Improving water use efficiency in grapevines: potential physiological targets for biotechnological improvement

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Running title: Biotechnology improvement of water use efficiency in grapevines
Abstract

Background and Aims: Improving water-use-efficiency (WUE) in grapevines is necessary for vineyard sustainability under increasing aridity, which occurs due to global climate change. From a physiological perspective, WUE reflects the ratio between the carbon assimilated by photosynthesis and the water lost in transpiration. Although at the expense of decreased photosynthesis rates and decreased grape yield, to keep stomata partially closed by means of regulated deficit irrigation (RDI) or partial root drying (PRD) represents an opportunity to increase WUE. It may be even more interesting – although not so readily available – to achieve increases in WUE by means of increasing photosynthesis without increasing water loses. Potentially, this could be achieved by genetic engineering.

Methods and Results: Here we review current knowledge and results relevant for the goal of improving WUE in grapevines by biotechnology and genetic engineering. The expected benefits of these manipulations on WUE of grapevines under water stress conditions are modelled.

Conclusions: Two ways are proposed to achieve this goal: (1) to improve CO₂ diffusion to the sites of carboxylation without increasing stomatal conductance, and (2) improving the carboxylation efficiency of Rubisco. The first way would be achieved increasing mesophyll conductance to CO₂ which partly depends on aquaporins. The second way could be achieved if grapevine Rubisco, whose specificity for CO₂ over O₂ is 100 mol mol⁻¹, could be replaced by Rubisco from other C₃ species reaching a specificity of 110 mol mol⁻¹.

Significance of the Study: The physiological bases for improving grape yield and WUE under drought are established. Future prospects to improve grapevine WUE by using these physiological features are discussed.

Keywords: Drought, mesophyll conductance to CO₂, photosynthesis, Rubisco, water-use-efficiency

Introduction

The wine industry and viticulture are currently enduring a period of profound changes worldwide. The viticulture expansion worldwide and the increasing environmental impacts of crop practices are of particular interest for the sustainability of the cropping areas. Within this frame, Bisson et al. (2002) have highlighted that, in addition to a product enjoyable in all sensorial aspects, consumers expect wines to be healthy and produced in an environmentally sustainable manner. They suggested that, as consumers become more aware of the vulnerability of our global environment, the demand for sound agricultural production practices will increase. Hence, in the near future wine industry will not only depend on wine quality, but also on maintaining (or cropping) environmentally friendly vineyards.

Among environmental problems related to viticulture, water scarcity is one of the major worldwide limitations to agriculture in general (Araus 2004; Morison et al. 2008), and to grape production in current viticulture areas in particular (Chaves et al. 2007). About two thirds of the major viticulture areas of the world present year precipitation below 700 L m⁻² (Figure 1). Moreover, a large proportion of vineyards in these areas are located in regions with a seasonal drought that coincides with the grapevine growing season (e.g. Mediterranean climate-type areas). In these areas, progressive soil water deficits and high leaf-to-air vapor pressure gradient, together with high irradiance and temperatures, exert large constraints in yield and quality. Climate change predictions suggest that the viticulture areas subject to water deficit limitations will even increase in the near future. For instance, for the whole viticulture area in
Europe, Schultz (2000) predicted that doubling atmospheric CO$_2$ will result in decreases in soil moisture content from 20% or more in Central Europe to 70% in the Iberian Peninsula and the Balearic Islands. Indeed, the number of dry days per year has increased in southern Europe (Chaves et al. 2007). Increasing water scarcity will have an impact in viticulture, from changing the optimum ranges for different grape varieties (Schultz 2000) to force viticulturists to rely on irrigation more often (Chaves et al. 2007). In turn, this will have impacts on the wine industry, because the effects of irrigation on grape quality are at least controversial, with several reports suggesting that excess water leads to reduced quality through decreases in colour and sugar content and imbalanced acidity (Matthews et al. 1990; Medrano et al. 2003; Salón et al. 2005), as well as interfering with the normal timing of flavonoid development (Castellarin et al. 2007).

Improving vineyards water use efficiency by crop management

In this scenario, reducing water use for irrigation and increasing water use efficiency (WUE) - i.e., improving the yield to water consumption ratio - becomes a major priority in agriculture (Costa et al. 2007; Collins et al. 2008; Morison et al. 2008). The efforts being done include improvements in agronomical management practices (Gregory 2004), developing regulated deficit irrigation (RDI) systems (Costa et al. 2007) which are often supported by physiologically-based monitoring tools, such as infra-red thermometry (Jones et al. 2002), trunk diameter sensors (Conejero et al. 2007) or sap flow meters (Fernández et al. 2008), or introducing physiologically-based agronomic techniques such as partial root drying (PRD, Chaves et al. 2007).

Other agronomical techniques have also been tested and developed in grapevines, and some are already being used in field trials and commercial vineyards. For instance, canopy management is an important agronomic technique being widely used in viticulture to regulate the microenvironment around the clusters, and hence fruit sanitary conditions, yield and quality, but also light absorption by the canopy, and hence canopy photosynthesis and water loss, i.e., WUE (Carbonneau 1980; Williams and Ayars 2005). Other techniques related to vineyard management have started to be tested as the use of mulching (Buckerfield & Webster 2001; Hatfield et al. 2001; Gregory 2004) or growing herbaceous species in vineyard inter-row cropping, which may help regulate soil evaporation and runoff, as well as root development and nutrient availability (Morlat and Jacquet 2003; Monteiro and Lopes 2007). Up to date, the effects of this later technique on yield, grape quality and WUE under Mediterranean conditions, where competition for water could lead to severe water stress, have been reported by few works (Monteiro and Lopes, 2007; Gulías et al. 2008). The results suggest that reduction of vegetative vigor by water and nutrient competition between grapevine and the cover crop could be a way to reduce grapevine water consumption late in the season, when this resource is more scarce. The use of PRD, a dripping system that irrigates both sides of the grapevine root system alternative is becoming more common, as it has been shown to induce root ABA synthesis in the dried zone (Dry and Loveys 1998; Dry et al., 2000). PRD induces partial stomatal closure without reducing leaf water status and increased WUE (Stoll et al. 2000; de Souza et al. 2005; Chaves et al. 2007). Trials in Australia using this type of irrigation have shown a significant reduction in vegetative growth while maintaining fruit yield (Dry et al. 2001) and increasing quality parameters such as colour (Bindon et al. 2008), therefore saving in irrigation while increasing quality. The applications of RDI in grapevines have recently been reviewed elsewhere (Keller 2005; Costa et al. 2007), and several different physiological indicators of potential use for RDI scheduling in grapevines have also been reviewed (Cifre et al.
Among them, sap flow meters were among the first to be proposed and tested in irrigated (Lu et al. 2003; Tarara and Ferguson 2006) and water stressed (Escalona et al. 2002) grapevines, obtaining a gradient of grape yield and qualities. The usefulness of trunk diameter sensors to assess water stress and water consumption in grapevines was first demonstrated by Escalona et al. (2002) in potted plants, while only recently studies have begun to develop under field conditions (Patakas et al. 2005; Intrigliolo and Castel 2007, 2008). Remote sensing indicators have also been proposed and are presently being evaluated in field trials, such as passive chlorophyll fluorescence (Flexas et al. 2002a), infra-red thermometry (Jones et al. 2002) and hyperspectral reflectance indices (Evain et al. 2004). Also stem water potential has been proposed as an indicator for irrigation scheduling (Carbonneau 1998, 2003; Patakas et al. 2005), being a widely used indicator despite the practical problems of their use for commercial vineyards.

Besides these managing techniques, in crops other than grapevines plant breeding and genetic engineering are also seen as pivotal in improving WUE (Boyer 1996; Condon et al. 2004). However, as pointed out by Vivier and Pretorius (2002), grapevine improvement has been untouched by classical breeding programs in the sense that relatively few new cultivars have become commercially successful, especially in the wine industry where commercial production relies in a few selected and ancient cultivars. Given the difficulties of acceptance of newly developed cultivars by traditional breeding, it is envisaged that it will be even more difficult to introduce genetically-transformed genotypes. Nevertheless, there is little doubt that feeding the increasing human world population will require, in the near future, large increases in crop yields, while doing it in an environmentally sustainable manner would require, among other issues, a parallel increase in WUE, which ultimately will depend on biotechnologically improved crops, including genetically-modified plants (Morison et al. 2008; Murchie et al. 2009). Viticulture cannot elude these requirements and indeed, drought resistance and increased water use efficiency have already been identified as the most important priority targets for grapevine biotechnology, together with pest and disease control and improved grape quality (Vivier and Pretorius 2002). Nevertheless, as pointed out by Blum (2005), drought resistance and water use efficiency are not synonymous, and it is important not to confound each other, although this is often the case in the literature (Morison et al. 2008). Indeed both characteristics are often conferred by plant traits that are mutually exclusive (Blum 2005). For instance, Arabidopsis mutants with an increased plant nuclear factor Y(NF-Y) present a higher drought-tolerance than wild-type plants, but with identical WUE (Nelson et al. 2007). Contrarily, ERECTA mutants, which present an enhanced WUE, do not present an improved drought tolerance (Masle et al. 2005). In other cases, however, increased drought-tolerance can be associated with higher WUE, like in PSAG12-IPT transgenic tobacco (Rivero et al. 2007) or transgenic Arabidopsis expressing the bZIP transcription factor ABP9 (Zhang et al. 2008). The present review focuses on traits that can potentially confer increased WUE, not drought resistance.

Although until now, no increase in WUE has been achieved in grapevines by genetic modification, and despite the fact that grapevine was initially proven to be recalcitrant to such manipulations, the success in grapevine transformation in recent years (Vivier and Pretorius 2002) and the current availability of the completely-sequenced grapevine genome (Jaillon et al. 2007) suggest that opportunities are open to achieve this goal in the near future (Troggio et al. 2008). In addition, initial studies have emerged describing water stress effects on gene expression and protein profiling
which may be viewed as a first step to identify potential gene targets for genetic engineering of WUE.

The aims of the present review are (1) to define the concept of plant water use efficiency (WUE) and describing its variability in grapevines, and (2) to identify and discuss, from a physiological perspective, the potential target processes whose genetic manipulation may lead to improved WUE.

Plant water use efficiency: concept and components

As stated above, making agriculture sustainable requires a major reduction in water use in many regions. Plant water-use efficiency (WUE) is a key parameter to determine how efficiently the agricultural sector is using water. WUE (i.e., the amount of carbon gained per unit water used) is currently a priority for the United Nations policy, in what is called the 'Blue Revolution' and summarised as 'more crop per drop' (Annan, 2000).

Figure 2 shows the components of WUE and their interdependencies. On one hand, WUE depends on total plant water consume during the growing season. This is the sum of the amount of water lost without being used by the plant, plus the transpired water. The former occurs through soil evaporation, runoff, etc., and can be avoided or reduced by agronomic methods previously described, such as drip irrigation, mulching or vineyard intercropping (Gregory 2004). Transpired water depends on aspects related to canopy growth and structure, such as leaf angle, leaf area index (LAI) or shoot positioning, which ultimately determine light interception or the energy load for transpiration, and on leaf transpiration (E), which depends on the atmospheric evaporative demand, represented by the leaf-to-air vapor pressure deficit (VPD), and on leaf conductance, i.e. cuticular ($g_c$) and, specially, stomatal ($g_s$) conductances. Canopy structure and light interception are commonly regulated by management techniques, including the selection of proper training system and pruning (Mullins et al. 1998).

Despite their interest for improving WUE, (Baeza et al. 2005) canopy structure depends on complex whole-canopy components interrelationships, which makes difficult to identify specific targets for genetic manipulation and hence are out of the scope of this review. Leaf conductance and its diurnal and seasonal regulation, in contrast, depends on well-identified physiological traits that may be targeted for transformation and WUE improvement.

The second component of plant WUE is the whole plant carbon and biomass acquisition, as well as its partition to yield components (i.e., fruits). Grapes are an extremely efficient carbon sink. Despite constituting only 20-30% of the total plant dry mass, they import about 80-90% of the total assimilates obtained in photosynthesis, and this strong sink capacity is maintained under water stress conditions (Bota et al. 2004).

Therefore, partitioning seems to be already optimized in grapevines, so it is unlikely to be an optimum target for genetically improving WUE. Instead, plant net carbon acquisition depends on two processes: photosynthesis and respiration that, unlike carbon partitioning, vary with grape genotypes (Flexas et al. 1999a; Bota et al. 2001) and are differentially affected by water stress (Flexas et al. 2006a). That makes them a more likely candidate to be good targets for genetic improvement of WUE.

As previously noted (Figure 2), a clear link emerges between leaf level WUE and whole plant WUE. The former is often approached using the instantaneous WUE, i.e. the ratio of net assimilation ($A_n$) to leaf transpiration (E), or the intrinsic WUE, i.e. the ratio of $A_n$ to stomatal conductance ($g_s$), which was introduced to compare photosynthetic properties at a common evaporative demand (Osmond et al. 1980). More recently, the carbon isotopic composition ($\delta^{13}C$) in leaf dry matter is used as a long-term
The definition of the intrinsic WUE improvement of WUE at the leaf level: net carbon gain in photosynthesis and stomatal conductance. It has to be pointed out that improving leaf-level WUE may not necessarily result in improving plant and yield WUE (Jones 2004), due to the interference of canopy and ambient processes (Figure 2). Nevertheless, comparing different grapevine cultivars grown in glasshouse, good correlations have been found between yield-based WUE and leaf intrinsic WUE (Gibberd et al. 2001a). Also, in a preliminary experiment of our group with five contrasted cultivars, we have found a good correlation between whole-plant WUE and leaf intrinsic WUE (Tomàs et al., unpublished). Although the relationship was positive and significant, it varied between irrigated and water-stressed plants (Figure 3), indicating the complexity of the leaf to whole-plant WUE relationship.

Genetic variability in water use efficiency in grapevines

Before considering theoretical aspects for genetically improving WUE, it is important to evaluate whether WUE presents some natural variation in grapevines and whether this has a genetic basis. In species other than grapevines, differences between genotypes in AN/gs and WUE have been reported to have a genetic basis (Martin et al. 1989, Masle et al. 2005), and breeding for high WUE has been and continues to be a main objective for many crops (Condon et al. 2004). In grapevines, despite the fact that they have not been object of intense breeding for high WUE, substantial genotypic variations in WUE have been described.

For instance, AN/gs in irrigated plants was shown to range between 38 and 64 mmol CO2 mol-1 H2O when comparing 20 Mediterranean cultivars (Bota et al. 2001), but higher values have been described in other cultivars such as Grenache and Syrah (Schultz 2003), the species Vitis riparia (Flexas et al., 1999a) or the rootstock R-110 (Pou et al. 2008). The highest reported values in irrigated grapevines, ca. 100 mmol CO2 mol-1 H2O, have been reported for the cultivar Rosaki (Rodrigues et al., 1993), while the lowest values, ca. 25 mmol CO2 mol-1 H2O, have been shown for cultivar Kékfrankos (Zsófi et al. 2009). AN/gs typically increases under water stress conditions, commonly to 100-200 mmol CO2 mol-1 H2O (Bota et al. 2001; Flexas et al. 2002b; Pou et al. 2008; Zsófi et al. 2009).

Also δ13C shows large variation among cultivars. Gaudillère et al. (2002) showed differences of up to 3‰ in fruit δ13C in a comparison of 32 cultivars, which were used to classify those cultivars in terms of their relative drought tolerance and WUE. Slightly lower variations (2‰) have been shown for leaf δ13C in different comparisons of 19 (Gibberd et al. 2001) and 5 (Tomàs et al., unpublished) different cultivars under irrigation. Similar variations (2-3‰) are observed for any single cultivar when subjected to water stress (Souza et al. 2003; Chaves et al. 2007; Pou et al. 2008), when δ13C becomes less negative, so that it correlates positively with AN/gs (Chaves et al. 2007).

Much less data is available in grapevines for whole-plant WUE, but variation is still present, ranging from 2.5 to 3.4 g of dry matter Kg-1 H2O for fruit-based WUE (Gibberd et al. 2001) and from 2.5 to 6 g of dry matter Kg-1 H2O for vegetative growth-based WUE (Tomàs et al., unpublished, see Figure 3). However, contrary to AN/gs and δ13C, whole-plant WUE decreases rather than increases under water stress (Figure 3), suggesting that variations in WUE resulting from reduced gs do not result in increased WUE at the yield level.
In summary, there is substantial evidence for genetic variability of WUE in grapevine cultivars (Bota et al. 2001; Gaudillére et al. 2002) and also in grapevine rootstocks (Satisha et al. 2006), which supports the possibility of improving WUE by genetic engineering.

Potential physiological targets for biotechnological improvement of WUE

As stated earlier, intrinsic WUE ($A_N/g_s$) is a good basis to summarize the potential targets for genetic improvement of WUE at the leaf level. Since $A_N$ shows a direct but curvilinear dependency of $g_s$ (Figure 4), any reduction in $g_s$ results in an increased WUE. Therefore, genetically manipulating $g_s$ would result in improved WUE at the leaf level, although associated to reduced photosynthesis and potential yield. Although it has been pointed out that genotypic variation in WUE often derives from variations in $g_s$ and not $A_N$ (Gibberd et al. 2001; Blum 2005), the results shown in Figure 4 demonstrate that higher $A_N$ is also a factor for increased WUE in grapevines at constant $g_s$. The data scattering in Figure 4 represents the genotypic and environmental variations in intrinsic WUE described in the previous section, but it also illustrates how WUE could be increased while maintaining or even increasing yield, which would require an increase of $A_N$ at a given value of $g_s$, i.e., genotypic modifications in $A_N$-$g_s$ relationship (Parry et al., 2005).

Since respiration occurs continuously in all living cells, its reduction may result in substantial increases in whole plant carbon gain. In turn, increased photosynthesis at any given $g_s$ can be potentially achieved by either increasing total leaf area or increasing leaf photosynthetic capacity (Sharma-Natu and Ghidiyal 2005; Long et al. 2006). However, in grapevines there is a limit for the capacity of improving photosynthesis by increasing total leaf area, and this may result in a penalty in terms of WUE. Indeed, Escalona et al. (1999a, 2003) showed that leaves occupying the inner part of the canopy constitute up to 35 to 50% of total leaf area, but contribute to less than 5% of total net canopy carbon gain. Theoretical calculations suggest that selective pruning of these leaves would result in a 4-5% increase in canopy WUE.

It may be more interesting to increase leaf photosynthetic capacity at any given $g_s$. Potentially, this could be achieved by diverse methods (reviewed by Long et al. 2006; Peterhansel et al. 2008; Murchie et al. 2009). These include improving carboxylation efficiency and/or improving CO$_2$ diffusion in the mesophyll through either inducing C$_4$-like photosynthetic metabolism in C$_3$ plants (Long et al. 2006) or increasing the mesophyll diffusion conductance to CO$_2$ from sub-stomatal cavities to chloroplasts ($g_m$, Flexas et al. 2008). In turn, carboxylation efficiency can be achieved through increased Rubisco catalytic rate (Parry et al. 2007) and/or specificity for CO$_2$ (Galmes et al., 2005; Parry et al. 2007), reducing or bypassing photorespiration (Long et al. 2006), increasing the capacity for RuBP regeneration (Pertehansel et al., 2008), or reducing the photoprotective state upon high to low light transitions (Long et al. 2006). However, considering grapevine physiology and its response to water stress, only a few of these appear to be really attainable targets for grapevine improvement.

Grapevine transpiration, photosynthesis, photorespiration, photoprotection and respiration responses to water stress have previously been described (Flexas et al. 2002b; Medrano et al. 2002, 2003). Some important features are shown in Figure 5, which average real data obtained for two different Mediterranean cultivars under commercial vineyard conditions. In the next sections, these realistic data will be taken...
as the basis for discussion and simulation of the potential effects of genetically engineering different physiological mechanisms in grapevines. First, water stress leads to progressive decreases in gs (Figure 5, right to left), which results in leaf temperature increases from 3 to 8°C (Flexas et al. 1999b). The diffusion of CO₂ through the leaf internal structure, or mesophyll conductance to CO₂ (gm), decreases concomitantly with gs (Flexas et al. 2002b), but the relationship between both conductances is curvilinear, so that the higher the intensity of water stress the higher is gm as compared to gs (Figure 5). Decreases of gs and gm as water stress intensifies result in less CO₂ available in the chloroplasts (Cc), which can be typically reduced to half or less of the values under irrigation (Figure 5). Together, decreased Cc, increased leaf temperature and sustained high irradiance lead to increased photorespiration and photosystem heat dissipation associated with xanthophylls de-epoxidation, both of which are thought to exert photoprotection of the photosynthetic apparatus (Flexas et al., 1999a, b, 2002a, b; Medrano et al. 2002). In contrast, leaf respiration is barely affected by water stress in grapevines (Escalona et al. 1999b).

Coming back to physiological targets to increase photosynthesis, only few appear to be reliable for grapevines subjected to water stress in view of the responses described above. For instance, since grapes often grow under high light conditions and only illuminated leaves contribute significantly to carbon gain (Escalona et al. 2003), both photorespiration and the photoprotective state associated to xanthophylls de-epoxidation may be necessary to avoid further damage to photosynthesis, particularly under water stress conditions (Medrano et al. 2002). Similarly, although increasing sedoheptulose-1,7-bisphosphatase (i.e., increasing the capacity for RuBP regeneration) in leaves has been shown to enhance photosynthesis in some species under irrigation (Lefebvre et al. 2005) and salt stress (Feng et al. 2007), grapevine photosynthesis operates in the Rubisco-limited region (i.e., photosynthesis limited by CO₂ availability in the chloroplasts) and not the RuBP-limited region, particularly under water stress (Flexas et al. 2002b, 2006a). For the same reason, increasing Rubisco specificity for CO₂ may be preferable to increasing its abundance, activation state or catalytic rate (Galme et al., 2005; Parry et al. 2007). Instead, increasing mesophyll conductance to CO₂ (gm) may result in increased Cc without increasing the water expenses that would be associated to increased gs, and C₄ metabolism would be favorable due to increased leaf temperature under water stress conditions (Figure 5). Figure 6 summarizes the known effects of combined water stress and increased leaf temperature on photosynthesis, showing the logics for transforming C₃ into C₄ plants, increasing gm, and improving Rubisco specificity for CO₂ as the most interesting choices for genetically improving photosynthesis and WUE under water stress in grapevines.

In summary, we will focus on discussing four potential targets for genetically engineering improved WUE in grapevines: stomatal physiology, plant respiration, mesophyll conductance to CO₂ and Rubisco specificity for CO₂. Although C₄ metabolism could be an interesting target as well, particularly in view of the high leaf temperatures achieved by grapevines under water stress, the scarce success achieved in other species in such kind of transformation suggests that this would be a goal too far to be achieved in grapevines.

Regulation of transpiration
Cuticular and night conductance

The amount of water transpired by leaves depends on cuticular conductance and stomatal conductance. Water transpired through the cuticle contributes to lowering WUE because this is almost totally impermeable to CO₂, so that water is lost without
Nevertheless, cuticular conductance is insignificant. In contrast, stomatal conductance is high and strongly regulated, an intrinsic property that is linked to photosynthesis, therefore constituting a potential target for biotechnological improvement of WUE.

It is important to highlight that stomatal conductance, contrary to previous thoughts, can be maintained at night, when no photosynthesis occurs, constituting a significant source of water losses and reduced WUE (Caird et al. 2007). Therefore, night conductance values ($g_{\text{night}}$) are often higher than what would be expected if all the conductance under these conditions was cuticular ($g_c$). In several *Vitis* species, $g_{\text{night}}$ has been shown to range from 0.03-0.06 mol H$_2$O m$^{-2}$ s$^{-1}$ in *V. berlandieri* and *V. rupestris* to values as high as 0.205 mol H$_2$O m$^{-2}$ s$^{-1}$ in *V. riparia* (Caird et al. 2007). Considering that the maximum values of daytime stomatal conductance in grapevines are often lower than 0.4 mol H$_2$O m$^{-2}$ s$^{-1}$, these values of $g_{\text{night}}$ are quite high. To the best of our knowledge, no data for $g_{\text{night}}$ in *Vitis vinifera* have been previously reported. Here we show (Table 1) data of $g_{\text{night}}$ and leaf transpiration in the night ($E_{\text{night}}$) for two different cultivars of *Vitis vinifera*, Tempranillo and Manto Negro. They were subjected to various irrigation regimes, as recorded in a vineyard in Mallorca in August 2000 (Flexas et al., unpublished). The values range from 0.009 mol H$_2$O m$^{-2}$ s$^{-1}$, i.e. close to cuticular conductance values, to 0.107 mol H$_2$O m$^{-2}$ s$^{-1}$, i.e. similar to typical day-time values for mild to moderate water stressed plants (Flexas et al. 2002b). $E_{\text{night}}$ ranges from 0.3 to 1.2 mmol H$_2$O m$^{-2}$ s$^{-1}$. This represents a substantial loss of water. For instance, during a 9-hour night a typical individual grapevine plant with 6 m$^2$ of leaves would lose from 1 to 4 L of water.

Remarkably, differences in $g_{\text{night}}$ and $E_{\text{night}}$ were apparent between cultivars and treatments. While the values were generally lower in Manto Negro, a cultivar with drought-resistant reputation, than in Tempranillo, they were much strongly responsive to water stress in the latter, so that under severe water stress Tempranillo consumed less water by night than Manto Negro. These results suggest that $g_{\text{night}}$ is a regulated character with a genetic basis, and therefore a potential target for biotechnological improvement. Unfortunately, although it is known that $g_{\text{night}}$ responds to similar factors as daytime $g_s$, including CO$_2$, abscisic acid (ABA) and water stress (Caird et al. 2007), the physiological basis of $g_{\text{night}}$ are poorly understood, so that possible molecular targets for genetic engineering are unknown. Clearly more efforts are needed to characterize the importance of $g_{\text{night}}$ in grapevines and to understand the molecular mechanisms underlying its regulation.

**Daytime stomatal conductance**

Much more is known about the regulation of daytime $g_s$. The well-known declining response of $g_s$ to water stress, and the concomitant increase in intrinsic WUE have allowed the development of management practices such as RDI and PRD, and of $g_{\text{c}}$-based or $g_{\text{c}}$-dependent physiological indicators for irrigation scheduling like sap flow or infra-red thermometry (Cifre et al. 2005). But in addition to this empirical approach, the substantial knowledge about the molecular basis for $g_s$ regulation opens the possibility of genetic engineering of this leaf trait. Indeed, in species other than grapevines, several mutants and transgenic plants have been described with altered $g_s$ and/or altered $g_s$ responses to environment (Merlot et al., 2007; Nilson and Assmann, 2007). In grapevines, this goal has yet to be achieved, but efforts have been just initiated (Matus et al. 2008).
In grapevines, gas is well known to be regulated by the concentration of ABA (Loveys and Kriedemann 1974; Rodrigues et al. 2008), either synthesized by roots in response to water stress and transported in the xylem to leaves (Correia et al. 1995; Lovisolo et al. 2002; Pou et al. 2008) or locally synthesized in buds and leaves (Soar et al. 2004, 2006). The capacity for ABA biosynthesis and ABA-mediated stomatal closure depends on the cultivar, being higher in isohydric cultivars like Grenache, with high WUE, and lower in anisohydric cultivars like Shiraz (Soar et al. 2006). Many different genes and proteins involved in ABA synthesis, ABA catabolism and ABA-mediated stomatal closure are known, which have been used in other species to genetically modify the control of transpiration (Saez et al. 2006; Verslues & Bray 2006), and that can potentially be used in grapevines as well. Genes involved in ABA synthesis include 9-cis-epoxycarotenoid dioxygenase (especially NCED3), while genes involved in ABA catabolism include four cytochrome P450 monooxygenases, of which CYP707A3 is specifically induced under water stress (Nilson and Assmann, 2007). Among genes involved in ABA signaling and ABA-mediated stomatal closure, protein kinases (like OST1) act as positive regulators while type 2C protein phosphatases (PP2C) and farnesyltransferases (like ERA1) act as negative regulators (Schroeder et al. 2001). In addition to ABA, drought-induced variations in xylem pH have also been related to changes in gs (Rodrigues et al. 2008). Although the molecular mechanism for these changes is unknown, Wilkinson and Davies (2008) have shown that xylem pH can be manipulated using buffered foliar sprays, resulting in modified gs, and have therefore proposed this method for managing WUE in a horticultural context.

In addition to chemical signaling, losses of hydraulic conductivity of xylem vessels have been suggested to induce down-regulation of gs in grapevines under water stress (Lovisolo and Schubert 1998; Rodrigues et al. 2008), but especially during recovery after water stress (Lovisolo et al. 2008a; Pou et al. 2008). In particular, root hydraulic conductivity (Vandeleur et al. 2009) and the conductivity of leaf petioles (Lovisolo et al. 2008a) have been related to gs. Differences in root hydraulic conductivity and vulnerability among cultivars of Vitis vinifera have been described, which can be either that of the cultivar itself (Vandeleur et al. 2009) or the rootstock on which the cultivar is grafted (Alsina et al. 2007; Lovisolo et al. 2008a, b). Although part of hydraulic losses may be due to embolism-induced cavitation of xylem vessels, an important regulatory role of aquaporins has been demonstrated (Galmés et al. 2007; Lovisolo et al. 2008b; Vandeleur et al. 2009). Indeed, during water stress, drought-adapted Vitis berlandieri x V. rupestris rootstocks present a higher proportion of aquaporin-dependent, rapidly reversible down-regulation of hydraulic conductivity and a lower proportion of embolism-dependent loss of conductivity than drought-sensitive Vitis berlandieri x V. riparia rootstocks (Lovisolo et al. 2008b). The genes for several plasma membrane intrinsic protein aquaporins (PIP1 and PIP2) as well as tonoplast intrinsic proteins (TIPs) are well-characterized. In leaves, all these aquaporin genes are down-regulated during water stress and up-regulated after re-watering (Galmés et al. 2007). In roots, instead, some are unresponsive and some are up-regulated during water stress and down-regulated after re-watering (Galmés et al. 2007). This differential behavior illustrates the different function of aquaporins in leaves and roots, and the complex nature of their regulation (Kaldenhoff et al. 2008). A recent study (Vandeleur et al. 2009) has demonstrated that the low stomatal control associated with anisohydric behavior in Chardonnay depends on the maintenance, during water stress, of constant amplitude in the diurnal variations of root hydraulic conductance, which is instead reduced in isohydric cultivar Grenache. These differences are associated with different expression patterns of aquaporins. In addition, aquaporins have also been suggested to
be directly involved in guard cell movements, although this has yet to be demonstrated in mesophyll conductance to CO₂. Thus, aquaporins have diverse important roles and are therefore a key target for genetically improving WUE.

Clearly, aquaporins have diverse important roles in processes associated with WUE and are therefore a key target for genetically improving WUE.

In summary, genetic engineering for regulated gs and improved WUE can be achieved based on genes involved in ABA synthesis and signaling, as well as in aquaporins. Nevertheless, as discussed in previous sections, improving leaf-level WUE by means of reducing gs may result in decreased photosynthesis and yield and, sometimes, in decreased whole-plant WUE. Therefore, increasing WUE by means of decreasing plant respiration or increasing leaf photosynthesis at any given level of transpiration are likely to be more successful.

Decreasing plant respiration

Although plant respiration is a fundamental process for plant life, sustaining plant growth (growth respiration) and maintenance, which includes ion transport and compartmentation, protein turnover and tissue acclimation to environmental change (Amthor 2000; Sharma-Natu and Ghildiyal 2005), it is also associated with important carbon losses which decreased net carbon gain and hence WUE. In absolute values, the rates of respiration are much lower than photosynthesis rates. However, since photosynthesis occurs in leaves and during the day only while respiration occurs in all plant organs and through the entire plant life, the overall carbon losses by respiration are substantial, and the percentage of carbon gained in photosynthesis that is lost by whole plant respiration to on a seasonal basis typically ranges from 30 to 90%, depending on the plant types and environmental conditions (Amthor 2000). Such estimates have not been properly done in grapevines, because very few studies have addressed respiration rates in this species, especially in roots (Comas et al. 2000; Huang et al. 2005). For leaves, it has been shown that respiration rates do not differ between sun leaves with high photosynthesis rates and shade leaves with low photosynthesis (Zufferey et al. 2000; Escalona et al. 1999a, 2003), contributing to low WUE in the latter leaves and in the whole plant. Moreover, contrary to photosynthesis, leaf respiration is not impaired under water stress (Escalona et al. 1999b, 2003), and indeed it is strongly enhanced by increased leaf temperature (Zufferey et al. 2000), a condition occurring under water stress (Figure 5).

For all these reasons, and because maintenance respiration uses a substantial proportion of the total carbon assimilated, it has been suggested that crop production and WUE could be increased by reducing this component of respiration in favor of growth respiration. In particular, since ca. 40% of total maintenance respiration is associated with cyanide-resistant alternative oxidase (AOX, Florez-Sarasa et al. 2007), and this pathway does not contribute to ATP synthesis and growth, it has been suggested that reducing AOX could result in significant increases of plant carbon balance and WUE (Loomis and Amthor 1999; Sharma-Natu and Ghildiyal 2005). However, measurements of AOX activity in grapevines are lacking, and its potential role in more fundamental processes including photosynthesis (Juszczuk et al. 2007) suggest that further studies are needed to properly characterize plant respiration in grapevines prior to selecting it as a target for improving WUE.

Increasing leaf photosynthesis

Mesophyll conductance to CO₂: improving CO₂ availability for photosynthesis
pathway through stomata is regulated by stomatal conductance ($g_s$), which affects both photosynthesis and transpiration so that increasing photosynthesis by increasing $g_s$ results in reduced WUE (Figure 4). Leaf internal CO$_2$ diffusion component is determined by the so-called mesophyll conductance ($g_m$), which can be divided in at least three components, i.e., conductance through intercellular air spaces ($g_{ia}$), through cell wall ($g_w$) and through the liquid phase inside cells ($g_{liq}$). Concerning $g_m$, evidence has accumulated showing that, contrary to early thoughts, this is finite and variable, and that it can change as fast as $g_s$ in response to environmental variables (reviewed in Flexas et al., 2008). While $g_{ia}$ and $g_s$ mostly depend on complex leaf structural traits that may not change in the short term, $g_{liq}$ has been shown to partly depend on rapidly regulated proteins, particularly aquaporins (Hanba et al. 2004; Flexas et al. 2006b).

Several authors have already suggested that increasing $g_m$ would increase WUE (Flexas et al., 2008), because it would result in increased $C_s$ and hence photosynthesis ($A_S$) without any effect on leaf transpiration, i.e., will displace the $A_S/g_s$ relationship vertically (Figure 4). Moreover, one environmental variable typically inducing strong decreases of $g_m$ is water stress (Flexas et al. 2002b; Warren, 2008). A recent study by Miyazawa et al. (2008) in tobacco showed that water stress-induced decreases of $g_m$ could be mimicked by adding HgCl$_2$ to well-watered plants. Since HgCl$_2$ is an inhibitor of gating some aquaporins, and there were no evidences for water stress-induced changes in the amounts of aquaporins, it was suggested that water stress led to decreased $g_m$ by means of aquaporin closing (Miyazawa et al. 2008). Therefore, we propose that increasing $g_m$ at any given $g_s$ (i.e., increasing the ratio $g_m/g_s$) will result in increased $A_S/g_s$ and, hence, potentially in increased plant WUE (Figure 2).

This would certainly be the case under water stress in field grown grapevines, under the conditions described in Figure 5 (Figure 7). With decreasing $g_s$ due to progressive drought, both $g_m/g_s$ and $A_S/g_s$ increased simultaneously (Figure 7A), except at very severe water stress (i.e., the lowest $g_s$) in which $A_S/g_s$ decreased due to metabolic impairment of photosynthesis (Flexas et al. 2002b) while $g_m/g_s$ still increased. With the exception of this value, a high degree of correlation was observed between $g_m/g_s$ and $A_S/g_s$ (Figure 7B). Similar simultaneous increases in $g_m/g_s$ and $A_S/g_s$ were shown in water stressed tobacco or in irrigated tobacco after mercurial addition (Miyazawa et al. 2008).

Therefore, it appears that $g_m$ is a potential target for breeding and/or genetic engineering of WUE. That $g_m$ has a genetic basis in grapevines was evidenced by Patakas et al. (2003a, b), who described important differences among cultivars in the leaf anatomical characteristics typically affecting $g_{ia}$, $g_w$ and, perhaps, $g_{liq}$ (i.e., $g_m$), such as palisade and spongy parenchyma thickness, the fraction of the intercellular air spaces, or the surface of mesophyll cells exposed to the intercellular air spaces per unit leaf area. These anatomical differences were related to differences in photosynthetic efficiency, but $g_m$ was not determined in that study.

However, Bota et al. (2001) performed simultaneous measurements of gas exchange and chlorophyll fluorescence in up to 20 different grapevine cultivars, subjected to either full irrigation or water stress. From these experimental data, a preliminary, rough estimate of $g_m$ was calculated using the variable chlorophyll fluorescence method already developed for grapevines (Flexas et al. 2008). The estimations of $g_m$ are defined as $g_m$ because several input parameters of this method (i.e., leaf absorptance and light distribution between photosystems I and...
Rubisco specificity factor and leaf respiration) were not determined specifically for each cultivar and treatment, but a common constant value was used after determinations in cultivars Tempranillo and Manto Negro (Flexas et al. 2002b). Although differences among cultivars in Rubisco specificity are probably low (Bota et al. 2002, see next section), slight inter-cultivar differences in leaf absorptance and respiration may bias results to some extent (indeed, of the 20 cultivars originally evaluated only 18 were included in the present study, the other two displaying unreliable $g_m$ values). Despite their preliminary character, the results are promising since significant differences in $g_m/g_s$ were strongly and positively correlated with the already described differences in $A_{N}/g_s$, both considering irrigated plants only or including water stressed plants (Figure 8).

In summary, differences in $g_m$ among grapevines cultivars exist, associated to leaf anatomical differences (Patakas et al. 2003a, b) and, perhaps, to patterns of aquaporin expression or activity, which translate in differences in $A_{N}/g_s$, both under irrigation and water stress (Figure 8). While anatomical differences may depend on complex gene interactions, aquaporins depend on single genes and their post-translational regulation may be more complex, for which they are primary target candidates to attempt genetic manipulation aimed to increase WUE. Studies are urgently needed to extent our knowledge on genotypic variations in $g_m$ and WUE, to evaluate whether these translate into whole-plant and yield-based differences in WUE, and to identify specific aquaporins responsible of these differences.

### Rubisco: improving CO$_2$ availability for photosynthesis

Because grapevine photosynthesis operates in the Rubisco-limited region and decreased $g_s$ and $g_m$ during water stress results in lowered chloroplast CO$_2$ availability ($C_o$), increasing Rubisco specificity for CO$_2$ may be more effective in increasing WUE in grapevines under Mediterranean conditions than increasing Rubisco abundance, activation state or catalytic rate (Parry et al. 2007). Nevertheless, increased Rubisco activity should not be totally ruled out as a way to improve WUE. In a recent study with the Vitis rootstock Richter-110 (Flexas et al. 2009), changes in the maximum velocity of carboxylation ($V_{c,max}$, which relates to Rubisco activity) occurred as a result of water stress and re-watering and were related to WUE. In these plants, $V_{c,max}$ decreased during water stress (while $A_{N}/g_s$ increased as a consequence of increased $g_m/g_s$), but acclimation to water stress during one week resulted in increased $V_{c,max}$ above control values after re-watering, so that $A_{N}/g_s$ in previously-stressed plants was kept higher than in irrigated plants during weeks. Therefore, although further studies are needed to verify this trend, increasing Rubisco capacity could be a means of increasing WUE during water stress re-watering cycles in grapevines.

Rubisco specificity for CO$_2$ ($\tau$) was determined in two grapevine cultivars, Tempranillo and Manto Negro. As expected, an identical value (100 mol mol$^{-1}$) was obtained (Bota et al. 2002). It is very unlikely that evolution under human selection resulted in relevant differences in the $rbcL$ sequence among cultivars. Nevertheless, contrary to early thoughts, there is now evidence that substantial variability in $\tau$ exists even within $C_3$ plants, and values larger than those of grapevines (i.e., up to 110 mol mol$^{-1}$) have been described for the Mediterranean species Limonium gibertii (Galmes et al. 2005). Even higher values (up to 240 mol mol$^{-1}$) are found in some red algae, the highest known value corresponding to Galdieria partita (Uemura et al. 1997). Therefore, there are better forms of Rubisco outside grapevines, which may be used to improve carboxylation efficiency and WUE. Moreover, such differences have a clear genetic basis. For instance, the large subunits of Rubisco in spinach ($\tau = 80$), grapevines
present up to 39 non-conserved aminoacids (not with Limonium), Limonium gibertii present up to 39 non-conserved aminoacids (not with Vitis). Although this result may suggest that improvement of $\tau$ could be achieved by site-directed mutagenesis, this goal seems yet too far because of the lack of complete knowledge on the relationship between specific aminoacids and Rubisco kinetics. Therefore, trying to replace a native Rubisco with a better foreign one seems to be the most likely option to succeed, at least in the short term.

Advances in chloroplast transformation have lead to successful replacement of the native Rubisco large subunit of tobacco by other higher plants versions (Kanevski et al., 1999; Whitney & Andrews, 2001; Sharwood et al. 2007). So far, transformed plants present low amounts of Rubisco due to the lack of proper chaperones and other molecules necessary for correct transcription, translation and assembling of the enzyme (Sharwood et al. 2008). However, these seem to be minor problems and there are good perspectives towards the possibility of obtaining improved plants by transferring Rubiscos within higher plants.

Bearing this in mind, a simulation of the theoretical effects of placing Rubisco from Limonium gibertii into grapevines is presented. Parameters from real conditions, as those described in Figure 5 (i.e., assuming that transformation will not affect conductances to CO$_2$ and leaf temperature), and the model by Farquhar et al. (1980) have been used. Applying this model, values of $A_N$ (Figure 10A) and of $A_N/g_s$ (Figure 10B) are calculated for each water stress condition in Figure 5. We have considered three different scenarios: a grapevine plant with a Rubisco specificity factor ($\tau$) equal to that of Limonium but with a maximum velocity of carboxylation ($V_{\text{c,max}}$) of (1) 80 $\mu$mol m$^{-2}$ s$^{-1}$, as described for Vitis vinifera by Schultz (2003), (2) 100 $\mu$mol m$^{-2}$ s$^{-1}$, as described for Vitis vinifera by Flexas et al. (2006a), or (3) 120 $\mu$mol m$^{-2}$ s$^{-1}$, as described for Limonium gibertii by Galmés et al. (unpublished). Depending on the scenario considered, such transformation would result in potential increases of maximum net photosynthesis ($A_N$) by 15 to 80% as compared to current values (Figure 10A). This would result in similar increases of intrinsic WUE under irrigation, but these increases will become much larger as water stress intensifies, potentially reaching increases of up to 100% or higher (Figure 10B).

Therefore, the expected benefits of Rubisco engineering on WUE are high, and hence this should be a priority area of research for the immediate future. In particular, Rubiscos from grape cultivars should be fully characterized both molecularly (sequence) and biochemically ($\tau$), and the first steps for chloroplast transformation in grapevines attempted.

Concluding remarks

The reduction of watering necessities and the improvement of the water use efficiency by grapevines is an important target for present and will be even more in the future according to the climate change predictions, to secure an environmentally friendly and sustainable viticulture.

As this review paper resumes, there are different agronomic ways to improve the WUE (summarized in Table 2) which, moreover, also lead to the improvement of grape quality. Important progress can be achieved from agronomic practices addressed to reduce direct soil evaporation and runoff as mulching, vegetative growth contention by spring competence for water by cover crops, and green pruning, but a precise evaluation...
In terms of contribution to water saving and WUE improvement in different environments is urgent. Most recently, long term improvements of grapevine’s genetic capacity for water saving and increasing plant water use efficiency (Table 2) have been highlighted as a promising way. These relay on a better control of water losses (reducing night transpiration, improvement of stomatal control), and recent biotechnological achievements in other plants species open interesting areas to explore a tighter transpiration control, even though the concomitant reductions in carbon gain should be taken in to account to evaluate the results.

Alternatively, WUE may be increased by genetically improving net plant carbon uptake, through either reducing carbon losses in respiration or improving photosynthesis gains. Recent reports on the role of mesophyll conductance in photosynthesis limitations and the relationships of CO2 diffusion with the aquaporin expression suggest that these may be a potential target to achieve such goal. On the other hand, recent identification of plants with superior Rubisco specificity factor, which could be transferred to crops as grapevine, seems an exciting way which merits to be explored.

As the present review shows, improving WUE is an unavoidable subject of research for sustainable viticulture, and offers fascinating areas of research which merits to be explored in near future.

Acknowledgements

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The control of transpiration. Insights from ARS.- 579-580.


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The data were collected using a Li-6400 (Li-Cor Inc, NE), in complete darkness, an atmospheric CO₂ concentration of 365 μmol CO₂ mol⁻¹ air, air temperature 23°C, and leaf-to-air vapor pressure deficit 1.5 KPa. Two cultivars (Tempranillo and Manto Negro) and three different irrigation treatments (consisting in applying drip irrigation twice a week at, respectively, 100%, 30% or 0% the past week potential evapotranspiration, ET₀) were analyzed.

<table>
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<tr>
<th></th>
<th>g_{night}</th>
<th>E_{night}</th>
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<tbody>
<tr>
<td></td>
<td>(mol H₂O m⁻² s⁻¹)</td>
<td>(mmol H₂O m⁻² s⁻¹)</td>
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<tr>
<td>Tempranillo 100% ET₀</td>
<td>0.107 ± 0.009</td>
<td>1.19 ± 0.07</td>
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<tr>
<td>Tempranillo 30% ET₀</td>
<td>0.036 ± 0.007</td>
<td>0.52 ± 0.09</td>
</tr>
<tr>
<td>Tempranillo 0% ET₀</td>
<td>0.009 ± 0.001</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>Manto Negro 100% ET₀</td>
<td>0.033 ± 0.003</td>
<td>0.45 ± 0.03</td>
</tr>
<tr>
<td>Manto Negro 30% ET₀</td>
<td>0.029 ± 0.006</td>
<td>0.41 ± 0.08</td>
</tr>
<tr>
<td>Manto Negro 0% ET₀</td>
<td>0.019 ± 0.003</td>
<td>0.29 ± 0.04</td>
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Table 2. Summary of the different ways proposed in the present review to improve WUE, with their corresponding techniques and targets, including those achievable by biotechnology. For agronomic techniques, two references are given as examples of their application in grapevines, while for biotechnological techniques the first reference is general and the second refers specifically to grapevines.

<table>
<thead>
<tr>
<th>WAY TO IMPROVE WUE</th>
<th>TECHNIQUE / TARGET</th>
<th>REFERENCE (s)</th>
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<tbody>
<tr>
<td><strong>AGRONOMY / MANAGEMENT</strong></td>
<td></td>
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<tr>
<td>Reducing soil evaporation and runoff</td>
<td>Mulching</td>
<td>Buckerfield &amp; Webster (2001); Hatfield et al. (2001)</td>
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<td></td>
<td>Vineyard inter-cropping</td>
<td>Monteiro &amp; Lopes (2007); Gulfs et al. (2008)</td>
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<tr>
<td>Modifying root growth patterns + inducing partial stomatal closure and reduced plant transpiration</td>
<td>Regulated Deficit Irrigation (RDI)</td>
<td>Cifre et al. (2005); Costa et al. (2007)</td>
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<td></td>
<td>Partial Root Drying (PRD)</td>
<td>Dry et al. (2001); Chaves et al. (2007)</td>
</tr>
<tr>
<td>Optimizing light interception by the canopy and radiation use efficiency</td>
<td>Training system</td>
<td>Carbonneau (1980); Williams &amp; Ayars 2005</td>
</tr>
<tr>
<td></td>
<td>Prunning</td>
<td>Escalona et al. (1999a); Escalona et al. (2003)</td>
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<tr>
<td><strong>BIOTECHNOLOGY</strong></td>
<td></td>
<td></td>
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<tr>
<td>Reducing leaf (and canopy) transpiration</td>
<td>Reducing cuticular conductance</td>
<td>------- (target genes unknown)</td>
</tr>
<tr>
<td></td>
<td>Reducing night and/or day stomatal conductance (ABA-genes)</td>
<td>Nilson &amp; Assmann (2007); Matus et al. (2008)</td>
</tr>
<tr>
<td>Optimizing water uptake, transport and transpiration</td>
<td>Aquaporins</td>
<td>Kaldenhoff et al. (2008); Vandeleur et al. (2009)</td>
</tr>
<tr>
<td>Reducing carbon losses in respiration</td>
<td>Reducing alternative oxidase</td>
<td>Loomis &amp; Amthor (1999); present review</td>
</tr>
<tr>
<td>Increasing photosynthesis at any given rate of transpiration</td>
<td>Increasing CO₂ availability (aquaporin-mediated gₘ)</td>
<td>Flexas et al. (2008); present review</td>
</tr>
<tr>
<td></td>
<td>Increasing carboxylation efficiency (Rubisco specificity for CO₂)</td>
<td>Parry et al. (2007), present review</td>
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Figure 1: Global distribution of the major viticultural areas (gray and black) with those suffering from moderate to severe drought (i.e. those with a current annual rainfall inferior to 700 L m$^{-2}$, black).
Figure 2. Theoretical diagram showing the dependency of plant water use efficiency (WUE) on different processes and its links with leaf-level WUE (some important parameters related to different processes are indicated in parenthesis). See text for details.
Figure 3. The relationship between intrinsic leaf WUE ($A_{N}/g_{s}$) and whole plant WUE (based on vegetative growth, first-year plants not producing grapes) in 5 different cultivars of *Vitis vinifera* growing outdoors in pots during summer in Mallorca (Balearic Islands, Spain). Closed symbols represent irrigated treatment at field capacity and open symbols to non-irrigated treatment defined by the leaf maximum daily $g_{s}$ (about 0.05 mol H$_2$O m$^{-2}$s$^{-1}$). Different cultivars symbols are: Grenache (circles), Callet (squares), Tempranillo (triangles up), Malvasia of Banyalbufar (triangles down) and Cabernet Sauvignon (diamonds). Values represent means ± SE of 6 ($A_{N}/g_{s}$) or 4 replicates (whole plant WUE). Regression lines are displayed with their $R^2$. 
Figure 4. The relationship between net photosynthesis ($A_N$) and stomatal conductance ($g_s$) in grapevines. Empty circles are real data for many different grapevine cultivars and conditions (modified after Cifre et al. 2005) and gray lines are the idealized upper and lower limits for this relationship. Black arrows indicate two potential ways to increase WUE (see text for details).
Figure 5. Common responses of several leaf parameters to progressive water stress in field-grown grapevines in Mallorca (Balearic Islands, Spain). Water stress progression in intensity is represented as the decline of stomatal conductance ($g_s$, from right to left in the X-axis). The represented parameters include leaf temperature (gray triangles), mesophyll conductance to CO$_2$ ($g_m$, solid circles) and the mean CO$_2$ concentration in the chloroplasts ($C_c$, empty circles). The data are averaged for discrete intervals of $g_s$ and for two cultivars (Tempranillo and Manto Negro) showing similar response to water stress. The original data for each of the two cultivars are published elsewhere (Flexas et al. 2002).
Water stress + high temperature

Stomatal closure and decreased \( g_m \)

\[ \text{[CO}_2\text{]} \text{ at the Rubisco locus} \]

To increase \([\text{CO}_2]\) in the chloroplast

Increasing \([\text{CO}_2]\) assimilation efficiency

Improving Rubisco specificity factor for \( \text{CO}_2 \)

Figure 6. Diagram showing the physiological bases for decreased photosynthesis under combined water stress and high temperature (as often found in many semi-arid viticultural areas), and highlighting (bold) the theoretical targets to improve photosynthesis under these conditions.
Figure 7. A. The response of leaf intrinsic WUE ($A_N/g_s$, filled circles) and the ratio of mesophyll to stomatal conductance to CO$_2$ ($g_m/g_s$, empty circles) during progressive water stress in field-grown grapevines in Mallorca (Balearic Islands, Spain). Environmental conditions and original data are as for Figure 3. B. The relationship between $A_N/g_s$ and $g_m/g_s$ during the drought cycle (the outlier corresponds to the lowest absolute $g_s$, when metabolic impairment of photosynthesis occurred, see Flexas et al. 2002).
Figure 8. The relationship between $A_{\text{N}}/g_{\text{s}}$ and $g_{\text{m}}/g_{\text{s}}$ in 18 different cultivars of *Vitis vinifera* growing outdoors in pots during summer in Mallorca (Balearic Islands, Spain), and either daily irrigated at field capacity (filled circles) or six days after withholding water (empty circles, corresponding to a 40% decline in substrate water content). Data re-calculated after Bota et al. (2001).
**Figure 9.** Alignment (Vector NTI, Invitrogen) of the amino acid sequences for the Rubisco large subunit from *Limonium gibertii* (τ = 110, GenBank accession number CAH10354), *Vitis vinifera* (τ = 100, accession number YP67084) and *Spinacia oleracea* (τ = 80, accession number NP054944). Yellow boxes indicate fully conserved residues among species. Blue boxes indicate partially conserved residues, while the exceptions are indicated in green (similar type of amino acids) or white (different type of amino acids).
Figure 10. Model simulation of the effects of replacing grapevine native Rubisco by Rubisco from Limonium gibertii on (A) net photosynthesis ($A_N$) and (B) intrinsic WUE ($A_N/g_s$) at different water stress intensities (i.e., different stomatal conductance, $g_s$). The environmental conditions and original data (black circles) are as for Figure 3. Three different scenarios are considered, representing the achievement of a grapevine plant with a Rubisco specificity factor ($\tau$) equal to that of Limonium but with a maximum velocity of carboxylation ($V_{c,max}$) of (1) 80 $\mu$mol m$^{-2}$ s$^{-1}$ (dark gray squares), as described for Vitis vinifera by Schultz (2003), (2) 100 $\mu$mol m$^{-2}$ s$^{-1}$ (pale gray rhomboids), as described for Vitis vinifera by Flexas et al. (2006a), or (3) 120 $\mu$mol m$^{-2}$ s$^{-1}$ (pale gray rhomboids), as described for Limonium gibertii by Galmes et al. (unpublished). All the $V_{c,max}$ values are scaled to a leaf temperature of 35°C using the temperature functions described by Bernacchi et al. (2002).