were highly similar for the same query, the hits were identified as alleles of the same gene. In total, 46 ABA metabolism genes were identified from the

primary (alternative (alt1) or secondary (alt2) CS haplotig sequences (Supplementary Data 2). Alleles are referred to as alternatives without a designated haplotype because the phased chromosome-scale assembly was not complete for the CS clone 8 v1.0 genome. Alternative names were assigned as "alt1" if only 1 paralog was identified based on V3 annotation. Alternatives were assigned as "alt1" and "alt2" when two paralogs were identified with "alt1" corresponding to the primary contig and "alt2" corresponding to the haplotig denoted as "P" and "H", respectively, in CS gene names. When multiple paralogs were identified corresponding to multiple V3 genes with the same annotation symbol, "alt1_1" and "alt2_1" were named to correspond to CS alleles with the same V3 gene name. Paralogs with only one allele with the same annotation symbol as other V3 genes are indicated as "alt1_2" and "alt1_3".

5. RNA-Seq analysis

Reads were trimmed as previously described using trimmomatic v0.36 (Bolger *et al.*, 2014), and sample quality was confirmed with FastQC v0.11.5 (Babraham Bioinformatics, 2010). PRJNA516950 was analysed with the CS clone 8 v1.0 genome (Chin *et al.*, 2016) being the only phased high-quality and the most contiguous *Vitis* genome available at the time. Salmon (Patro *et al.*, 2017) version 0.14.1 was used to estimate the transcript abundance from the trimmed fastq files with settings specified as --gcBias, --seqBias, --fldMean=76, --fldSD=1, --validateMappings --rangeFactorizationBins 4. An augmented hybrid fasta file was built from the *Vitis vinifera* cv. Cabernet Sauvignon genome using generateDecoyTranscriptome.sh from salmontools. This file was used to build the index file used for the quantification with a k-mer size of 15. The salmon output (quant files) was imported into DESeq2 v1.26.0 (Love *et al.*, 2014). Contrasts of interest included comparing each species x week x organ Control and WD as well as comparing WD RM x organ x week to those of CS, RG, and SC. All heatmaps were drawn with ComplexHeatmap v2.2.0 (Gu *et al.*, 2016), dendextend v1.13.4 (Galili, 2015), and circlize v0.4.8 (Gu *et al.*, 2014) to extract contrasts of interest in RStudio with R. The CS alignment produced similar results to that of previously reported PN40024, and a detailed report and figures of ABA metabolism-related transcripts can be found in (Cochetel *et al.*, 2020).

WGCNA is a correlation network methodology used to 1) identify clusters (modules) of interconnected nodes, 2) summarise node profiles of a module using a highly connected hub node (Eigengene) 3) identify significant modules and annotate the network nodes (Langfelder and Horvath, 2008). In this case, the WGCNA was performed to identify genes most connected to Eigengenes that were most associated with WD, [ABA], and [ABA-related metabolite] with a focus on ABA metabolism-related genes associating transcript and metabolite quantitative data in an integrative analysis. WGCNA version 1.41 (Langfelder and Horvath, 2008) was performed per organ as previously described (Cochetel *et al.*, 2020). Topological Overlap Matrix (TOM) was used to detect modules using the DynamicTreecut

algorithm with a minimum module size of 30 and a branch merge cut height of 0.25. The module eigengenes were used to evaluate the association among the modules for species, treatments, weeks, and various metabolites. GO enrichment analysis was performed with TopGO v2.38.1 (Alexa *et al.*, 2006) using biological process terms corresponding to the CS clone 8 v1.0 genome. WD and control modules (Supplementary Figure 6) were generally preserved except the blue, midnight blue, and turquoise leaf control modules with the Salmon WD module, but these modules did not contain ABA metabolism-related genes.

6. Statistical analysis

Statistical analysis was performed comparing multiple means, including one-, two-, three-, and four-way ANOVAs after parametrical assumptions were met. Outliers were identified and removed for values greater than the quartile ($Q3+1.5*\text{interquartile range (IRQ)}$) and $Q1-1.5*\text{IRQ}$). Additional outliers were not removed after one round of outlier removal, and outliers were not removed if removal would result in experimental groups with fewer than three replicates. Non-normal data were Box-Cox transformed to meet normality and homoscedastic assumptions. Post Hoc tests were performed with Tukey's test HSD for comparisons between species, treatments, and time points after assumptions were met. Asterisks indicate statistical

significance from ANOVA (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$). Letters indicate statistical significance between the multiple comparisons. The error rate $\alpha = 0.05$ was used in all comparisons. Linear regression models and associated statistics were performed in R. Statistical analyses and all other R-based analyses were performed using R version 3.6.3 in RStudio version 1.2.13335.

7. Availability of data and material

RNA-Seq data were deposited in the Sequence Read Archive (SRA) database with the accession number PRJNA516950 in previous work (Cochetel *et al.*, 2020). All Figures, Supplementary Data, and Supplementary Figures are available at <https://figshare.com/s/38cd732935dc6141c7b8>. All other data, including additional DEA contrasts, are available upon request to the corresponding author.

RESULTS

1. ABA and related metabolite concentrations differed in grapevine species and organs during one- and two-week moderate WD

ABA and the ABA-related metabolites (ABA-GE, 7'OH ABA, NeoPA, PA, and DPA) were quantified from total leaf and root samples (Figures 1 and 2) collected from a small pot; the moderate WD experiment was published previously

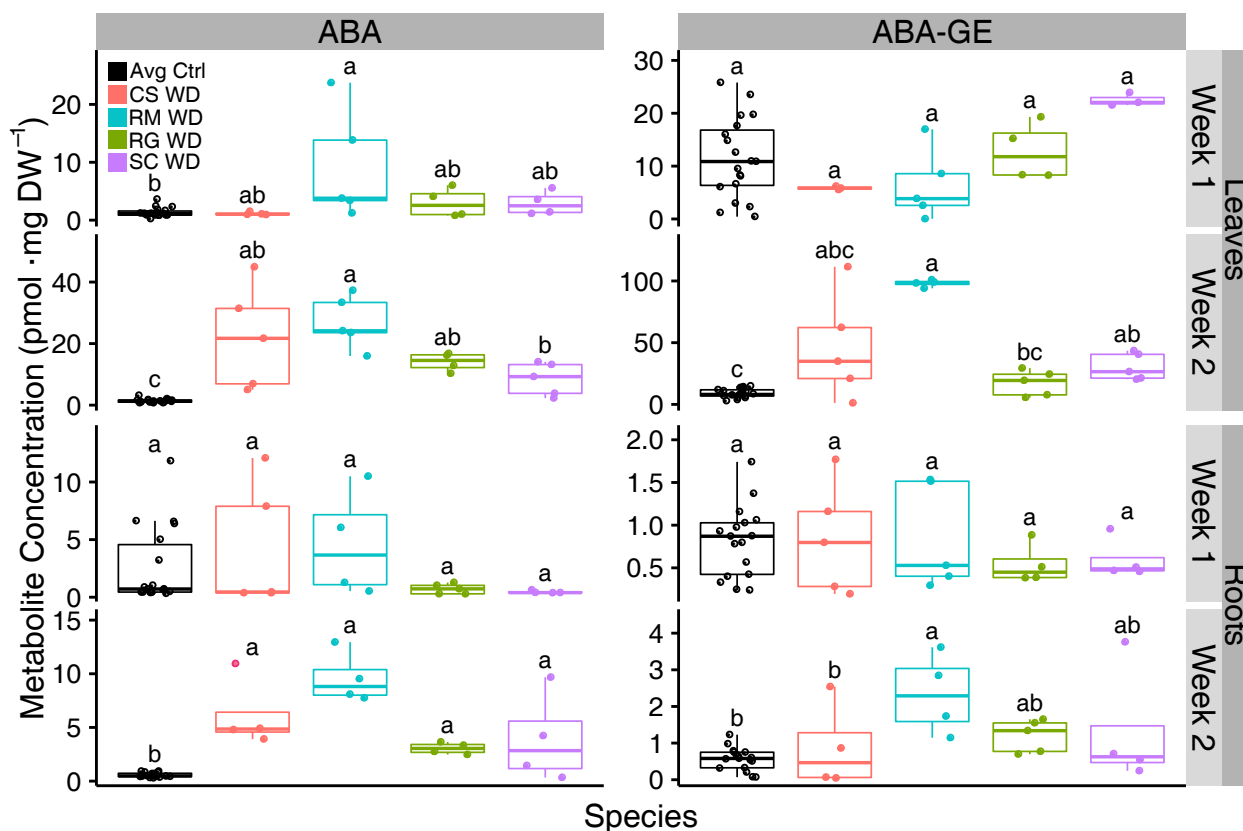


FIGURE 1. ABA and ABA-GE concentrations after one and two weeks of moderate WD.

ABA (left) and ABA-GE (right) concentrations ($\text{pmol} \cdot \text{mg DW}^{-1}$) in leaves (top) and roots (bottom) after one and two weeks of control (Ctrl) or WD treatment. Control represented all species per organ per time; there was no significant difference between control species per organ per week. Two-way ANOVA Tukey's HSD ($p \leq 0.05$) letters for each metabolite per organ per week. Black corresponds to the control values for all species. Pink, blue, green, and purple correspond to CS, RM, RG, and SC, respectively. Each point represents an individual measurement. $N = 3$ to 5 individual vines.

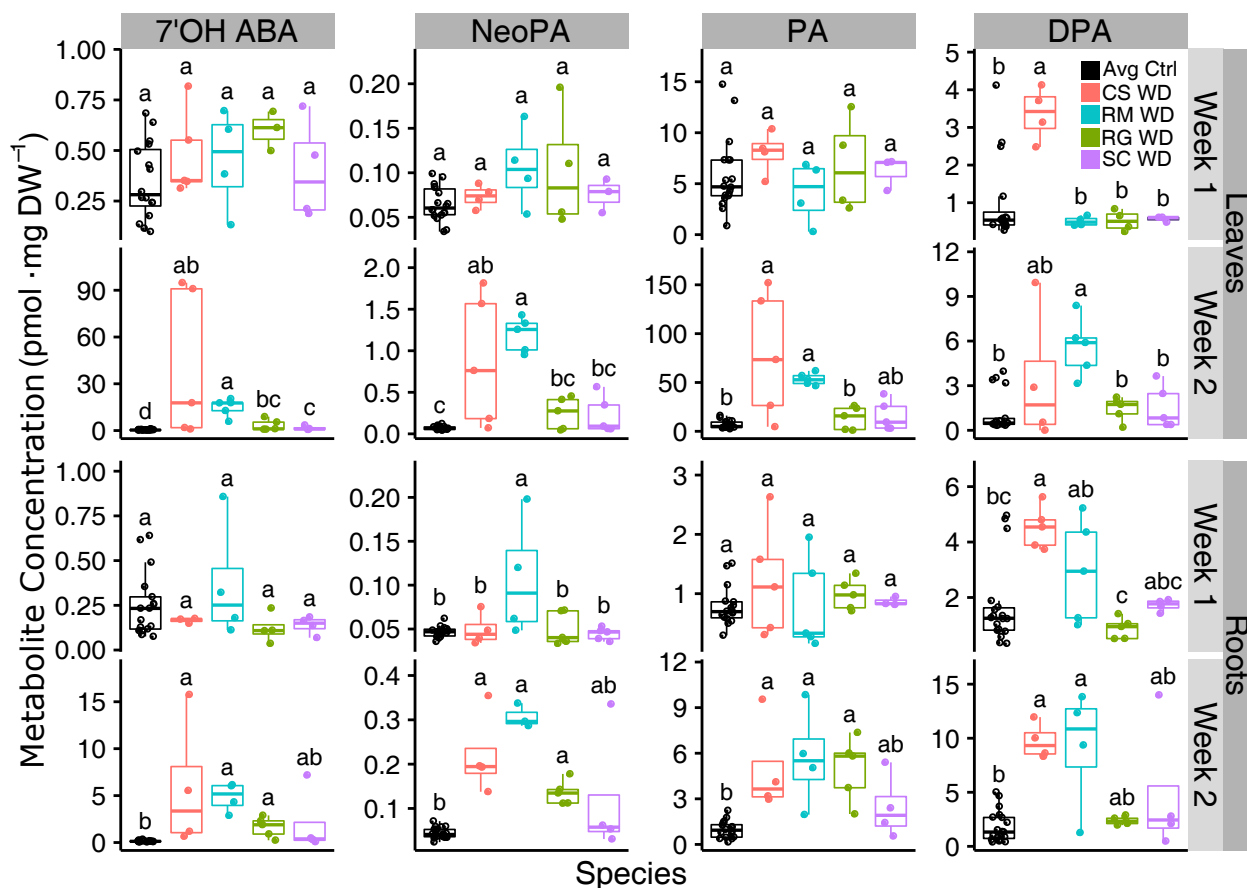


FIGURE 2. ABA catabolite concentrations after one- and two-weeks of moderate WD.

7'OH ABA, NeoPA, PA, and DPA (left to right) concentrations ($\text{pmol} \cdot \text{mg DW}^{-1}$) in leaves (top) and roots (bottom) after one- and two-weeks of control (Ctrl) or WD treatment. Control represented all species per organ per time; there was no significant difference between controls per species per organ per week. Two-way ANOVA Tukey's HSD ($p \leq 0.05$) letters for each metabolite per organ per week. Black corresponds to the control values for all species. Pink, blue, green, and purple correspond to CS, RM, RG, and SC, respectively. Each point represents an individual measurement. $N = 3$ to 5 individual vines.

(Cochetel *et al.*, 2020). Averages and other summarised values are in Supplementary Data 1. Briefly, CS, RM, RG, and SC were subjected to one or two weeks of well-watered control or a natural dry-down moderate WD. The moderate WD was accurately determined by measuring pot weights and average stem water potentials that decreased from approximately -0.49 MPa in the first week of WD to approximately -0.82 MPa by the second week (Cochetel *et al.*, 2020). There were no statistically significant differences in stem water potentials among the species at either individual time point (Cochetel *et al.*, 2020), indicating the different grapevines were experiencing the same level of stress. The term “significant” will be used here to mean statistically significant at a p -value of 0.05 or less.

After two weeks of WD, ABA, and ABA-related metabolites were significantly more abundant in the leaves and roots of the four species (Figures 1 and 2). The control values of all species were averaged together in these figures to simplify the visual comparisons because there were no significant differences among the species' controls. On average, for all species, the ABA accumulation in response to WD was higher in the second week relative to the first week and resulted in a higher ABA concentration in leaves than roots. Herein, ABA-related metabolite concentrations in picomole per milligram of dry weight ($\text{pmol} \cdot \text{mg DW}^{-1}$) will be bracketed (e.g., [ABA]).

Strikingly, [ABA] increased significantly above that of the controls only for RM in response to WD in leaves after just one week of treatment (Figure 1). There was some variability at this early stage of WD (Supplementary Data 1); [ABA] in roots of some individual RM and CS vines increased, but the averages of all WD-treated individuals were not statistically significant from controls. The leaves and roots of RM had higher [ABA] relative to that of the drought-sensitive RG (Figure 1). After two weeks of treatment, RM retained higher [ABA] compared to RG (and SC), while CS had more variable and intermediate [ABA] (Figure 1). Unlike leaves, the significant accumulation of ABA in roots at two weeks of WD was not significantly different among the species. As a general rule, [ABA] can be ranked by species during this moderate WD as $\text{RM} > \text{CS} > \text{RG} > \text{SC}$ in both leaves and roots.

Consistent with [ABA], [ABA-GE] increased with WD and was much higher in leaves than roots. There were no significant increases of [ABA-GE] in the leaves or roots of any of the species after one week of WD. However, by the second week of WD, RM, and SC had significantly higher [ABA-GE] in leaves relative to controls, and RM had significantly higher [ABA-GE] in roots relative to controls (Figure 1). [ABA-GE] was significantly higher than [ABA]

in leaves and lower than [ABA] in roots (Figure 1) for all species per treatment per week.

Among the ABA catabolites (7'OH-ABA, NeoPA, PA, and DPA), RM had significantly higher [NeoPA] and [DPA] relative to controls in roots and significantly higher [DPA] in leaves after one week of WD (Figure 2). None of the other species had significant increases in any ABA catabolite concentrations relative to controls after one week of WD in roots or leaves. At two weeks of WD, all species had significantly higher [7'OH-ABA] relative to controls in leaves; CS had significantly higher [NeoPA] and [PA] in leaves, but RG and SC had no significant increases in the other ABA catabolites. However, by two weeks of WD in roots, all the ABA catabolites increased significantly in CS, all but DPA significantly increased in RG, and there was no significant increase in any ABA catabolite in SC. Several CS vines accumulated NeoPA, PA, and DPA to a comparable level as RM in both leaves and roots. However, [ABA catabolites] were more variable in CS than RM. [ABA catabolites] were comparable between the organs at one week of WD, but [ABA catabolites] were generally higher in leaves than roots at two weeks of treatment (Figure 2). For example, RM had a large increase in [PA] in leaves relative to roots at two weeks of WD. RM was the only species that had significantly increased concentrations of all four ABA catabolites in both organs in response to two weeks of WD.

Like [ABA] and [ABA-GE], all [ABA catabolite] increased in response to the moderate WD with $RM > CS > RG \geq SC$ (Figure 2). As expected (Nambara and Marion-Poll, 2005), the products of the main ABA catabolic pathway, [DPA] and [PA], were much higher than [7'OH ABA] and [NeoPA]. Interestingly, [PA] was higher than [DPA] in WD leaves. However, the reverse was true in WD roots, where [DPA] was equivalent to or higher than [PA]. NeoPA had very low concentrations relative to the other catabolites.

To further understand ABA metabolism in the plant as a whole, the distribution of ABA and ABA-related metabolites was examined for the whole plant. "Total ABA Metabolites" was defined as the sum of the leaf and root total [ABA] and [ABA-related metabolites] (Supplementary Figure 1) for the whole plant (leaves + roots) and individual organs. This approach revealed similar patterns as the [ABA-related metabolites] individually; RM had an earlier accumulation of [Total ABA Metabolites] at one week of WD and retained higher [Total ABA Metabolites] than the other species at two weeks of WD. Generally, leaves and roots had comparable [Total ABA Metabolites] at one week of WD, but leaves had higher [Total ABA Metabolites] than roots at two weeks WD. Altogether, ABA metabolism in RM appears to respond earlier and more significantly than in the other species.

To examine possible changes in the distribution of the ABA-related metabolites, [ABA] and each [ABA-related metabolite] per organ were also divided by the summed [Total ABA Metabolites] per the whole plant to estimate the percentage that each metabolite represented in the whole metabolic pathway (Supplementary Figure 2).

ABA never averaged above 20% of the total ABA metabolites regardless of species, organ, treatment, or time. ABA-GE and PA represented the two major portions of the leaf ABA metabolites in controls in the first week, followed by ABA. However, after one week of WD, RM leaves had a significant decrease in the proportion of ABA-GE from 0.48 ± 0.15 to an average of 0.20 ± 0.08 , indicating that ABA metabolism was shifted from ABA-GE to other ABA metabolites such as ABA and DPA. This reduction of ABA-GE pools was specific to an early response of RM leaves to WD because there was no significant difference in RM control and WD leaves at two weeks, nor did the other species show the same shift in ABA-GE distribution in WD leaves. Likewise, the proportion of ABA-GE in WD roots in the second week decreased relative to control in all of the species except for CS. These changes in metabolite proportions indicate that ABA metabolism was complex, dependent upon the species, the organ, and the duration of the WD.

2. [ABA] increased during severe WD

To further understand the ABA metabolism of the distinct species in response to WD, [ABA] was quantified from another experiment that was performed previously (Cochetel *et al.*, 2020). Briefly, the four *Vitis* species underwent a natural dry-down over a week that achieved a lower stem water potential (~ -1.5 Mpa) (see Cochetel *et al.*, 2020 Additional File 5) than that of vines that experienced the two-week moderate WD treatment (~ -0.8 Mpa). Like in the moderate WD experiment, there was no difference in stem water potential among the species (Cochetel *et al.*, 2020) in the severe WD experiment, indicating the different grapevines were experiencing the same level of stress. In the severe WD experiment, also performed in small pots, RG was significantly impacted by the WD treatment; the majority of RG shoots withered and died (Cochetel *et al.*, 2020); shoots of the other species wilted but did not die. Only surviving vines were used in subsequent analyses.

Previously, it was found that *NCED3* transcript abundance was significantly increased in response to WD in all species except for the surviving RG (Cochetel *et al.*, 2020); RM and CS had the highest *NCED3* transcript abundance in the leaves and roots in response to WD (see Cochetel *et al.*, 2020 Figure 6b and Additional File 11). [ABA] after one week of severe WD increased and were comparable to those of two weeks of moderate WD (Figures 1 and 3 and Supplementary Data 1). All WD leaves, except those of RG, were significantly different from respective controls (Figure 3), which paralleled *NCED3* normalised relative quantity (see Cochetel *et al.*, 2020 Additional File 11). RM leaves had a significantly higher [ABA] than those of RG and SC in response to WD, which was also observed for the *NCED3* transcript abundance. Additionally, RM was the only species to have significantly higher [ABA] in both leaves and roots in response to WD (Figure 3). The similarity in [ABA] between the week-two moderate and one-week severe WD experiments indicated that ABA metabolism was not only dependent on the severity of WD stress but also the duration.

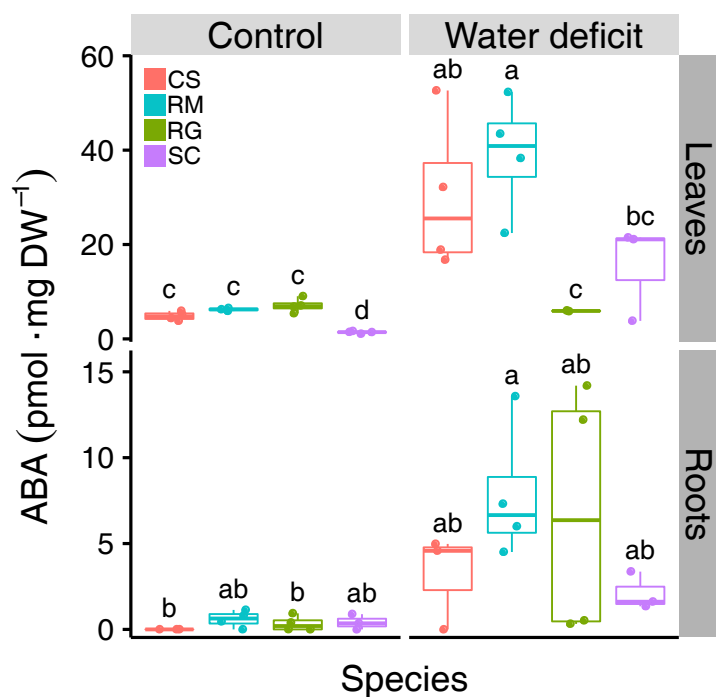


FIGURE 3. ABA concentrations after one week of severe WD.

ABA concentrations ($\text{pmol} \cdot \text{mg DW}^{-1}$) in leaves (top) and roots (bottom) after one-week of Control (left) or WD (right) treatment. Two-way ANOVA Tukey's HSD ($p \leq 0.05$) for each metabolite per organ per week. Pink, blue, green, and purple correspond to CS, RM, RG, and SC, respectively. Each point represents an individual measurement. $N = 3$ to 5 individual vines.

Increasing [ABA] correlated to the declining stem water potential of the vine (Figure 4) for individual vines from both the moderate and severe WDs (Figure 4). Data are from vines where both ABA and stem water potentials were measured on the same vine. In some cases, ABA concentrations and stem water potentials were not measured for the same vine, thus reducing the total number of samples used in this figure. In each experiment and the combination of the experiments (Figure 4), there was an observable trend of increasing [ABA] with decreasing stem water potential. Furthermore, [ABA] was generally higher for WD-treated RM and lower for WD-treated RG (Figure 4) despite comparable stem water potentials. Similar comparisons of [ABA] to photosynthetic rate and stomatal conductance support the unique ABA metabolism among the species despite comparable vine mass and leaf surface area (Supplementary Figure 3). Surprisingly, RG generally had much lower photosynthesis and stomatal conductance at much lower [ABA], reflecting not only differences in ABA metabolism, but may also reflect species-specific differences in ABA sensitivity (Supplementary Figure 3).

3. Transcript abundances of several ABA transporters were increased by WD

To investigate the ABA metabolism regulation of the four *Vitis* species in response to WD, a targeted analysis of an RNA-Seq dataset was performed using the sequence

annotation for the CS genome (see Materials and Methods). Average transcripts per million (TPM) values for annotated genes are in Supplementary Data 1. Statistics for contrasts of interest for the differential expression analysis (DEA) are in Supplementary Data 3–6; due to the complexity of the comparisons, statistics are not visualised, but the discussed genes are significantly differentially expressed for contrasts of interest (Ctrl vs. WD, Leaves vs. Roots, RM vs. RG, etc.). Forty-six ABA metabolism-related genes were identified with the protein basic local alignment search tool (BLASTP) using known ABA metabolism PN40024 protein sequences as a query (Supplementary Data 2). The alleles were classified as alt1 or alt2 based on the contig origin of this heterozygous genome (primary or haplotig); no haplotype was attributed because the phased chromosome-scale assembly for this genome was not available at the time.

4. *NCED3* transcript abundance increased significantly after one- and two-week moderate WD

Many of the 46 metabolism-related genes identified in CS were significantly differentially expressed and described, and visualised previously in Cochetel *et al.*, 2020. Of note, *NCED3* (see Cochetel *et al.*, 2020 Figure 6b) was the only increased differentially expressed gene (DEG) specifically linked to ABA metabolism in leaves and roots of all four species in response to two weeks of WD (Supplementary Data 3). *NCED3* was also the ABA metabolism-related DEG that had the highest average TPM for all WD species' leaves. *NCED3* had the highest expression level of the five annotated *NCEDs* in both leaves and roots at one and two weeks of WD in all species, with the highest level of expression in RM (Supplementary Data 3). The other *NCEDs*, like *NCED6* alternatives, were lowly expressed but also had higher expression levels in RM compared to the other species. Other ABA metabolism DEGs were identified (Supplementary Data 3–6,) including genes involved in ABA biosynthesis and catabolism (Supplementary Figure 4) and deconjugation (Supplementary Figure 5), but their expression was not to the extent of *NCED3*. The transcript abundance of *BG1* alt1 and alt2 (Supplementary Figure 5) decreased for WD only in RM in both leaves and roots. The transcript abundance of *BG3* alt1 decreased for WD for all species and organs. Overall, *NCED3* appears to be the major ABA metabolism-related gene contributing transcripts to downstream ABA biosynthesis during WD.

5. ABA metabolism genes were correlated with multiple WGCNA modules

WGCNA was performed to identify clusters of genes (modules) associated with the different experimental conditions (organ, time, [ABA] and [ABA-related metabolite]) (Supplementary Figures 6–7 and Supplementary Data 7). The 46 ABA metabolism-related genes were spread across multiple modules. In the leaves, 30 modules were identified (Supplementary Figure 6). WD was positively correlated with five modules: lightyellow, darkgreen, brown, saddlebrown, and green. Generally, the ABA-related metabolites were positively correlated with the same five modules as WD.

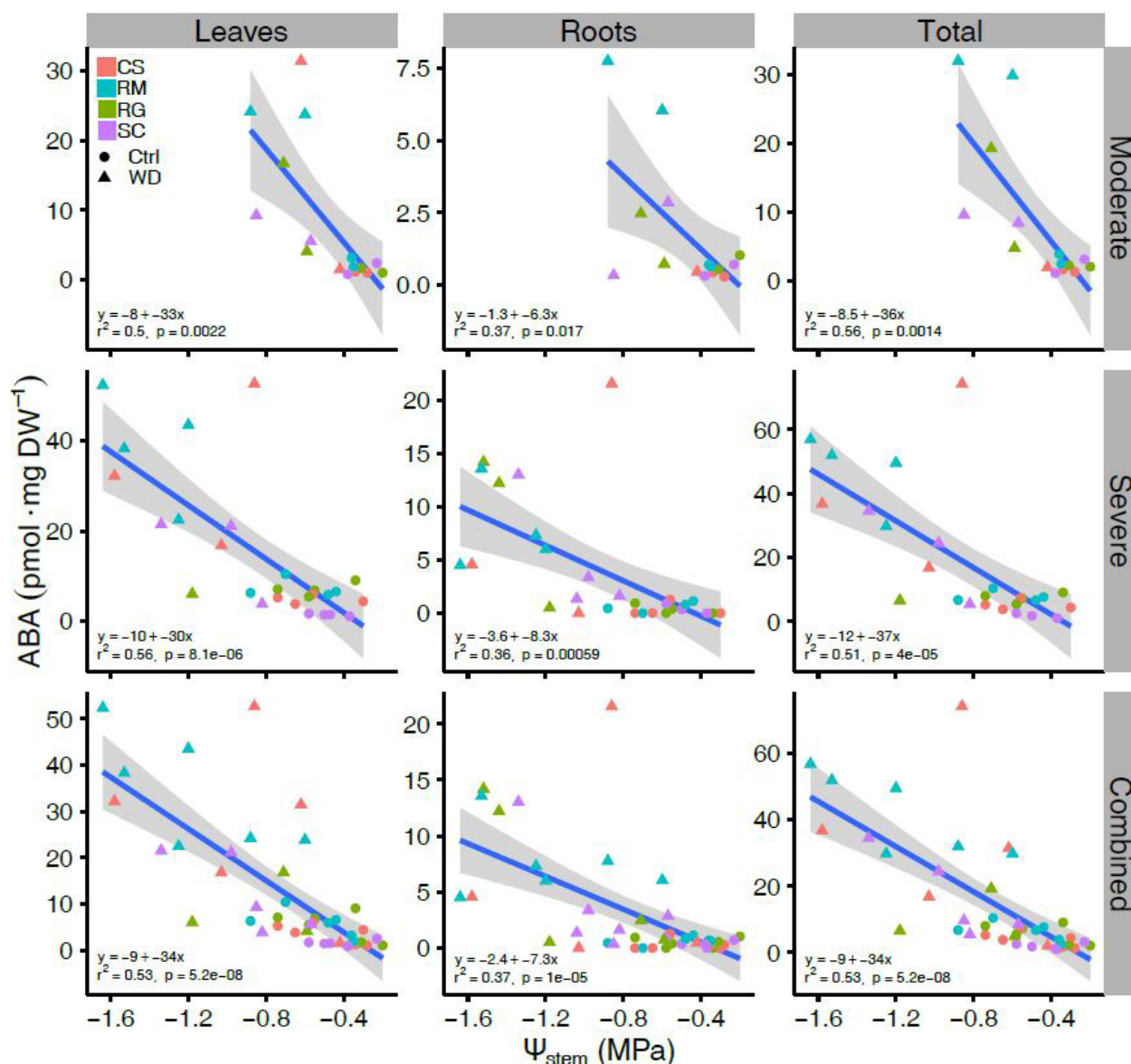


FIGURE 4. ABA concentrations related to stem water potential.

ABA concentrations ($\text{pmol} \cdot \text{mg DW}^{-1}$) in leaves (left), roots (middle), and total (combined leaves and roots) in the moderate (top), severe (middle), or combined moderate and severe WD experiments (bottom). Individual WD-treated vines are shown as triangles and control (Ctrl) treated samples are represented by circles. Linear regression was performed per organ \times experiment combination; the resulting equation, r^2 , and p -value are shown. Pink, blue, green, and purple correspond to CS, RM, RG, and SC, respectively. Each point represents an individual vine. $N \geq 2$ individual vines per organ \times experiment combination.

However, [DPA] did not correlate with the same modules as WD nor the other [ABA-related metabolites] in the leaves. In fact, [DPA] had an opposing correlation compared to the other [ABA-related metabolites] for numerous modules, indicating [DPA] may be uniquely regulated or involved in a different or complementary signalling pathway in the leaves.

NCED3 was a hub gene in the lightyellow module, being the 15th most correlated gene to the eigengene representing this module. Disruption of hub genes disturbs the gene expression of numerous other genes in a module. *NCED3* was closely connected to ABA signalling genes in this module, including the top-ranking gene, *HIGHLY ABA INDUCED 1 (HAI1)* (VvCabSauv08_P0061F.ver1.0.g440640). Other top-ranking, hub genes in the light-yellow module included

RAS-RELATED PROTEIN 18 (RAB18) (VvCabSauv08_H0004F_076.ver1.0.g050030), *HOMEBOX-7 (HB-7)* (VvCabSauv08_P0060F.ver1.0.g439740), and *PP2C-8* (VvCabSauv08_P0452F.ver1.0.g610510) (Supplementary Data 7). The rank of *NCED3* in the lightyellow module far exceeded that of any other ABA metabolism-related gene in any module; the second-highest-ranking gene was *UGT71C4 alt2* (rank 90) in the red module (Supplementary Data 7). In the light-yellow module, *NCED6 alt1* was the second-ranking ABA metabolism-related gene (rank 259; Supplementary Data 7).

In the roots, 34 modules were identified (Supplementary Figure 7). In total, ten modules were positively correlated with WD. Among the modules positively correlated with

WD, four were also positively correlated with [ABA] and the [ABA-related metabolites] (Supplementary Figure 7 and Supplementary Data 7): royalblue, lightgreen, midnightblue, and pink. The ABA metabolism-related genes were spread across 20 modules in the roots. The midnightblue module contained the greatest number of ABA metabolism-related genes (ten), including *NCED3*, *NCED5* alt2, *NCED5* alt1, and *NCED6* alt1 (module membership (kME) of 0.95, 0.80, 0.75, 0.65, respectively) (Supplementary Data 2 and 7). Of all ABA metabolism-related genes and all root modules, *NCED3* was the only hub gene corresponding to the midnightblue module (rank 19; Supplementary Data 7). Like in the leaves, *NCED3* was closely connected to ABA signalling genes in the midnightblue module in the roots; these genes included: *RD26* (VvCabSauv08_H0024F_036.ver1.0.g115900), SEVEN IN ABSENTIA OF ARABIDOPSIS 2 (*SINAT2*; VvCabSauv08_P0027F.ver1.0.g381860), *NAC DOMAIN CONTAINING PROTEIN 47* (*NAC047*; VvCabSauv08_P0024F.ver1.0.g368680), and *GALACTINOL SYNTHASE 1* (*GolSI*; VvCabSauv08_P0018F.ver1.0.g349170 and VvCabSauv08_P0018F.ver1.0.g349150).

To better understand the roles of these genes in the modules, gene ontology enrichment was performed using biological process terms for the brown, green, and lightyellow modules in the leaves and the midnightblue and pink modules in the roots (Supplementary Data 8). These modules were selected for high correlation to WD and the metabolites, as well as for the number of ABA metabolism-related genes contained in each module. These modules were enriched for “response to stress” and “response to stimulus” GO terms (Supplementary Data 8). All modules except the green module in the leaves included the “response to abiotic stimuli” GO term (Supplementary Data 8). The lightyellow module in the leaf was enriched for the “biosynthetic process” GO term (Supplementary Data 8), and lightyellow had the highest correlation with the ABA biosynthetic genes *NCED3* and *NCED6* in the leaves (Supplementary Data 2), supporting this grouping. The ABA metabolism-related gene functions were enriched with GO terms assigned to the modules most correlated with WD and the [metabolites], supporting a link between transcript abundance and [metabolite].

DISCUSSION

1. Grapevine species differ in physiological and ABA metabolism pathway response to WD

In these experiments, there were significant differences in ABA metabolism among the species. CS generally had the most variable [ABA], [ABA-related metabolite], and *NCED3* transcript abundance in response to various water deficits, along with high variability in physiological responses (Cochetel *et al.*, 2020). RG quickly closed stomata (Cochetel *et al.*, 2020) and retained low stomatal conductance throughout the WD while having a small increase in [ABA], [ABA-related metabolites], and ABA metabolism-related transcript abundances relative to CS and RM. This may reflect a greater sensitivity of RG stomata to ABA during

WD. SC demonstrated a similar biochemical response to RG with lower [ABA], [ABA-related metabolites], and associated transcript abundances. In moderate and severe WD, RM had higher [ABA] in both leaves and roots relative to the other species. RM had the highest *NCED3* transcript abundance during WD. Additionally, RM had the highest levels of *NCED3* transcripts and [ABA] at the earliest time point during the moderate WD, indicating RM may be the most sensitive to respond to WD. RM maintained higher physiological function during the moderate and severe WD (Cochetel *et al.*, 2020) despite higher levels of ABA than the other species. These observations indicate that some physiological functions of RM may be less sensitive to [ABA] over time (e.g., stomatal closure) or that RM may be acclimating better to WD (e.g., modified rooting depth, root suberisation, or hydraulic conductance) relative to the other species as described in other plants (Mamrutha *et al.*, 2017; Thameur *et al.*, 2011; Tombesi *et al.*, 2015), and these changes can occur early in water deficit responses (Barrios-Masias *et al.*, 2015; Zhang *et al.*, 2020). Several aquaporin genes display differential and unique responses to WD in RM leaves, and genes involved in cysteine biosynthesis and metabolism are also constitutively higher in the roots of RM (Cochetel *et al.*, 2020); this observation linked the gene expression and physiological responses to other known ABA effectors that were not investigated here. The physiological and sensitive transcriptomic response that occurs at milder longer-term WD in RM indicates RM may be able to take advantage of moderate or longer-term WD by maintaining open stomata longer than the other species despite decreasing water availability.

The data from this study were collected from natural dry-down pot experiments. Variation in such experiments can result from experimental conditions, plant size, or individual physiology. Although efforts were made to minimise differences (similar plant size, foil-covered pots, soil media volume, randomised design, etc.), there was biological variation between individual grapevines, which may be largely attributed to individual morphology, anatomy, physiology, and biochemistry. The relative soil water content was the same at each time point, and stem water potentials were not significantly different among species for a particular stress or time. Although not statistically significant, RG had lower stem water potentials than RM at any water deficit level other than control. Many factors can affect the stem water potential, such as leaf area, stomatal conductance, hydraulic conductance, rooting depth, etc. Nevertheless, there were quantifiable differences in ABA metabolism between the species, with RM having the highest [ABA] and *NCED3* transcript abundance of the species at any given level of WD tested in this study.

2. Leaves and roots differ in [ABA], [ABA-related metabolite], and *NCED3* transcript abundance

The primary site of ABA biosynthesis (leaves versus roots), as well as the initial site and signal to trigger a stress response during WD, is a controversial topic (Hussain *et al.*, 2020;

McAdam *et al.*, 2016; Takahashi *et al.*, 2018). Leaves and roots had different concentrations of ABA, ABA-related metabolites, and ABA metabolism-related transcripts in these experiments. Additionally, the leaf and root WGCNA *NCED3*-containing gene modules did not contain the same ABA metabolism genes, indicating a difference in ABA metabolism and signalling in leaves and roots as observed previously in grapevine (Khadka *et al.*, 2019; Rosssdeutsch *et al.*, 2016) and other plant species (Min *et al.*, 2020). However, whole organs were used for these analyses. Specific cell types like the guard cell (Assmann and Jegla, 2016) or root endodermis (Duan *et al.*, 2013) have specialised responses to ABA and likely have unique regulation of *NCED3* and ABA metabolism, each of which requires further investigation in these grapevine species.

In both the moderate and severe WD experiments, WD leaves generally had a higher [ABA] than roots, as was expected (Lovelli *et al.*, 2012; Manzi *et al.*, 2015), but WD leaves and roots had comparable *NCED3* transcript abundance. The differences in [ABA], [ABA-related metabolite], and ABA metabolism-related transcript abundance between leaves and roots may reflect different sensitivity to ABA between the organs (Correia *et al.*, 2014; Finkelstein, 2013). It is likely that the [ABA] threshold to elicit a specific ABA response is organ-specific as well (Rattanakon *et al.*, 2016) and may involve ABA and ABA-related metabolite transport between organs (Kuromori *et al.*, 2018).

ABA is biosynthesised and transported throughout a plant (Kuromori *et al.*, 2018). ABA transport from vascular cells into guard cells is well documented with several identified transporters. There is evidence that root ABA may be in part shoot-sourced (Goodger and Schachtman, 2010; Ikegami *et al.*, 2009), and ABA transport into the root has been described (Ikegami *et al.*, 2009) in addition to ABA-vascular unloading and transport into guard cells (Kuromori *et al.*, 2010) and endosperm-ABA unloading into the seed embryo (Kang *et al.*, 2015). In the moderate WD experiment, *ABCG25* and *ABCG40* had higher transcript abundance in roots than leaves for all species, treatments, and time points, indicating a possible role for these transporters in the root, perhaps in long-distance vascular transport pathways.

ABA-GE is highly abundant (Hansen and Dörffling, 1999) and is rapidly deconjugated during water deficit to form a separate pathway for ABA production (Han *et al.*, 2020). In this study, leaves had significantly higher [ABA-GE] than roots in the moderate WD experiment, supporting the conclusions of others (Acanda *et al.*, 2020; Manzi *et al.*, 2015; Thameur *et al.*, 2011) that the organs may have different needs for this metabolite. *BGL1* had significantly higher transcript abundance in roots than leaves for most species for both treatments and times in the moderate WD experiment indicating the possible importance of ABA-GE deconjugation in the roots. Despite low membrane permeability (Baier *et al.*, 1990) and attempts to identify an ABA-GE transporter (Kang *et al.*, 2010), this potential ABA transport pathway remains unsolved. ABA-GE transport may be one source of shoot-derived ABA in roots that have not

been well characterised (Kuromori *et al.*, 2018). Furthermore, the metabolism of ABA-GE during WD by RM appears to be enhanced as compared to the other species. ABA-GE in the leaves of RM decreased in proportion to the other ABA-related metabolites after one week of moderate WD and was produced to a much greater extent in the leaves after two weeks of moderate WD as compared to the other species.

3. Higher *NCED3* transcript abundance is a core WD response in *Vitis*

Throughout these experiments, *NCED3* stood out as a key WD response gene in both leaves and roots of the four grape species differing in drought tolerance (Cochetel *et al.*, 2020; Fort *et al.*, 2017; Suarez *et al.*, 2019). From the WGCNA, two modules contained the most ABA metabolism-related genes in leaves and roots and were most correlated with WD and [ABA-related metabolites]. *NCED3* was the sole ABA metabolism hub gene in these modules. Additionally, *NCED3* transcript abundance increased in both WD experiments described here regardless of severity, duration, organ, and species. *NCED3* is a key transcript in grapevine WD response (Pilati *et al.*, 2017; Sussmilch *et al.*, 2017) and other plants (Liu *et al.*, 2016; Qin and Zeevaart, 1999). In addition to ABA metabolism and signalling genes, the *NCED3*-containing modules include numerous genes involved in plastid function like the chloroplastic 50S ribosome subunit, as well as aquaporins, ion transporters, ascorbate oxidase, galactinol synthase, and cysteine and sulphur regulatory genes, which have known associations to ABA (Batool *et al.*, 2018; Lamarque *et al.*, 2019; Li *et al.*, 2020).

Two levels of the biochemical regulation of ABA were examined in this study (transcripts and metabolites). However, numerous other steps may impact [ABA], [ABA-related metabolite], and the physiological responses a plant has to WD. For these reasons, the response of a plant to a short, long, moderate, or severe WD may be vastly different. Many levels of biochemical regulation of WD response and ABA biosynthesis, conjugation, and catabolism require further characterisation, including alternative splicing, signal peptides like CLE25 (Takahashi *et al.*, 2018), miRNA, siRNA, and other enzyme activity regulators.

The results in this study support the hypothesis that ABA metabolism varies in different grapevine species that vary in drought tolerance. RM, a drought-tolerant species, had significantly higher [ABA], [ABA-related metabolites], and transcript abundance of ABA transporters, *NCED3*, and other ABA metabolism-related genes during WD than that of RG, a drought-sensitive species. The ABA metabolism response to WD was dependent upon the species, organ, and duration of WD. ABA metabolism in *Vitis* species is complex, utilising multiple pathways; further research is needed to elucidate the underlying regulatory mechanisms.

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