Abscisic Acid Down-Regulates Hydraulic Conductance of Grapevine Leaves in Isohydric Genotypes Only1[OPEN]

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Plants evolved different strategies to cope with water stress. While isohydric species maintain their midday leaf water potential ($\Psi_M$) under soil water deficit by closing their stomata, anisohydric species maintain higher stomatal aperture and exhibit substantial reductions in $\Psi_M$. It was hypothesized that isohydry is related to a locally higher sensitivity of stomata to the drought-hormone abscisic acid (ABA). Interestingly, recent lines of evidence in Arabidopsis (Arabidopsis thaliana) suggested that stomatal responsiveness is also controlled by an ABA action on leaf water supply upstream from stomata. Here, we tested the possibility in grapevine (Vitis vinifera) that different genotypes ranging from near isohydric to more anisohydric may have different sensitivities in these ABA responses. Measurements on whole plants in drought conditions were combined with assays on detached leaves fed with ABA. Two different methods consistently showed that leaf hydraulic conductance ($K_{leaf}$) was downregulated by exogenous ABA, with strong variations depending on the genotype. Importantly, variation between isohydry and anisohydry correlated with $K_{leaf}$ sensitivity to ABA, with $K_{leaf}$ in the most anisohydric genotypes being unresponsive to the hormone. We propose that the observed response of $K_{leaf}$ to ABA may be part of the overall ABA regulation of leaf water status.

Maintaining adequate tissue water content through efficient controls of water supplies and losses is a key requirement for crop performance and plant survival in dry environments. Accordingly, plants evolved with varied capacities to close stomata in response to soil drying, thereby limiting the drop of water potential along the transpiration path yet at the expense of carbon assimilation and growth. Optimization between carbon gain and water loss has resulted in the evolution of a continuum of strategies among species, ranging from isohydry to anisohydry. Anisohydric species exhibit a substantial decrease in their midday leaf water potential ($\Psi_M$) as soil water deficit develops, while isohydric species maintain higher $\Psi_M$ through stomatal closure at incipient stages of soil drying (Tardieu et al., 1996). A wide spectrum of behaviors has also been observed between varieties of the same species, as in apple tree (Malus; Massonnet et al., 2007) and grapevine (Vitis vinifera; Schultz, 2003; Soar et al., 2006; Prieto et al., 2010). In grapevine, genomic regions (QTLs) have been identified that control the maintenance of $\Psi_M$ under moderate water deficit (Coupel-Ledru et al., 2014). Although dependent on environmental conditions (Franks et al., 2007), variation from iso- to anisohydry has therefore a clear genetic basis.

How stomata coordinate with plant hydraulics to optimize $\Psi_M$ in response to drought and how this may vary between species remain a matter of debate. Yet, it has been frequently reported that stomatal response parallels the dynamics of hydraulic conductance in roots (Zulferey and Smart, 2012; Vandeleur et al., 2014), leaves (Cochard et al., 2002), or whole plants (Meinzer, 2002; Zulferey and Smart, 2012). This observation has been mostly interpreted as the result of a biological coupling between water supply (hydraulic conductance) and water demand (transpiration), preventing water potential along the transpiration path from dropping to damaging levels (Cochard et al., 1996; Sack and Holbrook, 2006; Franks et al., 2007; Simonin et al., 2015). On the one hand, water deficit may impact on water supply via either the development of cavitation in xylem conduits (Tyree and Sperry, 1989), thereby reducing hydraulic conductance from the inner root tissues to the leaf petiole, or down-regulation of...
aquaporin activity, which controls water transfer through membranes of living cells in both roots and leaves (Chaumont and Tyerman, 2014). On the other hand, stomata primarily control the response of transpiration to water deficit. The physiological mechanisms underlying stomatal response most likely involve both hydraulics and biochemistry with the accumulation of the drought hormone abscisic acid (ABA). Water deficit draws down water potential in all plant tissues and may directly impair turgor pressure in the guard cells surrounding the stomatal pores, thus reducing stomatal aperture (Buckley, 2005; Peak and Mott, 2011). In parallel, accumulation of ABA, whether synthesized in roots (Simonneau et al., 1998; Borel et al., 2001) or leaves (Christmann et al., 2005, 2007; Ikegami et al., 2009; McAdam et al., 2016), directly impacts on guard cells to close stomata (Kim et al., 2010; Joshi-Saha et al., 2011).

The relative contribution of ABA signaling and hydraulics to drought-induced stomatal closure varies depending on species (Tardieu et al., 1996; Brodribb and Jordan, 2011; Brodribb and McAdam, 2013; McAdam and Brodribb, 2013, 2014; Brodribb et al., 2014). Interestingly, leaf water potential appears to sensitize guard cells to the effect of ABA, thus resulting in a feedforward effect on stomatal closure upon water stress (Tardieu and Davies, 1992). Moreover, this feedforward effect is only observed in isohydic species (Tardieu and Simonneau, 1998). Although such apparent interaction between hydraulics and ABA accounts for the distinction between isohydic and anisohydic behaviors, the biological basis for this observation remains unknown.

Recent studies on the isohydic species Arabidopsis (Arabidopsis thaliana) challenged the classical view that ABA induces stomatal closure by solely acting at the guard cell level. First, ABA reduces leaf hydraulic conductance ($K_{leaf}$) through the down-regulation of aquaporin activity in the bundle sheath around leaf veins (Shatil-Cohen et al., 2011). Second, xylem-fed ABA induces parallel reductions in $K_{leaf}$ and stomatal conductance in leaves of mutants that are insensitive to ABA at the guard cell level (Pantin et al., 2013). These results gave rise to the proposal that ABA promotes stomatal closure in a dual way, via its local, biochemical effect on the guard cells, but also via a remote, hydraulic impact of a drop in water permeability within the bundle sheath. Bundle sheath cells were thus assigned a role of “control center” for water flow, able to convert ABA signaling into feedforward hydraulic signals up to guard cells. We surmise that such hydraulic effect of ABA may underlie the apparent interaction between hydraulics and ABA on stomatal control of isohydric species (Tardieu and Simonneau, 1998) and thus originate the genetic differences between isohydric and anisohydric behaviors.

Here, we tested this hypothesis by examining the relationship between $K_{leaf}$ sensitivity to ABA and (an)isohydric behavior of grapevine genotypes obtained from a cross between two contrasting cultivars, the near-anisohydric Syrah and the near-isohydric Grenache (Schultz, 2003; Soar et al., 2006; Prieto et al., 2010). A wide range of variation for (an)isohydry was evidenced within the offspring, ranking far beyond the parental behaviors (Coupel-Ledru et al., 2014). We selected a panel of contrasting genotypes and combined experiments on whole plants under two watering regimes with measurements on detached leaves fed with various ABA concentrations. The inhibiting effect of ABA on $K_{leaf}$ was observed only in those genotypes that showed strong stomatal closure and typical isohydric behavior upon water deficit. By contrast, $K_{leaf}$ of more anisohydric genotypes was insensitive to ABA. These results support a major role for genetic variation in $K_{leaf}$ sensitivity to ABA in determining (an)isohydric behavior in grapevine.

RESULTS

Two Independent Methods Reveal Differential Sensitivity of $K_{leaf}$ to ABA between Syrah and Grenache

To investigate the putative link between ABA, leaf hydraulics, and plant (an)isohydric behavior, we assessed the effect of ABA on $K_{leaf}$ on two grapevine cultivars reputed to be isohydric (Grenache) and anisohydric (Syrah). $K_{leaf}$ response to ABA was first characterized using the Evaporative Flux Method (EFM) on detached leaves that were xylem-fed for 1 h with a control solution or with a solution of exogenous ABA at a concentration of 2, 4, 8, 16, or 32 mmol m$^{-2}$s$^{-1}$. $K_{leaf}$ displayed a strong sensitivity to ABA in Grenache, declining from 12 mmol m$^{-2}$s$^{-1}$MPa$^{-1}$ in the control solution to 5 mmol m$^{-2}$s$^{-1}$MPa$^{-1}$ in the 32 mmol m$^{-3}$ ABA solution (Fig. 1A). By contrast, $K_{leaf}$ of Syrah was much less sensitive to ABA (Fig. 1B), with a slight decrease that was not found significant ($P > 0.1$).

To consolidate these results, we measured $K_{leaf}$ sensitivity to ABA with a second, independent method. We used the High Pressure Flowmeter (HPFM) to measure $K_{leaf}$ in detached leaves of Grenache and Syrah fed for 1 h with control or ABA solutions (Fig. 1, C and D). Overall, whatever the method used, $K_{leaf}$ was strongly and significantly reduced by ABA feeding in the near-isohydric cultivar Grenache ($P < 0.01$) but not in the near-anisohydric Syrah. $K_{leaf}$ values obtained in control conditions with the HPFM were highly consistent with those previously reported by Pou et al. (2013), who operated similarly. However, $K_{leaf}$ values were 3-fold higher when measured with the HPFM as compared to the EFM, which suggests that $K_{leaf}$ might be overestimated by the HPFM because of the higher hydrostatic pressure imposed to water within the leaf (Prado and Maurel, 2013) while negative pressures develops in transpiring leaves. For this reason, the EFM most likely mimicked the natural pathway of water in leaves through the transpiration flow (Sack and Scoffoni, 2012), even though the flow rate was of the same order of magnitude whatever the method used (between 0.5 and 3 mmol m$^{-2}$s$^{-1}$; see also Supplemental Fig. S2).
The HPFM method also made it possible to distinguish the conductance between petiole and lamina. For that purpose, just after $K_{\text{leaf}}$ measurement with the HPFM, the leaf was cut at the junction between petiole and lamina, and hydraulic conductance of the petiole ($K_{\text{petiole}}$) was determined. The hydraulic conductance of the lamina ($K_{\text{lamina}}$) could then be derived considering that water pathways in petiole and lamina operate in series. Irrespective of the cultivar, the conductance in the petiole was much stronger (about 10-fold higher) than in the lamina when calculated at the whole organ level, indicating that most of the resistance to water.
transfer takes place in the lamina. No effect of ABA was observed for Syrah on either part of the leaf (Fig. 1, F and H) as could be expected from the absence of effect on overall K_{leaf} (Fig. 1D). By contrast, in the nearly isohydric Grenache, ABA feeding markedly reduced K_{lamina} (Fig. 1G; P < 0.01) while K_{petiole} was not significantly affected (Fig. 1E).

Measurements at the leaf and lamina levels also revealed a significant difference in K values recorded before ABA perfusion: Grenache displayed higher initial K_{leaf} (and K_{lamina}) than Syrah (Fig. 1). K_{leaf} at maximum ABA concentration in Grenache reached about the same value as the initial K_{leaf} in Syrah.

Stomatal responses to ABA (measured by porometry) were much closer to each other between Syrah and Grenache (Supplemental Fig. S1) than differences in K_{leaf} response to ABA that were detected using the HPFM method.

Variability in (An)isohydry and ABA Accumulation for Ten Selected Offspring Genotypes and the Parental Cultivars

Analysis was then extended to a panel of genotypes with contrasting (an)isohydric behaviors. Ten offspring genotypes were selected in the Syrah × Grenache population based on the change in Ψ_M previously observed between well-watered (WW) and water-deficit (WD) conditions (Coupel-Ledru et al., 2014). Ψ_M measured in the selected genotypes under WD and controlled atmospheric conditions ranged from −1.1 to −0.85 MPa (Fig. 2A). The drop in leaf Ψ_M (ΔΨ_M) between WW and WD regimes displayed a highly significant effect of the genotype (P < 0.001), ranging from −0.5 MPa for the most anisohydric genotype to −0.1 MPa for the most isohydric one (Fig. 2B). Change in Ψ_M induced by drought in the parents was intermediate, with a slightly better maintenance in Grenache (ΔΨ_M of −0.15 MPa) compared to the more anisohydric Syrah (ΔΨ_M of −0.25 MPa).

We also assessed the variability in ABA accumulation in response to soil drying. For this purpose, ABA was assayed in xylem sap that was collected on leaves of 2 intact plants per genotype (10 offspring and 2 parental) under soil water deficit and standardized transpiring conditions. Genotype had a significant effect on ABA concentration in the xylem sap of WD plants (P < 0.01). Across genotypes, ABA concentration in the xylem sap ranged from 0.5 to 2.8 mmol m⁻³ (Fig. 3A). Syrah displayed much lower ABA concentration in the xylem sap (about 0.8 mmol m⁻³) than Grenache (1.9 mmol m⁻³). ABA concentration in the xylem sap did not match with the ranking of genotypes according to (an)isohydry level and did not correlate with ΔΨ_M (Fig. 3B). This rules out a simple role of genetic variation of ABA accumulation induced by water deficit in the determinism of (an)isohydry. We also examined whether higher ABA content could be responsible for reduced K_{leaf}, even under well-watered conditions, specifically in those genotypes where K_{leaf} did not respond to further treatment with exogenous ABA. ABA concentration was therefore determined in xylem sap extruded from leaves of Syrah and Grenache grown under well-watered conditions. Average ABA content was lower in Syrah (0.5 ± 0.2 mmol m⁻³, n = 4) than in Grenache (1.1 ± 0.5 mmol m⁻³, n = 4). This result rules out a possible role of high initial ABA levels in leaves on the absence of K_{leaf} response for genotypes like Syrah.

Genetic Variation in K_{leaf} Sensitivity to ABA Correlates with the Degree of Isohydry

K_{leaf} response to xylem-fed exogenous ABA was characterized on detached leaves of the 10 offspring genotypes and the parents using the EFM. In the absence of ABA, genetic variability was observed for K_{leaf} (P < 0.001), ranging from 2.5 to 13.5 mmol m⁻³ (Fig. 4). Feeding with ABA solutions at various concentrations had contrasting effects depending on the genotype (Fig. 4). Grenache and Syrah responses were consistent with those reported in the previous experiment (Fig. 1). Most of the change in K_{leaf} was observed for xylem ABA varying between 0 and 5 mmol m⁻³, corresponding to actual concentrations reported in the xylem sap of grapevines under various soil water conditions (e.g. Rogiers et al., 2012). Five genotypes exhibited a Grenachelike response to ABA, with a strong, significant (P < 0.001) reduction of K_{leaf} when ABA concentration was increased (Fig. 4, A–E). By contrast, the seven other genotypes showed a Syrah-like response, with nonsignificant effect of ABA on K_{leaf} (Fig. 4, F–L).

Linear models were then fitted to semilogarithmic transformed data (Supplemental Fig. S3), and K_{leaf} sensitivity to ABA was calculated as the slope of this regression, giving the expected change in K_{leaf} for any e-fold increase in ABA concentration. The more negative the slope, the more sensitive K_{leaf} was to ABA.

Figure 2. Characterization of (an)isohydric behaviors for a selection of 10 offspring genotypes as compared to the whole progeny and the parents Syrah and Grenache. Distribution of the genotypic means recorded in the whole Syrah × Grenache population for: (A) leaf water potential measured in the daytime under WD conditions (Ψ_M,exp), and (B) reduction in leaf water potential in the daytime under WD as compared to WW conditions (ΔΨ_M), as an indicator of the (an)isohydric behavior. A and B. Data are genotypic means calculated for the experiment conducted in 2012 presented in Coupel-Ledru et al. (2014) for n = 188 genotypes. Values for the panel of 10 offspring genotypes and for the parental genotypes Syrah and Grenache (notified as ‘Syr’ and ‘Gren’ respectively) are indicated with black arrows.
Sensitivity was thus calculated as the opposite of the slope. Although confidence intervals on slopes were quite large (Fig. 5B), analysis of covariance revealed a significant effect of the genotype on $K_{leaf}$ sensitivity to ABA ($P < 0.01$). Moreover, $K_{leaf}$ sensitivity to ABA strongly correlated with the initial level of $K_{leaf}$ before ABA inhibition ($K_{leaf\,max}$; Fig. 5C).

We next tested the relationship between $K_{leaf}$ sensitivity to ABA and (an)isohydry by examining the correlation with $\Delta \Psi_M$ for all selected genotypes. The correlation was highly significant and positive (Fig. 5D). This confirmed that the genotypes with the most sensitive $K_{leaf}$ to ABA had a better capacity to maintain $\Psi_M$ at high level under soil water deficit. By contrast, the genotypes with $K_{leaf}$ being hardly responsive to ABA exhibited a substantial drop in $\Psi_M$ under dry soil conditions.

It is somewhat counter-intuitive that a stronger reduction in $K_{leaf}$ may result in a better maintenance of leaf water potential upon water deficit. A drop in $K_{leaf}$ is rather expected to lower leaf water potential, provided leaf transpiration rate ($E_{leaf}$) is constant. However, ABA feeding also induced a strong reduction in $E_{leaf}$ in detached leaves (Supplemental Figs. S4–S6). This result supports the assumption that a drop in $K_{leaf}$ upon ABA increase could be responsible for a substantial stomatal closure, which could dominate on moderating the drop in leaf water potential (Supplemental Fig. S7).

**Predicting Genotypic Response to Water Deficit from ABA Accumulation and Sensitivity to ABA**

We then examined the hypothesis that genetic variation in $K_{leaf}$ sensitivity to ABA correlated with the reduction of $E_{leaf}$ in intact plants submitted to water deficit. For this purpose, we combined the sensitivities of $K_{leaf}$ and $E_{leaf}$ to ABA, as estimated in detached leaves of each genotype (Fig. 5B; Supplemental Figs. S3, S5, and S6), with the native ABA concentration that was measured in the xylem sap (Fig. 3A) of plants under soil water deficit. In addition, $E_{leaf}$ and $K_{leaf}$ of well-watered plants were estimated as the maximum values observed in detached leaves fed with ABA-free solution assuming that they were representative of leaves attached on well-watered plants. Changes in $E_{leaf}$ and $K_{leaf}$ induced by water deficit were then related to these maximum values determined for each genotype, yielding percent reduction in $K_{leaf}$ (% reduction $K_{leaf}$) and in $E_{leaf}$ (% reduction $E_{leaf}$).

The correlation between % reduction $E_{leaf}$ and % reduction $K_{leaf}$ could then be examined (Fig. 6A). Overall, $E_{leaf}$ predicted from ABA concentrations showed higher sensitivity to water deficit than $K_{leaf}$ yet the predicted changes in $E_{leaf}$ (% reduction $E_{leaf}$) covered a smaller range of genetic variation within the panel of genotypes (between 38% for the less responsive genotype and 50%; Fig. 6A) compared to the genetic range observed for the % reduction $K_{leaf}$ (between 0 and 38%; Fig. 6A). Despite this difference, % reduction $E_{leaf}$ positively correlated with % reduction $K_{leaf}$ (Fig. 6A). Similar results were obtained when plotting % reduction $K_{leaf}$ against relative changes in transpiration rate directly measured on the whole plants (Fig. 6B) during the high-throughput experiment (% reduction $E_{Plant}$). This suggested that the more $K_{leaf}$ was reduced by ABA accumulation under water deficit, the more $E_{leaf}$ was reduced. In agreement with our initial assumption, a better maintenance of plant water potential could be expected for those genotypes with $K_{leaf}$ and thus $E_{leaf}$ more sensitive to ABA.

**DISCUSSION**

This study demonstrates that ABA may down-regulate $K_{leaf}$ in grapevine with a variable effect depending on the
genotype. Previous works reported a role for ABA on plant hydraulics in Arabidopsis by means of mutants (Shatil-Cohen et al., 2011; Pantin et al., 2013). Here, we used two grapevine cultivars contrasting for their water use strategies in drought conditions (i.e. Syrah and Grenache), plus 10 offspring from a population obtained from their cross (Coupel-Ledru et al., 2014). Natural variations for K\(_{\text{leaf}}\) sensitivity to ABA could thus be detected within a species largely cultivated in drought-prone areas (Schultz, 2000; Jones et al., 2005). We observed that genetic variation in the sensitivity of K\(_{\text{leaf}}\) to ABA correlated with variation in (an)isohydric behavior in grapevine. We propose that the dual effect of ABA on stomata, via its direct biochemical effect on guard cells and the indirect consequence of K\(_{\text{leaf}}\) down-regulation on guard cell turgor, may underlie part of this genetic variation.

Differential K\(_{\text{leaf}}\) Sensitivity to ABA: A Physiological Process Involved in the Variability of Plant Response to Drought

In our study, genetic variation was found for ABA-induced responses to drought at two levels: (1) ABA accumulation in the xylem sap of intact plants exposed to drought, and (2) leaf sensitivity to the hormone by using detached leaves. On the one hand, ABA accumulation in the xylem sap of intact grapevine plants was highly dependent on the genotype, suggesting that ABA biosynthesis capacity or catabolism varied across genotypes. These variations may be due to differential expression of genes associated with ABA synthesis, such as \(\text{NCED1}\) and \(\text{NCED2}\), or genes coding for enzymes involved in ABA degradation to inactive compounds, including the ABA 8\(^{-}\)-hydroxylases (Riahi et al., 2013; Speirs et al., 2013). On the other hand, exogenous ABA application to detached leaves using a high concentration \((32 \text{ mmol m}^{-2} (+)-\text{ABA})\) reduced K\(_{\text{leaf}}\) by up to 50% for the most sensitive grapevine genotype (Fig. 4). A plateau was observed at the highest concentrations, indicating that maximal effect of the hormone on K\(_{\text{leaf}}\) was reached at an ABA concentration far above the physiological range observed even under severe drought (Rogiers et al., 2012). Most importantly, a strong variability in K\(_{\text{leaf}}\) sensitivity to ABA was observed between genotypes. Combining these values of K\(_{\text{leaf}}\) sensitivity to ABA as observed on detached leaves with native ABA
concentration made it possible to predict changes in $K_{\text{leaf}}$ ($\%$ reduction $K_{\text{leaf}}$) induced for each genotype by the water deficit scenario. Overall, genetic differences in $K_{\text{leaf}}$ response to drought ($\%$ reduction $K_{\text{leaf}}$) were much more influenced by differences in sensitivity of $K_{\text{leaf}}$ to ABA than in ABA accumulation. A wide range of genetic variation was obtained for a drought-induced drop in $K_{\text{leaf}}$, going from no reduction in the less sensitive genotypes to a decline by up to 38% for the most responsive ones under the moderate water deficit (Fig. 6). The initial, endogenous ABA content (prior to exogenous ABA feeding) was lower in Syrah than in Grenache ($0.5 \pm 0.2$ mmol m$^{-2}$ s$^{-1}$ MPa$^{-1}$ and $1.1 \pm 0.5$ mmol m$^{-3}$, respectively), while Syrah was less sensitive to addition of exogenous ABA than Grenache. This rules out a possible role of initial ABA concentration as responsible for a basal down-regulation of $K_{\text{leaf}}$ in the less sensitive genotype Syrah. The range of $K_{\text{leaf}}$ sensitivities observed within the panel of genotypes significantly correlated with their capacities to maintain leaf water potential in conditions of water deficit (Fig. 5D). The more sensitive $K_{\text{leaf}}$ was to ABA, the better water potential was maintained in leaves under water deficit conditions, that is, the more isohydric was the genotype. By contrast, in more anisohydric genotypes $K_{\text{leaf}}$ was hardly sensitive to ABA. Although variation from iso- to aniso-hydry has been shown to depend on environmental conditions, making their genetic origin debated (e.g. Franks et al., 2007), the genetic contrast between Syrah and Grenache was consistently reinforced across three independent experiments in our study (on whole potted plants in greenhouse, on leaves detached from plants cultivated in the vineyard, and leaves detached from potted plants cultivated outdoors).

Assuming that the ABA-induced reduction in $K_{\text{leaf}}$ was stronger in isohydric than anisohydric genotypes, it was possible to generate the different behaviors in silico by amending a simple model of leaf hydraulics with the observed effect of ABA on $K_{\text{leaf}}$ (see Methods and Supplemental Methods S8–10). The model predicted that the water potential of guard cells decreased more rapidly when $K_{\text{leaf}}$ was more responsive to ABA, leading to stomatal closure and maintenance of bulk leaf water potential. Figure 7 presents the results of two simulations during progressive soil drying, where...
hypothesized, isohydic, and anisohydric genotypes were built with all parameters maintained identical apart from $K_{\text{leaf}}$ sensitivity to ABA, based on observed values within the whole set of 12 genotypes. The simulations were run with the most extreme values combining the highest sensitivity observed and the widest range of variation for $K_{\text{leaf}}$ between min and max values. They confirm that differential down-regulation of $K_{\text{leaf}}$ by ABA can account for part of the contrasted (an)isohydic behaviors, where genotypes with the most responsive $K_{\text{leaf}}$ to ABA better control their transpiration rate, thus reducing the drop in leaf water potential under drought corresponding to an isohydic behavior.

Stronger down-regulation of $K_{\text{leaf}}$ by ABA occurred in those cultivars with higher $K_{\text{leaf,max}}$ (Figure 5C). Genetic variation in $K_{\text{leaf,max}}$ may arise from difference in intrinsic activity of aquaporins among genotypes or from variation in the vascular relative to the transmembrane, extra vascular water pathways. Multiple leaf and vein traits can influence $K_{\text{leaf}}$ (Sack and Scoffoni, 2013). As an example, larger conduit lumens might provide greater xylem conductivity and $K_{\text{leaf}}$, while the whole vein diameters also promote differences in transport capacity when they contain greater sizes and numbers of xylem vessels (Russin and Evert, 1985; Coomes et al., 2008; Taneda and Terasohma, 2012). Assuming that ABA downregulates $K_{\text{leaf}}$ by lowering aquaporin activity, those genotypes with higher aquaporin activity or a greater proportion of extra-vascular pathways would consistently display higher $K_{\text{leaf}}$ sensitivity to ABA.

Other processes that occur during dehydration may modulate the responses of $K_{\text{leaf}}$ to ABA and create genetic variability. Several factors result in a strong decline of $K_{\text{leaf}}$ during drought, including cavitation, collapse of xylem conduits, and loss of permeability in the extra-xylem tissues due to mesophyll and bundle sheath cell shrinkage (Trifílo et al., 2003; Cochard et al., 2004; Blackman et al., 2010). Xylem resistance to cavitation has been shown to vary in a series of conifer species and to correlate with prolonged stomatal opening after a period of 30 d without water (Brodribb et al., 2014). This suggests that variation between anisohydric and isohydric behaviors may also be ruled by differences in xylem resistance to cavitation. Cavitation unlikely occurred here on detached leaves that had their petiole immersed in water. Endogenous ABA that accumulates in leaves under water stress may have different effects from the one observed in our study with exogenous ABA fed to leaves of irrigated plants. Embolism repair, triggered by ABA, does not apply for water-fed leaves of irrigated plants but could occur in leaves of plants under water deficit (Kaldenhoff et al., 2008; Perrone et al., 2012). This possible action of ABA may introduce some discrepancies between what we observed in leaves detached from irrigated plants and what governs leaf hydraulics and indirectly influences stomatal response in plants under water deficit. Other discrepancies may originate in up-regulation of certain aquaporin gene expression when ABA accumulates in leaves under water deficit conditions (Kaldenhoff et al., 1996). Overall, ABA-triggered decrease in $K_{\text{leaf}}$ as described in our work for early stages of soil drying, may combine with further decrease in $K_{\text{leaf}}$ due to cavitation under more severe drought, with possible overlapping of mechanisms. This may explain why the genetic variation in $K_{\text{leaf}}$ sensitivity to ABA as determined on detached leaves did not strictly correlate with the genetic variation in (an)isohydr as determined on whole plants under water deficit.

The Variable Sensitivity of $K_{\text{leaf}}$ to ABA Was Confirmed by Two Independent Measurement Methods and Two Independent Experiments

Measuring $K_{\text{leaf}}$ with the EFM as the flow rate (i.e. transpiration rate $E_{\text{leaf}}$) divided by the leaf water potential ($\Delta V_{\text{sat}}$) has long been a matter of debate when exploring the relationship between $E_{\text{leaf}}$ and $K_{\text{leaf}}$ (Flexas et al., 2013). By contrast with other methods that use pressurized water to force water flow through the leaf, the EFM respects the native paths of water flow together with the range of negative values for water potentials within the leaf (Sack and Scoffoni, 2012). However, calculation of $K_{\text{leaf}}$ rests on $E_{\text{leaf}}$ that causes some partial correlation between variables and hinders the analysis of their relationship. A second method was therefore used in an independent experiment to provide alternative evidence of ABA acting as a regulator of $K_{\text{leaf}}$. We reiterated our ABA-feeding experiment on detached leaves of the two parental cultivars, Syrah and Grenache, and measured $K_{\text{leaf}}$ with the HPFM. This
method forces water flow through the leaf by pushing water out of the lamina with a controlled pressure gradient across the leaf (Sack et al., 2002). \( K_{\text{leaf}} \) values that were obtained in control conditions with this second method were highly consistent with those previously reported by Pou et al. (2013) who operated similarly (Syrah displayed \( K_{\text{leaf}} \) of about 20 mmol m\(^{-2}\) s\(^{-1}\) MPa\(^{-1}\) in both studies). However, \( K_{\text{leaf}} \) measured with the HPFM method was about 3-fold higher than the values we obtained for the same cultivars with the EFM (Fig. 1). \( K_{\text{leaf}} \) might be overestimated by the HPFM because of the higher hydrostatic pressure imposed to water within the leaf while negative pressures develop in transpiring leaves. The leaf is thus flooded with a liquid solution and leaf airspaces might rapidly become infiltrated, implying novel pathway for water movement, hence yielding \( K_{\text{leaf}} \) values not reflecting in vivo context (Prado and Maurel, 2013). By contrast, the EFM was proposed to more closely follow the natural pathway of water in leaves through the transpiration flow (Sack and Scoffoni, 2012). Despite differences in \( K_{\text{leaf}} \) magnitude, both methods and both independent experiments consistently evidenced a marked difference in \( K_{\text{leaf}} \) measured in control conditions between cultivars. Importantly, the HPFM allowed uncoupling \( K_{\text{leaf}} \) response to ABA from \( E_{\text{leaf}} \) response: because the leaf is flooded in water during the experiment, transpiration rate is nonexistent; thus, the observed effect of ABA is exclusively a direct one on \( K_{\text{leaf}} \). ABA feeding similarly impacted \( K_{\text{leaf}} \) whatever the method used. The high repeatability across methods and experiments observed for the variable response of \( K_{\text{leaf}} \) to ABA among cultivars thus strengthens the novel outputs of this work.

The use of the HPFM method made it possible to dissociate the effects of ABA on both components of \( K_{\text{leaf}} \): the lamina and the petiole. Differences in petiole hydraulic conductance between grapevine cultivars have been proposed as a cause of differences in (an)isohydric behaviors between cultivars, although variation with drought was not mentioned (Schultz, 2003). In our study, \( K_{\text{petiole}} \) was slightly (although not significantly) reduced by the ABA treatment in Grenache, whereas it was not in Syrah. Although this result merits further verification, it is consistent with a possible role of the petiole in causing differences in hydraulic behaviors between Syrah and Grenache. Regulation of
hydraulic conductance in petioles likely rests on a similar mechanism as in the lamina, since substantial expression of plasma membrane aquaporins in petioles have been reported (Baiges et al., 2001; Chen et al., 2008), where they may facilitate transcellular water transport. However, the major resistance to water resides in the lamina, so that the petiole may play only a marginal role. By contrast, K_{lamba} offers multiple sites of regulation from the petiole-leaf junction to the sites of evaporation, through apoplasmin and symplasm pathways. A significant reduction of K_{lamba} by ABA feeding was observed for Grenache, but not in Syrah (Fig. 1). We are now seeking the mechanism underlying these contrasting behaviors, and aquaporins are good candidates.

Toward the Understanding of ABA Action on Leaf Hydraulic Conductance

By which mechanism could ABA differentially affect K_{leaf} between genotypes or species remains a key question. Based on the major role of aquaporins in the water permeability of the bundle sheath cells, Shatil-Cohen et al. (2011) suggested that the ABA-induced decrease in K_{leaf} may occur via the down-regulation of aquaporin activity therein. Further work showed that silencing a family of plasma membrane intrinsic proteins (PIPs) specifically in the bundle sheath decreases K_{leaf} by a factor of three (Sade et al., 2014). However, experimental evidence is still missing that synchronously links ABA to the macroscopic regulation of K_{leaf} and to the cellular-level regulation of aquaporins. Different studies tried to ascribe physiological responses to water deficit with expression profile of aquaporins, but contrasting results have been obtained depending on both intensity and dynamics of water deficit (Tyerman et al., 2002; Galmés et al., 2007; Prado and Maurel, 2013). Pou et al. (2013) reported lower expression of aquaporin genes VvTIP2;1 and VvPPIP;2;1 and reduced activity of aquaporins in leaves under water deficit coinciding with a decrease of K_{leaf}. Decline of K_{leaf} in dehydrating leaves could also be correlated with low aquaporin activity in the mesophyll (Kim and Steudle, 2007). Importantly, ABA treatment could decrease C-terminal phosphorylation and thus activity of aquaporin AtPIP2;1 within 30 min of application in Arabidopsis seedlings (Kline et al., 2010). This timescale is compatible with the ABA-induced decrease in K_{leaf} described in our study like in previous works (Shatil-Cohen et al., 2011; Pantin et al., 2013). Altered phosphorylation of aquaporins may act on their trafficking and gating (Tornroth-Horsefield et al., 2006; Prak et al., 2008; Eto et al., 2010) to adjust leaf hydraulics in response to drought, as was described under changing light (Prado et al., 2013) and upon exposure to ABA in guard cells (Grondin et al., 2015).

Other proposals have arisen that may explain the contrasts between isohydry and anisohydry. Vandeuleur et al. (2009) showed that Grenache strongly reduced root hydraulic conductivity under drought, contrasting with the more anisohydic Chardonnay, and that this difference was reflected in different responses to drought in transcript abundance of PIP1;1 aquaporin. Whether this relationship was due to changes in water transport and aquaporin expression in roots only or to concomitant changes in leaves as evidenced in our study remains to be elucidated. Such an action of ABA on root hydraulic conductance could have been implemented in our model instead of the direct effect of ABA on K_{leaf} to yield similar results. Thus, these different mechanisms still have to be deciphered together with their respective importance in the determinism of (an)isohydry.

Contrary to most observations in leaves, ABA tends to increase hydraulic conductivity in roots (Ludewig et al., 1988; Zhang et al., 1995; Hose et al., 2000; Thompson et al., 2007). In spite of the opposite response to ABA, change in root hydraulic conductivity remains consistent with a role of aquaporins that are down-regulated at transcriptional and posttranscriptional levels when ABA concentration increases (Wan et al., 2004; Zhu et al., 2005; Parent et al., 2009). Opposite reactions in roots and leaves could be associated with selective action of ABA on specific members of the aquaporin family to alleviate the effects of water stress.

CONCLUSION

This study reveals a substantial genetic variation in the responsiveness of K_{leaf} to ABA and supports that it may impact isohydry in grapevine. Further studies will inform us whether this relationship is conserved in other species, and whether genetic variations in aquaporin regulation are responsible for the existing variability in isohydry.

MATERIALS AND METHODS

Assessing the Response to Exogenous ABA Feeding of the Cultivars Grenache and Syrah Cultivated in the Field Using Two Independent Methods

Plant material and preparation

This experiment was performed on Syrah and Grenache plants from the Coombe vineyard (Waite Campus, Adelaide, South Australia). Leaves were prepared as previously described in Pou et al. (2013). Briefly, shoots with mature leaves from the most exposed branches were collected on 5 to 10 plants per cultivar at the beginning of the night preceding measurements. Immediately after cutting, shoots were placed into a bucket with their cut ends immersed in distilled water, covered with black plastic bags, and taken to the laboratory. The shoots were then recut under degassed water and rehydrated overnight in full darkness until leaves were assigned to the perfusion solutions.

Response of transpiration rate on detached leaves of Grenache and Syrah cultivars cultivated in the field and determination of K_{leaf} using the Evaporative Flux Method

Mature leaves were chosen on similar position from the apex (8th–12th phyllometers). On the morning preceding measurements, leaves were excised from shoots, and their petioles were immediately immersed and recut in individual 5-mL containers filled with a filtered (0.2 μm), degassed control solution [2 mmol m⁻³ K₂HPO₄, 1 mol m⁻³ MES, 0.4 mmol m⁻³ Ca(NO₃)₂] adjusted to pH 6.5. A light source was suspended above the leaves providing approximately 400 μmol m⁻² s⁻¹ photosynthetically active radiation at leaf level.
Petioles were tightly sealed to the containers caps. As a precaution, initial transpiration rate was determined on each leaf by weighing leaves in their container every 20 min over 1 h 30 min. Measurements were stopped and discarded if the flow suddenly began to decline, likely due to blockage of water flow in the petiole by residual air bubbles. Synthetic (+)-ABA, solubilized with a negligible volume of ethanol, was then added to the solution to reach varying concentrations of (+)-ABA (2, 4, 8, 16, or 32 mmol m$^{-3}$). Final transpiration in ABA was determined once the weight declined at a stabilized rate (which occurred about 1 hour after adding ABA).

At the end of the experiment, each leaf was taken off its container and immediately set into a Scholander pressure chamber (Soil Moisture Equipment Corp) to measure its water potential ($\Psi_{leaf}$). Measurement of $\Psi_{leaf}$ with the pressure chamber was assumed to be close to the value for the transpiring leaf just before it is enclosed in the chamber, according to the principle basis of the Scholander chamber. Leaf hydraulic conductance ($K_{leaf}$) was then calculated following the evaporative flux method (EFM) as the flow rate divided by the leaf water potential taken as the driving force for water flow from the solution (Sack et al., 2002). The transpiration rate for this calculation was determined as the stable rate of weight loss measured at the very end of the experiment, just before measuring $\Psi_{leaf}$. $K_{leaf}$ was estimated on a leaf area basis as:

$$K_{leaf} = E / (\Psi_{leaf} \times LA)$$

where LA is the individual leaf area (m$^2$).

At the end of the measurement, the leaf was scanned and leaf area was determined on photographs using ImageJ (Rasband WS, 2009).

**Response of stomatal conductance on detached leaves of Grenache and Syrah cultivars cultivated in the field and determination of $K_{leaf}$ using High Pressure Flowmeter**

Another set of leaves were chosen and excised following the same protocol as described above. Each leaf was directly assigned to one of the ABA or control solutions (concentration of (+)-ABA 0, 2, 4, 8, 16, or 32 mmol m$^{-3}$) prepared as above.

After 1 h in perfusion, stomatal conductance ($q_g$) was measured using a porometer (Delta T AP4; Delta-T Devices Ltd) on each leaf fed with a different solution.

Leaf hydraulic conductance was then measured with a HPFM apparatus (HPFM-Gen3, Dynamax). This method first developed by Tyree et al. (2005) relies on pushing a solution into a plant part (here, a leaf) at a known delivery pressure. Measurements were performed using the transient method consisting in applying different pressures and recording flow rates to calculate the conductance as the slope of the regression line between flow rate and pressure (Sack et al., 2002). Leaves were attached to the flowmeter through the petiole using compression fittings. Filtered water was forced into the leaves at increasing pressure ($P$) up to 0.4 MPa, while measuring the instantaneous flow rate ($F$) every 2 s (Supplemental Fig. S2). Corresponding hydraulic conductances ($K_{petiole}$) were computed from the slope of the plot water flux versus pressure as:

$$K_{petiole} = \Delta F / (\Delta P \times LA)$$

where LA is the leaf area (m$^2$).

The lamina was then removed by excising the leaf at the junction with the petiole, and the leaf-specific petiole conductivity ($K_{petiole}$) was measured and computed according to (Sack et al., 2002) as:

$$K_{petiole} = \Delta F / (\Delta P \times LA \times Petiole length)$$

$K_{lamina}$ was then calculated from $K_{petiole}$ and $K_{petiole}$ according to the Ohm’s law analogy considering petiole’s and lamina’s pathways in series: $1 / K_{leaf} = 1 / (K_{petiole} \times petiole length) + 1 / K_{lamina}$.

During HPFM measurements, the leaves were submerged in a container filled with water to maintain constant leaf temperature and prevent transpiration. The temperature in the compartment was adjusted to 25°C with a regulated bath (Ministat, Peter Huber Kälte maschinenbau GmbH) which was continuously aerated. The HPFM apparatus corrects the hydraulic conductance for possible changes in temperature to account for corresponding changes in water viscosity. The leaves were exposed to the same light as the one used during perfusion.

At the end of measurement, the leaf was scanned and leaf area was determined on photographs using ImageJ (Rasband WS, 2009).

**Experiments on 10 Grapevine Offspring, and the Parents, from a Syrah × Grenache Mapping Population with Contrasting (An)isohydic Behaviors**

**Plant material and preparation**

A panel of 10 offspring was selected within the pseudo-F1 population of 186 two-year-old genotypes obtained as the first generation from a reciprocal cross between the grapevine cultivars Syrah and Grenache (Adam-Blondon et al., 2004; Coupel-Ledru et al., 2014; Coupel-Ledru et al., 2016). Offspring plus the two parental genotypes were grafted on 110 Richter rootstocks in 2010 and grown outside in 3-L pots for 2 years in Montpellier (France).

In winter 2012 (January), plants were transferred to a greenhouse in 9-L pots filled with a substrate calibrated for soil water management (Coupel-Ledru et al., 2014). In early spring 2012, the whole population was installed into a high throughput phenotyping platform (PHENOARCH platform hosted at the M3P, Montpellier Plant Phenotyping Platforms, http://bioweb.supagro.inra.fr/phenoard) in another greenhouse where two soil water conditions were applied using automated weighing stations and daily watering. Well-watered conditions, corresponding to a soil water content of 1.5 g water g$^{-1}$ dry soil, were imposed on one-half of the plants, while the other one-half was submitted to a moderate soil water deficit (1.05 g water g$^{-1}$ dry soil). Detailed information of PHENOARCH platform and measurement of environmental conditions is described in (Cabrera-Bosquet et al., 2016).

At the end of the high-throughput experiment in the greenhouse, the plants were transferred outside, where they were grown for one additional year and pruned to produce one, unbranched leaf axis with their inflorescences removed. Automated fertigation completed by occasional, individual weighing and watering ensured that all the plants were maintained well-watered (Coupel-Ledru et al., 2014). These plants were used in 2013 for experiments on detached leaves.

**Leaf water potential and transpiration rate of whole plants under controlled conditions in the phenotyping platform**

Potted plants were characterized for water relations under transpiring conditions during a 24-h cycle using a controlled environment chamber close to the phenotyping platform. For each genotype, leaf water potential was measured in the daytime ($\Psi_M$) on three plants per watering regime, using a pressure chamber (Soil Moisture Equipment Corp.) (Coupel-Ledru et al., 2014). Isohydric (respectively anisohydric) behavior was defined as the capacity (respectively inability) of a genotype to maintain leaf water potential in the daytime under water stress. The panel of 12 genotypes (10 offspring plus the 2 parents, Syrah and Grenache) was selected based on their contrasted behaviors for (an)isohydry.

Transpiration rate was determined in parallel in the same transpiring conditions and as was previously described (Coupel-Ledru et al., 2014, 2016). Each pot was weighed with 0.1 g accuracy (Sartorius balance, IB 34 EDEP) at the beginning and end of the light period in the controlled-environment chamber. Weight losses were used together with the estimated whole plant leaf area to calculate average transpiration rate on a leaf area basis ($E_{trans}$).

**Leaf area**

Individual whole plant leaf area was estimated through processed images taken every 2 d in the platform imaging cabin as previously described (Coupel-Ledru et al., 2014).

**ABA sampling in the phenotyping platform and quantification**

For each of the 12 genotypes (10 offspring plus 2 parental), xylem sap was extracted from two leaves on two water deficit plants following measurement of $\Psi_M$ with the pressure chamber. The same pressure chamber was used to pressurize the leaf at about 0.3 MPa above the balancing pressure that was imposed for $\Psi_M$ measurement, and about 30 μL of sap was expressed from the cut petiole exposed outside the pressure chamber. Expressed sap was collected in 0.5-mL microtubes and conserved at −80°C in a dedicated deep freezer (Herafreeze, HFU T series, Thermo Fisher Scientific) pending freeze-drying. Microtubes containing frozen sap samples were centrifuged in a centrifuge (Speed Vac Plus SC110A, Fisher Scientific) connected to a benchtop freeze-drier (Christ Alpha2-4, Fisher Scientific) that imposed a partial vacuum of 0.020
The water contained in the sap samples was sublimated and trapped on the cold condenser of the freeze-drier maintained at a temperature of -80°C. Analysis of ABA abundance in xylem sap was undertaken by liquid chromatography/mass spectrometry (LC MS/MS, Agilent 6410). Dried xylem sap samples were dissolved in 30 μL 10% acetonitrile containing 0.05% acetic acid plus a deuterated internal standard mix (containing D6-3,5,5',7,7',7-ABA at a concentration of 10 ng mL−1) before introduction into LC MS/MS. Separation was carried out on a C18 column (Phenomenex C18(2)75mm × 4.5mm × 5 μm) 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 were the same as previously described (Speirs et al., 2013).

Response of transpiration rate on detached leaves of the 10 offspring plus 2 parental genotypes under controlled conditions and determination of Kleaf using EFM

The potted plants used in the whole-plant experiment described above were further cultivated outdoors. In July 2013, one night prior to experiments, plants were transferred from outside to a controlled environment chamber to ensure initial, low-transpiration conditions. On the morning preceding measurements, leaves were excised from plants and their petioles were immediately immersed and recut in individual 5-mL containers filled with a filtered (0.2 μm), degassed control solution [2 mol m−3 KH2PO4, 1 mol m−3 MES, 0.4 mol m−3 Ca(NO3)2] adjusted to pH 6.5. Petioles were tightly sealed to the containers’ caps. Each leaf in its container was then placed in the chamber with VPD maintained at 2 ± 0.2 kPa and temperature at 27°C. Light was provided in the chamber by a bank of sodium lamps that maintained the PPFD at approximately 480 μmol m−2 s−1 at the leaf level. All leaves were chosen as well-exposed, fully expanded, generally on the eighth phytomer from the apex.

The protocol then followed for ABA perfusion (determination of transpiration rate, of Kleaf with the EFM method, and of their response to ABA) was the same as described in the section “Response of stomatal conductance on detached leaves of Grenache and Syrah cultivar cultivated in the field and determination of Kleaf using High Pressure Flow Meter.”

Statistical Analyses

Statistical analyses were performed with R (R Development Core Team, 2012). The Kruskal-Wallis test at the 5% alpha level was used for comparison of means. Semi-In transformations were used to fit linear models to Kleaf response to ABA and extract the slopes. Regression slopes were compared by analysis of covariance. Tests for significant differences among all pairs of slopes were further achieved using R compSlopes package with the FDR correction method for their technical support in plant preparation, high-throughput experiments, and measurements on detached leaves. We are also grateful to Wendy Sullivan for helping with the HPFM experiments and to Annette Boettcher for running ABA analysis with the LCMS.

In memory of our dear colleague and friend Eric Lebon.

Received May 30, 2017; accepted September 7, 2017; published September 12, 2017.

LITERATURE CITED

Leaf Hydraulic Conductance, ABA, and Isohydrophy


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Plant Physiol. Vol. 175, 2017 1133