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Expression of ABA synthesis and metabolism genes under different irrigation strategies and atmospheric VPDs is associated with stomatal conductance in grapevine (*Vitis vinifera* L. cv Cabernet Sauvignon)

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Abstract

The influence of different levels of irrigation and of variation in atmospheric vapour pressure deficit (VPD) on the synthesis, metabolism, and transport of abscisic acid (ABA) and the effects on stomatal conductance were examined in field-grown Cabernet Sauvignon grapevines. Xylem sap, leaf tissue, and root tissue were collected at regular intervals during two seasons in conjunction with measurements of leaf water potential (Ψ_{leaf}) and stomatal conductance (g_s). The different irrigation levels significantly altered the Ψ_{leaf} and g_s of the vines across both seasons. ABA abundance in the xylem sap was correlated with g_s . The expression of genes associated with ABA synthesis, *NCED1* and *NCED2*, was higher in the roots than in the leaves throughout and highest in the roots in mid January, a time when soil moisture declined and VPD was at its highest. Their expression in roots was also inversely related to the levels of irrigation and correlated with ABA abundance in the roots, xylem sap, and leaves. Three genes encoding ABA 8'-hydroxylases were isolated and their identities confirmed by expression in yeast cells. The expression of one of these, *Hyd1*, was elevated in leaves when VPD was below 2.0–2.5 kPa and minimal at higher VPD levels. The results provide evidence that ABA plays an important role in linking stomatal response to soil moisture status and that changes in ABA catabolism at or near its site of action allows optimization of gas exchange to current environmental conditions.

Key words: Absciscic acid, ABA, ABA 8'-hydroxylase genes, grapevine, irrigation, NCED genes.

Introduction

The plant hormone abscisic acid (ABA) is associated with regulating many developmental and physiological processes in plants including responses to a number of stresses (Zeevaart and Creelman, 1988). It has been well established that reductions in soil water availability increase levels of ABA in the xylem and apoplastic sap which, in turn, are associated with decreasing stomatal conductance (reviewed in Dodd, 2005). However, ABA may not be the only factor involved in stomatal regulation and there are good arguments proposing hydraulic and/or chemical signals other than ABA in the same role (Christmann *et al.*, 2007; Wilkinson *et al.*,

2007; Neumann, 2008; Rodrigues *et al.*, 2008; Schachtman and Goodger, 2008). It is also unclear whether signalling from the roots initiates the stomatal response or whether the key signal(s) originate in the leaf, for example as a function of leaf water potential (Rodrigues *et al.*, 2008), *de novo* synthesis of ABA in the leaf (Zeevaart, 1980; Pierce and Raschke, 1981; Tan *et al.*, 1997; Soar *et al.*, 2006) as distinct from synthesis in the root (Cornish and Zeevaart, 1985; Lachno and Baker, 1986; Davies *et al.*, 1987), redistribution of sequestered leaf ABA in response to changes in pH (Stoll *et al.*, 2000; Rodrigues *et al.*, 2008), changes in ABA transport in response

to changes in xylem sap pH (Sauter and Hartung, 2002; Else *et al.*, 2006), or re-activation of esterified ABA (ABA-GE) by increased glucosidase activity (Lee *et al.*, 2006).

Increases in ABA abundance in water stressed tissues have been linked with the expression of one or more of the ABA biosynthetic genes, in particular the gene(s) encoding the 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzyme (Tan *et al.*, 1997; Qin and Zeevaart, 1999). Concurrent with increased levels of ABA in stressed tissues are increased levels of its catabolic products, primarily phaseic acid (PA) (Zeevaart, 1980; Pierce and Raschke, 1981) which is regulated by the cytochrome P450 enzyme ABA 8'-hydroxylase (reviewed by Cutler and Krochko, 1999). Genes encoding ABA 8'-hydroxylases have been identified and characterized in a number of plants (Kushiro *et al.*, 2004; Saito *et al.*, 2004; Millar *et al.*, 2006; Yang and Zeevaart, 2006; Saika *et al.*, 2007). Using molecular probes to determine the expression of the genes associated with ABA synthesis and catabolism in grapevine, this work examined aspects of its function in field-grown grapevines under different irrigation regimes. The main objectives were to determine the effects of varied irrigation on vine physiology, the primary source of the ABA found in the xylem sap, how reduced irrigation affected the synthesis and abundance of the ABA in the sap, and evidence of any association between the expression of the genes encoding the ABA catabolism enzyme 8'-hydroxylase and the different irrigation levels. This study also looked for an association between expression of these 8'-hydroxylase genes and changes in ambient vapour pressure deficit (VPD), since high humidity rapidly induced two ABA 8'-hydroxylase genes in *Arabidopsis* (Okamoto *et al.*, 2009).

Materials and methods

Plant material

Mature, 12-year-old Cabernet Sauvignon vines on Ramsey rootstocks were made available for irrigation trials at the Yalumba Oxford Landing vineyard in the South Australian Riverland. At the end of the 2006–2007 season, 60-cm deep trenches were dug alongside selected groups of three vines and backfilled with washed sand to facilitate collection of root samples. Three trenches were installed in each of the three irrigation treatments. In seasons 2007–2008 and 2008–2009, the vines were irrigated from mid September until after berry harvest. All vines were irrigated at the vineyard standard rate (equivalent to approximately 3.3 $\text{ML ha}^{-1} \text{ season}^{-1}$, 0.64 crop evapotranspiration, ET_c) until fruit-set. Three different irrigation treatments were then applied between fruit-set and harvest in late February. T_0 vines were irrigated in excess of ET_c requirement (1.27 ET_c , 6.6 $\text{ML ha}^{-1} \text{ y}^{-1}$). The other two treatments were T_1 (0.65 ET_c , 3.3 $\text{ML ha}^{-1} \text{ y}^{-1}$) and T_3 (0.25 ET_c , 1.3 $\text{ML ha}^{-1} \text{ y}^{-1}$).

Weather conditions and evapotranspiration (ET_0 based on Penman Monteith) were monitored using an Automatic Weather Station (MEA, Magill, SA, Australia), located within the trial. Soil moisture measurements were obtained using gypsum blocks situated in the loamy sand of the vineyard, adjacent to the trenches. Crop evapotranspiration, taking into account effective rainfall and crop coefficients (K_c) for grapevines, was calculated based on optimizing production according to Pudney *et al.* (2004) and FAO irrigation guidelines (Allen *et al.*, 1998). Each treatment was applied to five contiguous rows, with only the central row used for measurements.

Leaf physiology

Measurements of stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) of trenced vines, and sampling of leaf, root, and xylem saps was carried out at approximately weekly intervals between the onset of the irrigation treatments and harvest.

Measurements of g_s were taken of sun-exposed fully expanded leaves using an AP4 porometer (Delta-T Devices, Cambridge, U.K.) on the northern side of the rows. Fifteen leaves were measured per panel, three panels per treatment.

Measurements of Ψ_{leaf} were made using a 3000 series Plant Water Status Console (Soilmoisture Equipment, Santa Barbara, USA). Leaves were enclosed in a plastic bag before excision and the bagged leaves were pressurized within 20 seconds. Ψ_{leaf} was measured on five leaves per panel, three panels per treatment. After recording Ψ_{leaf} , an overpressure of 100 kPa was applied and exuded sap was collected, transferred to pre-weighed microcentrifuge tubes, and snap frozen over liquid nitrogen. Samples were stored at -40°C prior to analysis of ABA and metabolites.

ABA sampling and analysis

The five leaves per plot used for Ψ_{leaf} were pooled, snap frozen under liquid nitrogen and stored at -80°C for future analysis. Approximately 15 g root tissue was removed from the sand filled trenches, washed, dried with paper towels then snap frozen, and stored as for the leaves.

Tissue was ground to a powder under liquid nitrogen and 50–100 mg of frozen tissue was extracted overnight at 4°C in 500 μl 20% aqueous methanol. After centrifugation, a deuterated internal standard (400 μl , containing D3-7',7',7'-PA and -dihydrophaseic acid, D5-4,5,8',8'-ABA-GE and D6-3',5',5',7',7',7'-ABA, all at a concentration of 10 ng ml^{-1}) was added to the supernatants. Phenomenex SPE columns (60 mg ml^{-1} : 8B-S100-UAK) were equilibrated with 1 ml methanol and 1 ml nanopure water as per the manufacturer's directions. The samples were loaded onto the columns, washed with 20% aqueous methanol (1 ml), and eluted with 90% aqueous methanol (1 ml). An aliquot (50 μl) of the eluate was dried in a vacuum centrifuge in preparation for analysis.

Analysis of ABA abundance in xylem sap, root, and leaf tissues was undertaken by liquid chromatography/mass spectrometry (LC MS/MS). The dried leaf and root extracts were dissolved in 50 μl aqueous acetonitrile (10% with 0.05% acetic acid) and 20 μl was analysed by LC-MS/MS (Agilent 6410). Separations were carried out on a Phenomenex C18(2) 75 mm $\times$ 4.5 mm $\times$ 5 μm column at 40°C . Solvents were nanopure water and acetonitrile, both with 0.05% acetic acid. Samples were eluted with a linear 15 min gradient starting at 10% acetonitrile and ending at 90% acetonitrile. Compounds were identified by retention times and multiple reaction monitoring. Parent and product ions are shown in Supplementary Table S1 (available at JXB online). Xylem sap samples were thawed, dried in a vacuum centrifuge, and dissolved in 30 μl 10% acetonitrile containing 0.05% acetic acid and the internal standard mix as described above, before introduction into LC MS/MS.

Gene expression

For RNA extraction, leaf and root tissues, three sets of each per treatment, were ground under liquid nitrogen and aliquots pooled subsequent to grinding. For each analysis, 50–100 mg of pooled leaf or root tissue was taken for RNA extraction. Total RNA was extracted, purified, and treated with DNAase using a Spectrum Plant Total RNA kit (Sigma-Aldrich). Purified RNA was stored under ethanol at -40°C .

First-strand cDNA was synthesized from 1 μg aliquots of DNAase-treated total RNA from leaf and root tissues using the reverse transcription component of the Phusion RT-PCR kit (Finnzymes). Primer pairs for quantification of the various gene products by real-time PCR are shown in Supplementary Table S2. After being diluted 1:20 with H_2O , 5 μl template cDNA was placed

in each reaction in a final volume of 15 µl containing 266 nM of each primer and 1 × Absolute QPCR SYBR Green Buffer (ABgene, Surrey, UK). Thermocycling conditions were as follows: an initial enzyme activation at 95 °C of 15 min, followed by 30–35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, followed by a melt gradient starting at 50 °C and heating to 96 °C at a rate of 0.2 °C s⁻¹. The reactions were carried out in a Rotor-Gene 2000 Real Time PCR instrument (Corbett Research, NSW, Australia).

The specificity of the product generated from each set of primers was determined by: (i) the melt gradient in which fluorescence decreases at a single discrete temperature indicating separation of the two strands of a single DNA species; (ii) agarose gel electrophoresis showing a single PCR product of the correct size; and (iii) sequencing the product extracted from an agarose gel. All cDNA samples to be compared for gene expression levels were analysed in a single batch for each primer pair and each set of analyses were conducted in triplicate.

Comparative abundances of the mRNAs (cDNAs) encoding the six gene products of interest were calculated as follows. Abundances of the target cDNAs in each sample relative to the abundance of *Ubiquitin* cDNA, taken as a stable internal standard, were determined and means and standard deviations from three separate sets of determinations for each target cDNA were calculated. Abundances of the target cDNAs relative to a second stable internal standard *EF1α* cDNA were also calculated. These closely reflected the abundances relative to the *Ubiquitin* cDNA. To visualize the results, the maximum expression of *NCED1* was taken as unity and expression levels of the other genes were expressed relative to this value.

Isolation and characterization of *Vitis vinifera* ABA 8'-hydroxylase genes

Initial work on isolation and characterization of the hydroxylase genes was undertaken using cDNA prepared from Shiraz grapevine leaf RNA. Isolation of the RNA and synthesis of the cDNA were done as already described. Redundant primers for amplification of potential ABA 8'-hydroxylase fragments from grape were constructed on the basis of conserved amino acid sequences of a number of ABA 8'-hydroxylases obtained from the NCBI database: *Arabidopsis* 1, 2, 3, and 4 (AB122149, NM_128466, AB122150, NM_112814), potato A1 and A2 (DQ206630, DQ206631), barley A1 and A2 (DQ145931, DQ145932), and rice A5 (DQ887714). Using these primers, three fragments of grape cDNA of approximately 300 bp were obtained with close similarity to each other and to the specific conserved regions of the published sequences. The 3'-ends of the complete cDNAs were determined using 3'-RACE

(Frohman *et al.*, 1988) using non-redundant primers based on the derived sequences of the grape fragments and the B25 primer (Frohman *et al.*, 1988). The 5'-ends of the three genes were obtained by designing primers based on homologous sequences in grape genomic contigs: *Hyd1* (AM426652), *Hyd2* (FN595769.1), and *Hyd3* (AM437620). Sequences of the Shiraz genes have been submitted to GenBank (accession numbers JX181977–JX181979, respectively). Gene identities were confirmed by functional expression in yeast cells (Supplementary Fig. S1). For this study on Cabernet Sauvignon vines, it was confirmed that the relevant regions used for the design of real-time PCR primers were fully conserved.

Statistics

Statistical analysis of leaf, xylem sap, and root [ABA] and metabolites was performed using SPSS version 16.0. Analysis of weekly measurements were undertaken using a general linear model univariate analysis of variance with irrigation treatment as the fixed variable and the measurement of interest as the dependent variable, with the output grouped by date. Differences within treatments across the season were also similarly analysed. *P*-values < 0.05 were taken as statistically significant.

Results

Sampling was undertaken on a weekly basis over two growing seasons (2007–2008 and 2008–2009) commencing at fruit-set; however, since results from both seasons are similar, only those from 2008–2009 are presented except where stated.

The different irrigation strategies affected mean soil water potential in the top 40 cm of the soil in both seasons (Table 1), with T₃ being significantly lower than the other treatments, but not significantly different between T₁ and T₀.

VPD_{max} was higher for much of the 2008–2009 season than the 2007–2008 season, ranging 9.10–0.76 kPa and 5.4–0.67 kPa, respectively (Fig. 1). VPD_{min} was similar throughout both seasons, only being notably higher on the hottest days of the 2008–2009 season.

Despite there being no significant difference in soil water potentials between T₀ and T₁ irrigation treatments, all three treatments had significant effects on Ψ_{leaf} and g_s in both seasons (Table 1). Under the T₀ treatment Ψ_{leaf} was the highest,

Table 1. Ψ_{leaf} and g_s of vines and soil water potentials under three different irrigation treatments in seasons 2007–2008 and 2008–2009. Irrigation treatments were: T₀ = 1.27 ET_c, 6.6 MI ha⁻¹ y⁻¹; T₁ = 0.65 ET_c, 3.3 MI ha⁻¹ y⁻¹; T₃ = 0.25 ET_c, 1.3 MI ha⁻¹ y⁻¹. Values are means of weekly averages across each season. Superscript letters indicate significant differences (*P* < 0.05) between irrigation treatments within each season as determined by the Tukey–Cramer multiple comparison test.

		2007–2008 (<i>n</i> = 6)	2008–2009 (<i>n</i> = 13)
		8 Jan–19 Feb	20 Nov–14 Jan
Soil water potential in top 40 cm (kPa ± SE)	T ₀	−16.0 ± 2.6 ^a	−13.7 ± 1.0 ^a
	T ₁	−21.7 ± 4.3 ^{a,b}	−18.0 ± 1.7 ^{a,b}
	T ₃	−33.8 ± 5.1 ^c	−33.2 ± 2.8 ^c
Leaf water potential (Ψ _{leaf} , MPa ± SE)	T ₀	−1.02 ± 0.070 ^a	−0.80 ± 0.054 ^a
	T ₁	−1.27 ± 0.072 ^b	−1.14 ± 0.073 ^b
	T ₃	−1.50 ± 0.042 ^c	−1.28 ± 0.058 ^c
Stomatal conductance (g _s ; mmol m ⁻² s ⁻¹ ± SE)	T ₀	326.2 ± 43.2 ^a	456.6 ± 41.2 ^a
	T ₁	166.6 ± 47.3 ^b	244.8 ± 25.7 ^b
	T ₃	84.6 ± 23.5 ^c	105.3 ± 17.5 ^c

Fig. 1. Maximum (solid line) and minimum (dashed line) vapour pressure deficits (VPDs) experienced by the experimental vines across seasons 2007–2008 (A) and 2008–2009 (B). Open and closed circles indicate tissue harvest days, with closed circles indicating also days on which analysis of the harvested tissue included analysis of gene expression.

with Ψ_{leaf} under T_1 being intermediate and under T_3 the lowest. Stomatal conductances of the vines in each irrigation treatment also reflected the differences in irrigation strategies (Table 1), differing significantly from each other across both seasons. The Ψ_{leaf} and g_s parameters were strongly correlated in both seasons, fitting an exponential relationship $g_s = a^{b\Psi}$. The individual correlations were marginally different, but sufficiently similar to give an R^2 of 0.646 when the data sets were combined (Fig. 2A), suggesting consistent sampling and season-to-season response.

Looking specifically at the 2008–2009 season, foliar ABA concentrations of the T_0 vines did not change significantly across the season except for a brief rise on 21 January (Fig. 3A). However, there were significant increases in the T_1 and T_3 vines after 30 December, peaking in late January to early February, with no difference between the two treatments for most of the

season. Concentrations of ABA in the xylem sap (Fig. 3B) varied significantly in all three treatments during the season with a significant decline occurring between mid December and early January in each case followed by a large significant rise in only the T_1 and T_3 treatments on 17 February. Despite the similar seasonal variation in concentration of xylem sap [ABA] in the three treatments, the actual concentrations were significantly different throughout much of the season, with T_3 being higher than T_1 and with T_1 being higher than T_0 , except between the 20 November and 30 December, where there was no difference between T_1 and T_3 . Root [ABA] of all three treatments (Fig. 3C) also varied significantly across the season, with higher concentrations from mid January onwards and the T_0 roots typically having lower concentrations than the other two treatments. The ABA metabolites PA, dihydrophaseic acid, and ABA-GE were also determined (Supplementary Fig. S2).

Fig. 2. (A) Correlation between g_s and Ψ_{leaf} in vines under different irrigation regimes and over both seasons; curve equation is $y = 5669.5e^{3.0163x}$. (B) Correlation between g_s and xylem sap [ABA] in vines under different irrigation regimes and over both seasons; curve equation is: $y = 591.4e^{-0.007x}$. Symbols refer to irrigation conditions and seasons as shown in the key: T_0 , 1.27 ET_c, 6.6 ML ha⁻¹ y⁻¹; T_1 , 0.65 ET_c, 3.3 ML ha⁻¹ y⁻¹; T_3 , 0.25 ET_c, 1.3 ML ha⁻¹ y⁻¹.

Fig. 3. Abundances of ABA in leaf, root, and xylem sap during season 2008–2009. Abundances were determined by LC MS/MS. Symbols are as described in the legend to Fig. 2. Error bars indicate standard deviations from separate analyses ($n = 3$). Asterisks indicate significant differences between T₁ and T₀ and between T₃ and T₀ at each harvest point, calculated using two-tailed t-test.

Of particular interest is the almost complete absence of PA and ABA-GE in the root throughout the season.

[ABA] in xylem sap, leaf, and root were all correlated with g_s . When pooling data from both seasons, the strongest correlation with g_s was for [ABA]_{xyl} ($R^2 = 0.6365$, Fig. 2B), but correlations with root ($R^2 = 0.5110$) and leaf ($R^2 = 0.4222$) were also significant.

Expression of the genes associated with regulation of synthesis and degradation of ABA in leaf and root tissues were determined by real-time PCR and are presented for ease of comparison, relative to the maximum expression of *NCED1* (Fig. 4B). The expression of *NCED1* was low in leaves from all three treatments and relatively constant (Fig. 4A). It was

similarly low in roots from all three treatments prior to 14 January, after which it increased markedly in the T₃ vines, to an intermediate extent in the T₁ vines, and to a lesser extent in the T₀ vines (Fig. 4B). Expression of *NCED2* in the leaves was an order of magnitude lower in all three treatments and also relatively constant (Fig. 4C). Its expression in roots mirrored the expression of *NCED1* but, again, was an order of magnitude lower (Fig. 4D). Expression of the zeaxanthin epoxidase-encoding gene (*Zep*) was also determined in both tissues, being at least two orders of magnitude lower in the root than leaf (Supplementary Fig. S3).

To examine the breakdown of ABA in the leaf and root tissues, the expression of genes encoding the ABA 8'-hydroxylase enzymes, which catalyse the primary (regulatory) catabolic step, was measured. To facilitate this, the three *V. vinifera* genes encoding the 8'-hydroxylases, *VvABA8'OH-1* (*Hyd1*), *VvABA8'OH-2* (*Hyd2*), and *VvABA8'OH-3* (*Hyd3*) were isolated and their identities confirmed by expression in yeast cell culture (Supplementary Fig. S1). In leaf, *Hyd1* was the most highly expressed of the genes, but was barely detectable in root (Fig. 5A and B). Expression of *Hyd2* in both leaf and root was an order of magnitude lower than *Hyd1* in leaf (Fig. 4C and D). The only grape tissue in which significant levels of mRNA from the *Hyd3* gene were detected was dry grape seed (data not shown).

In the 2008–2009 season, two peaks of expression of *Hyd1* were evident in the leaf tissues of all three treatments (Fig. 5A). Both peaks occurred in tissues which were harvested immediately following sudden drops in VPD (overlay in Fig. 5A). A similar peak in expression of *Hyd1* in leaf tissue in the 2007–2008 season (Fig. 5E) also coincided with a sudden drop in VPD (overlay in Fig. 5E), suggesting a causal relationship. Expression of *Hyd1* in root and *Hyd2* in both leaf and root (Fig. 5C and D) was low relative to that of *Hyd1* in the leaf and the fluctuations in expression did not appear to be related to those of *Hyd1* in leaf.

Using data from both seasons, the expression of *Hyd1* in leaves was compared with VPD levels either at harvest (Fig. 6) or averaged over the 1- or 2-h period prior to leaf harvest (data not shown). All three comparisons showed the bulk of *Hyd1* expression occurring at VPD levels at or below 2.5 kPa.

Discussion

In both seasons, the different irrigation strategies resulted in different soil water potentials, with decreased irrigation lowering both g_s and Ψ_{leaf} as is typical of water stress. Correlations between g_s and pre-dawn Ψ_{leaf} have been described for other grapevine cultivars (Schultz, 2003; Rodrigues *et al.*, 2008; Beis and Patakas, 2010) whereas here, g_s was correlated with mid-morning Ψ_{leaf} (Fig. 2A). This indicates that under the semi-stable conditions of this long-term irrigation trial, a relationship between the two factors was maintained well into the day, a conclusion supported by a similar correlation between data collected from grapevine at midday (Romero *et al.*, 2012).

Arguments for and against the role of ABA as a root-to-shoot chemical signal have previously been discussed

Fig. 4. *NCED1* and *NCED2* expression in leaves (A and C) and roots (B and D), respectively, as determined by real-time PCR. Results are normalized against the housekeeping gene ubiquitin and are expressed relative to the maximum expression of *NCED1* (Fig. 4B). Symbols are as described in the legend to Fig. 2. Error bars indicate standard deviations from separate PCR runs ($n = 3$).

(Introduction and Dodd, 2005; Li *et al.*, 2011) and it is now commonly accepted that a combination of chemical and hydraulic messages are associated with stomatal regulation. The correlations between $[ABA]$ and g_s (Fig. 2B) strongly support the involvement of ABA in stomatal regulation under field conditions and feeding ABA (at concentrations similar to those observed in this study) to detached vine leaves via the transpiration stream suggest that $[ABA]_{xyl}$ is effective in eliciting stomatal closure (Loveys, 1984).

In a number of plants under differing growth and stress conditions, relationships occurred between g_s and leaf $[ABA]$ (Zhang and Outlaw, 2001a; Beis and Patakas, 2010), g_s and leaf apoplast $[ABA]$ (Zhang and Outlaw, 2001a), g_s and guard cell $[ABA]$ (Zhang and Outlaw, 2001b), and between g_s and xylem sap $[ABA]$ (Wartinger *et al.*, 1990; Tardieu *et al.*, 1992, 1996). Although in the current study g_s was most highly correlated with xylem sap $[ABA]$ (Fig. 2B), relationships also existed between g_s and $[ABA]$ in both root and leaf, so no clear conclusions could be drawn regarding which was primarily associated with changes in stomatal aperture. Because ABA is mobile in plant tissues, all these sites are interrelated and the actual correlation may also be influenced by the catabolic activity at that site. Notably, all the data points from the vines with the highest level of irrigation (T_0) are clustered below xylem sap levels of 200 ng ml^{-1} and with relatively high g_s (Fig. 2B), suggesting a critical level of ABA in the xylem sap of around 200 ng ml^{-1} below which the stomata remain

open. Under the high levels of irrigation, this threshold was never exceeded.

Expression of the two main genes associated with ABA synthesis, *NCED1* and *NCED2*, was most pronounced in the roots (Fig. 4) and was inversely related to the amounts of irrigation being applied. Accumulation of ABA in the roots of the T_1 and T_3 vines increased in mid-January at a time when root *NCED* expression was peaking, but never attained the levels observed in the leaves (Fig. 3). In contrast, foliar ABA accumulation occurred in the absence of increased leaf *NCED* expression, but at a time when the root genes were highly active. At the same time, ABA abundance in the xylem sap increased in T_1 and T_3 vines consistent with the transport of ABA from root source to leaf sink. Alternatively, the rapid metabolism of the root-synthesized ABA and a slow accumulation of leaf-synthesized ABA seems unlikely as it would not explain the changes in $[ABA]_{xyl}$ and there is little evidence for increased levels of ABA metabolites in the roots during the period of high *NCED* expression (Supplementary Fig. S2). Increased $[ABA]_{xyl}$ in an acropetal direction (Soar *et al.*, 2004; Li *et al.*, 2011) could indicate a contribution of leaf-synthesized ABA directly into the xylem. However this could also result from a pH gradient along the stem influencing ABA availability (Li *et al.*, 2011), and a close correlation between $[ABA]_{xyl}$ sampled from the leaf and from the root (Dodd *et al.*, 2008) together with the enhanced gene expression in the root tissues supports the primary contribution originating from the root system.

Fig. 5. (A–D) *Hyd1* and *Hyd2* expression in leaves (A and C) and roots (B and D), respectively, as determined by real-time PCR. Results are normalized against the housekeeping gene ubiquitin and are expressed relative to the maximum expression of *NCED1* (Fig. 4B). (E) *Hyd1* expression in leaves in the 2007–2008 season. In A and E, VPD conditions have been indicated by grey lines with sampling dates shown by circles also in grey. Otherwise, symbols are as described in the legend to Fig. 2. Error bars indicate standard deviations from separate PCR runs ($n = 3$).

It seems likely that activation of the root *NCED* genes would be in response to changes in root water status. Under the different irrigation regimes, this would have been influenced by the soil water potential, but also by atmospheric VPD, as the likely increase in xylem flux would tend to lower root water potential relative to that of the soil. The root *NCED* expression reported here should reflect both of these variables but with a constant background level derived from the soil water potentials and hence the irrigation strategies. Jensen *et al.* (1998) identified a critical soil water potential of about -13.0 kPa, below which leaf ABA concentration increased and g_s decreased in field-grown lupins, while Zhang and Davies (1989) found that a critical soil water potential of between -20.0 and -30.0 kPa stimulated ABA accumulation in maize

roots. Critical soil water potentials were not determined in the current trial; however low levels of *NCED* expression were maintained throughout the season in the well-watered T₀ treatment, indicating a degree of induction, while in the T₁ and T₃ treatments, expression of the root *NCED* genes increased during late January when soil water potentials fell on occasions below -30 kPa (T₁) and -42 kPa (T₃). It appears therefore that root *NCED* expression and $[ABA]_{xyl}$ were highly responsive to changes in soil water potential which, in the case of $[ABA]_{xyl}$, was particularly the case in sandy soils (Dodd *et al.*, 2010) such as those in this trial.

While expression of two of the three grape genes encoding ABA 8'-hydroxylase was detectable in both leaf and root tissues throughout the periods examined, expression of *Hyd1*

Fig. 6. Expression of the *Hyd1* gene in leaf plotted against the vapour pressure deficit at the time of harvest in seasons 2007–2008 and 2008–2009. Similar plots were obtained using vapour pressure deficits averaged across 1 and 2 h prior to harvest. Symbols are as described in the legend to Fig. 2.

in the leaf tissue was most prominent. The peaks of *Hyd1* expression in leaves in all three irrigation treatments in 2008–2009 and the two drier treatments in 2007–2008 appeared to coincide with rapid drops in VPD immediately prior to leaf sampling. The absence of enhanced expression of the gene in the T₀ treatment in 2007–2008 may have been related to the lower abundance of ABA in the leaves and xylem sap of those vines (data not shown).

VPD is inversely related to the relative humidity of the ambient air. Comparing *Hyd1* expression in leaves of all three irrigation treatments over both seasons (Fig. 6) indicates that expression was low above a VPD of about 2.5 kPa but increased below that threshold. The scatter of expression levels also increased at a lower VPD, suggesting the influence of an additional factor, perhaps ABA concentration itself. Taken together these observations suggest that *Hyd1* in leaves responds to decreases in VPD which can relate to increases in the amount of moisture in the air. In *Arabidopsis*, two members of the *Arabidopsis* gene family encoding ABA 8'-hydroxylases (*CYP707A1* and *CYP707A3*) are rapidly induced in response to high humidity (Okamoto *et al.*, 2009). The resulting reduction (assumed) of ABA abundance in or near the guard cells, would open the stomata and consequently increase g_s . Such a response would allow greater carbon uptake with a minimal increase in transpiration as the evaporative demand of the air would be low on days of high humidity. However, it was not possible to determine either a decline in leaf [ABA] or an increase in g_s in the experiments reported here for two reasons. Firstly, grapevine contains 30–40-fold more ABA per g of leaf than *Arabidopsis*, much of it probably compartmentalized either in specific cell types such as the epidermal cells (Daeter and Hartung, 1995) or in organelles within cells (Cowan *et al.*, 1982). It would be difficult to observe subtle changes in guard cell or vascular ABA abundances against such a high background. Secondly, the weekly intervals between sampling likely precluded the

detection of subtle changes in ABA abundance. Much more frequent (hourly) sampling of tissue across an appropriate period of decreasing VPD would be required before an attempt could be made to correlate tissue hydroxylase activity with changes in ABA concentrations and g_s . Nevertheless, the results presented here suggest a role for regulation of ABA catabolism in fine-tuning stomatal response to environmental change.

In conclusion, these results provide strong evidence that ABA synthesized in the roots plays a key role in linking stomatal response to soil moisture status and that changes in ABA catabolism at or near its site of action allows optimization of gas exchange according to current environmental conditions. Future work is planned to examine possible linkages between grapevine leaf *Hyd* expression and rapid adaptation to environmental conditions in more detail.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Analytes, retention times and *m/z* values for parent and product ions of native and deuterated internal standards.

Supplementary Table S2. Oligonucleotide primer pairs used for real-time real-time PCR amplification.

Supplementary Fig. S1. Percentage conversion of ABA to PA in cultures of yeast cells transformed with inducible plasmids containing full-length cDNAs of the three grape ABA 8'-hydroxylases.

Supplementary Fig. S2. Abundances of ABA metabolites in leaf, root, and xylem sap during season 2008–2009.

Supplementary Fig. S3. Expression of the gene encoding zeaxanthin epoxidase in leaf and root tissues during season 2008–2009.

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