

Diffusional conductances to CO₂ as a target for increasing photosynthesis and photosynthetic water-use efficiency

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Abstract A key objective for sustainable agriculture and forestry is to breed plants with both high carbon gain and water-use efficiency (WUE). At the level of leaf physiology, this implies increasing net photosynthesis (A_N) relative to stomatal conductance (g_s). Here, we review evidence for CO₂ diffusional constraints on photosynthesis and WUE. Analyzing past observations for an extensive pool of crop and wild plant species that vary widely in mesophyll conductance to CO₂ (g_m), g_s , and foliage A_N , it was shown that both g_s and g_m limit A_N , although the relative importance of each of the two conductances depends on species and conditions. Based on Fick's law of diffusion, intrinsic WUE (the ratio A_N/g_s) should correlate

on the ratio g_m/g_s , and not g_m itself. Such a correlation is indeed often observed in the data. However, since besides diffusion A_N also depends on photosynthetic capacity (i.e., $V_{c,max}$), this relationship is not always sustained. It was shown that only in a very few cases, genotype selection has resulted in simultaneous increases of both A_N and WUE. In fact, such a response has never been observed in genetically modified plants specifically engineered for either reduced g_s or enhanced g_m . Although increasing g_m alone would result in increasing photosynthesis, and potentially increasing WUE, in practice, higher WUE seems to be only achieved when there are no parallel changes in g_s . We conclude that for simultaneous improvement of A_N and WUE, genetic manipulation of g_m should avoid parallel changes in g_s , and we suggest that the appropriate trait for selection for enhanced WUE is increased g_m/g_s .

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Introduction

By 2050, an increase in grain yields by 30 % is a prerequisite to fulfill the food requirements of the growing population (Borlaug 2000; Edgerton 2009). So, further improvement in photosynthesis is a necessity for increased production of food and fuel. With the Green Revolution starting in the sixties, grain yields have doubled worldwide (Tilman et al. 2002; Araus et al. 2008). This has been achieved not only by releasing genotypes with higher biomass partitioning to grains, i.e., higher harvest index (Araus et al. 2008), but also by increasing the use of pesticides, fertilizers, and, notably, water for irrigation (Tilman et al. 2002). Rockström et al. (2007) estimated that water use for irrigation would need to be increased by 100 % to achieve a 30 % yield increase with current agricultural systems and genotypes. However, the supply of fresh water is a factor limiting world agriculture, which already uses some 70 % of overall withdrawal of water resources globally (Borlaug 2000; Fereres and Connor 2004; Morison et al. 2008). Furthermore, it has been suggested that climate change may exacerbate the present shortage of water and is likely to increase drought in some Earth regions (IPCC 2007), although this has not happened yet at the extent predicted (Dai 2011; Sheffield et al. 2012). Therefore, increasing irrigation water by 100 % on a global scale is simply not possible. Moreover, large extractions of water for the purposes of agriculture have resulted in major ecological damage, as has field runoff of fertilizers and pesticides (Jackson et al. 2001; Jobbagy and Jackson 2004). Clearly, to make agriculture sustainable requires a major reduction in water use in many regions as well as taking great care in any further increases in the use of fertilizers. It is therefore imperative to improve not only yields but also water productivity or water-use efficiency (WUE), i.e., the amount of carbon gained per unit water used, and nitrogen-

use efficiency (NUE) too. According to Norman E. Borlaug, the father of the Green Revolution and 1970 Nobel Peace Prize Laureate, “humankind in the 21st century will need to bring about a ‘Blue Revolution’ to complement the ‘Green Revolution’ of the 20th century. In the new Blue Revolution, water-use productivity must be wedded to land-use productivity. New science and technology must lead the way” (Borlaug 2000).

Complementing agronomic approaches (Gregory 2004), WUE can also be improved at the physiological level (Boyer 1996; Parry et al. 2005), where the issue is often approached using the “intrinsic” WUE, i.e., the ratio of net assimilation (A_N) to stomatal conductance (g_s), which was introduced to compare photosynthetic properties at a common evaporative demand (Osmond et al. 1980). Differences between genotypes in A_N/g_s and WUE have been reported to have a genetic basis (Martin et al. 1989; Masle et al. 2005), and breeding for high WUE has become a main objective for many crops (Condon et al. 2004). But, genotypic variations in WUE often result mainly from variations in g_s and not A_N (Blum 2005). An increase in WUE due to reduction in g_s results in reduced photosynthesis and yield, and indeed increased yields speeded up by the Green Revolution have resulted in decreased WUE (Barbour et al. 2000; Rockström et al. 2007). Increasing WUE while simultaneously increasing yield 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). The feasibility of this option is demonstrated by the fact that increased photosynthetic capacity accompanied by decreased stomatal conductance and enhanced WUE has been observed simultaneously in the *Arabidopsis ERECTA* mutants (Masle et al. 2005), while in Drysdale wheat obtained by breeding for dry conditions, higher yields are concomitant with improved WUE in water-limited conditions (Rebetzke et al. 2002; CSIRO Plant Industry Communication Group 2004). However, the physiological basis for yield increase in these studies is largely unknown. Although some success has been undoubtedly achieved via natural mutation and conventional breeding, there is a growing consensus that further improvement in yields should still be possible via direct genetic manipulation of plants, albeit the success has been fairly moderate so far (Huang et al. 2002; Edgerton 2009).

At the leaf level, simultaneous increases of A_N and A_N/g_s could theoretically be achieved by improving the carboxylation rate through increased concentration of catalytically active Rubisco sites and/or improved enzyme's kinetic characteristics (Galmés et al. 2005; Whitney et al. 2011), or bypassing photorespiration (Kebeish et al. 2007; Peterhansel and Maurino 2011), and/or by improving CO_2 diffusion in the mesophyll through introducing a C_4 -like

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photosynthetic metabolism in C_3 plants (Leegood 2002), inserting cyanobacterial bicarbonate transporters in C_3 plants (Price et al. 2011), or increasing the mesophyll diffusion conductance to CO_2 (g_m) from sub-stomatal cavities to the site of fixation (Parry et al. 2005; Flexas et al. 2010; Zhu et al. 2010). Here, we focus on the latter potential way of improvement of photosynthesis.

While the biochemistry of the photosynthetic machinery depends on well-known specific genes, g_m is a complex feature involving different structural features and molecular controls, all of which have not yet been fully understood. Some specific genes (e.g., aquaporin NtAQP1) have been identified that apparently affect g_m (Uehlein and Kaldenhoff 2008; Flexas et al. 2006a; Evans et al. 2009), so that genetic manipulation of g_m may be possible. Evidence suggests that g_m is variable due to differences in leaf anatomy (Tosens et al. 2012; Tomás et al. 2013), but it can also change as quickly as g_s in response to environmental variables, perhaps because aquaporins and/or carbonic anhydrase are involved in its regulation (reviewed in Flexas et al. 2008; Warren 2008a; Evans et al. 2009). Variations of g_m have important implications for plant photosynthesis and productivity (Niinemets et al. 2009a, b) and may play an important role in vegetation distribution (Niinemets et al. 2011). Several authors have already suggested that modifying g_m would affect WUE and the relationship between WUE and ^{13}C discrimination (Parry et al. 2005; Warren and Adams 2006; Flexas et al. 2008; Seibt et al. 2008; Bickford et al. 2010). Moreover, the effects of genotypic differences in g_m on WUE were recently demonstrated in barley (*Hordeum vulgare*) (Barbour et al. 2010) and among wild balsam poplar (*Populus balsamifera*) genotypes sampled across a latitudinal gradient (Soolanayakanahally et al. 2009). However, the effects of varying g_m or g_m/g_s on A_N and A_N/g_s across a wide range of species have not been either theoretically or experimentally tested.

From Fick's first law of diffusion, at steady state, A_N can be expressed as (Gaastra 1959; Farquhar and Sharkey 1982; Long and Bernacchi 2003)

$$A_N = g_s(C_a - C_i) \quad (1)$$

where C_a is the CO_2 concentration in the ambient air (leaf boundary layer) and C_i is that in sub-stomatal cavities. In a steady state, the flux of CO_2 can also be written as the product of g_m and CO_2 concentration differential between sub-stomatal cavities and chloroplasts (C_c):

$$A_N = g_m(C_i - C_c). \quad (2)$$

Combining Eqs. 1 and 2, we can express the steady-state rate of net assimilation as the product of overall diffusion

conductance from ambient air to chloroplasts (the sum of two resistances in series) and the overall CO_2 concentration difference between ambient air and chloroplasts $C_a - C_c$ (Niinemets et al. 2009b):

$$A_N = \frac{1}{\frac{1}{g_s} + \frac{1}{g_m}}(C_a - C_c) \quad (3)$$

This equation demonstrates that the relative magnitude of g_s and g_m alters the distribution of overall resistance to CO_2 transfer between the stomata and mesophyll. As the stomatal diffusion component is also inevitably affecting the water use, while the mesophyll component is only affecting photosynthesis, enhancing g_m/g_s ratio can significantly improve both WUE and productivity.

Dividing both terms in Eq. [2] by g_s , or solving C_i and combining Eqs. [1] and [2], the following expressions are obtained:

$$A_N/g_s = g_m/g_s(C_i - C_c) \quad (4)$$

$$A_N/g_s = \frac{g_m/g_s}{1 + g_m/g_s}(C_a - C_c) \quad (5)$$

Although these expressions are simply directly derived from the Fick's first law, and [4] actually is identical to [2], it is still worthwhile expressing them in this way to highlight several aspects of the interdependency among different variables, and in particular that of A_N/g_s on g_m ¹:

- (1) While A_N depends directly on g_m (Eq. 2), A_N/g_s may not be expected to directly depend on g_m , but on the ratio of g_m to g_s .
- (2) Equation [4] resembles a linear relationship, but Eq. [5] describes a hyperbolic dependency of A_N/g_s on g_m/g_s . From Eqs. 1 and 2,

$$C_c = \frac{C_i(g_s + g_m) - g_s C_a}{g_m} \quad (6)$$

Thus, hyperbolic dependence results from the effects of both g_m and g_s on C_i and C_c and from the effect of C_c on A_N .

- (3) Although both equations suggest a direct, positive relationship between A_N/g_s and g_m/g_s , this may not necessarily hold because in both equations, the second term may not be constant. C_a can vary with long-term changes in atmospheric composition, while variations of C_i and C_c are more complex, depending on C_a , g_s , and g_m themselves (Niinemets et al. 2009a),

¹ Notice that g_s is expressed in $\text{mol } CO_2 \text{ m}^{-2} \text{ s}^{-1}$ in all the equations above to fulfill the internal units' requirements of the equations. However, when A_N/g_s is used as a surrogate for intrinsic WUE, g_s should be expressed in $\text{mol } H_2O \text{ m}^{-2} \text{ s}^{-1}$. While we will use A_N/g_s in $\mu\text{mol } CO_2 \text{ mol}^{-1} CO_2$ throughout the paper, values can be easily converted to the most common unit, $\mu\text{mol } CO_2 \text{ mol}^{-1} H_2O$, by simply dividing them by 1.6 (which is the ratio of H_2O/CO_2 diffusivities in air).

as well as on the photosynthetic activity, which in turn depends mostly on light availability, leaf temperature, and Rubisco amounts and kinetics (Farquhar et al. 1980). For the same reasons, the equivalent relationship between A_N and g_m may not necessarily hold.

- (4) Therefore, this *apparent* dependency can be circumvented depending on C_a and on how C_i and C_c are regulated. In particular, an inverse co-variation of g_m/g_s and either $C_a - C_i$ or $C_i - C_c$ could result in a lack of correlation between A_N and either g_s or g_m , and/or an unchanged A_N/g_s despite the variations in g_m/g_s .

Despite some complexity, the positive relationships between A_N and g_m and A_N/g_s and g_m/g_s , *if holding*, would demonstrate the potential of improving A_N and WUE by means of genetically manipulating g_m , a conclusion of great help for biotechnologists in their search for targets for genetic improvement of plants. By making use of the large database on g_m in the literature, we review some of these relationships in nature at different scales: (1) at the plant functional type and species scale, by comparing different plant groups and species (the evolutionary scale); (2) at the genotypic scale, by comparing different genotypes within given crops (the scale for agronomic selection); and (3) at the sub-genotypic or gene scale, by assessing results in plants with altered genes specifically affecting g_s or g_m (the scale for biotechnological approaches).

Methods

Literature dataset for wild plants and crop plants

The data used in this review were derived predominantly from publications reporting measurements made on multiple species subjected to experimental water stress (see Online Resource 1 for the data and Online Resource 2 for references of studies used). Data from both control (non-water-stressed) and water-stressed plants were used, including measurements made on sun and shade leaves, plants grown at different altitudes, and leaves of different ages, but excluding data involving any additional abiotic or biotic stress factor. In all these studies, basic gas-exchange parameters (A_N , g_s , and C_i) were determined at saturating light using gas-exchange systems, while C_c and g_m were determined by curve-fitting methods or simultaneous measurements of chlorophyll fluorescence and/or on-line isotope discrimination according to procedures described elsewhere (Warren 2006). Average data as presented in the original references were used. When one or more of the

five basic parameters used (A_N , g_s , g_m , C_i , or C_c) were not shown in the original reference, they were calculated from average data for the other parameters using $A_N = g_s (C_a - C_i) = g_m (C_i - C_c)$. Only studies where all five basic parameters could be derived were included in this study, and the data were filtered to keep only the data at saturating irradiance. The total number of measurements in non-stressed plants was 96 and in water-stressed plants 98. Altogether, data for 44 species (for some of them, a number of varieties or provenances were also included) were available. Among them, six species were woody gymnosperms and the rest were angiosperms. The latter included dicotyledonous annual herbs (7 species), perennial herbs (2 species), and woody perennials (29 species). For five species—rice (*Oryza sativa*) (Centritto et al. 2009), balsam poplar (*P. balsamifera*) (Soolanayakanahally et al. 2009), barley (*H. vulgare*) (Barbour et al. 2010), grapevine (*Vitis vinifera*) (Flexas et al. 2010; Tomas, unpublished), and tomato (*Solanum lycopersicum*) (Galmés et al. 2011)—intraspecific variability could be assessed.

Literature-based dataset for genetically modified genotypes and mutants

An additional dataset (Online Resource 3) comprised transgenic plants and natural mutations reported to specifically alter g_m , including transgenic rice plants expressing barley PIP2 aquaporin (Hanba et al. 2004), transgenic tobacco (*Nicotiana tabacum*) plants with altered levels of native PIP1 aquaporin (Flexas et al. 2006a), tobacco plants with reduced levels of chloroplast carbonic anhydrase (Price et al. 1994; Williams et al. 1996), tobacco plants with altered phytochrome responses having a modified chloroplast distribution (Sharkey et al. 1991), *Chup1* mutants of *Arabidopsis* showing reduced chloroplast exposure to intercellular air spaces (Tholen et al. 2008), cytoplasm male sterility II (CMSII) mutants of *Nicotiana sylvestris* showing impaired mitochondrial function and g_m (Priault et al. 2006), and mitochondrial rearrangement mosaic mutant MSC16 *Cucumis sativus* plants also showing altered g_m (Juszczuk et al. 2007). For comparison, data concerning short-term environmental or chemical manipulations of plants were also incorporated in this second dataset (Online Resource 3), including moderate water stress and addition of $HgCl_2$ to leaves (Miyazawa et al. 2008), rapid leaf desiccation and addition of exogenous ABA (Flexas et al. 2006b), changing leaf-to-air vapor pressure deficit (Warren 2008b), varying leaf temperature (Warren and Dreyer 2006), varying incident light and CO_2 concentration around leaves (Flexas et al. 2007), or filtering blue light to avoid chloroplast movements (Tholen et al. 2008).

Measurements in *Arabidopsis* mutants with varying ABA sensitivity

Additional data were measured for ABA-insensitive and -hypersensitive mutants of *Arabidopsis* (*ost-1*, *abil-2 pp2ca-1*, and *abil-2 pp2ca-1 ost-1-3*). Wild-type and mutant plants were grown in hydroponics under controlled environmental conditions in a growth chamber (12 h of light per day at ca. 150 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, air temperature of 26/22 °C at day/night, and air humidity of about 50 %), as described in Rubio et al. (2009). Leaf gas-exchange and chlorophyll fluorescence measurements at saturating light were conducted in at least four plants per genotype (35–40 days old) before and after adding 0.2 mM ABA to the hydroponic solution, with the measurements on ABA-treated plants being carried out 2 h after ABA application. The variable fluorescence method of Harley et al. (1992) was used to determine g_m . The *ost1* mutant used during this work was obtained from NASC and it corresponds to line SALK_008068. Homozygous individuals were identified using the following primers: Fost1, 5'-GAACGTCGAAGGATATTGGC and Rost1, 5'-GGATGTCTTA AGGACCTGTGG. The original *ost1* alleles described by Mustilli et al. (2002), *ost1-1* and *ost1-2*, were generated in La-er background. Therefore, the new allele described in this work, Columbia background, was named *ost1-3*. The double *abil-2 pp2ca-1* mutant has been described previously (Rubio et al. 2009) and it was crossed with *ost1-3* in order to obtain the triple *abil-2 pp2ca-1 ost1-3* mutant.

On the validity of g_m estimates used in this study

At the time of writing this paper, an important concern has been raised regarding the validity of mesophyll conductance estimates using the common methods from which all data included here arise (Tholen et al. 2012). The reason is that, because the measured A_N is a net flux composed of the sum of photosynthetic, photorespiratory, and respiratory fluxes, and because the distance traveled by CO_2 is not the same for all three fluxes (as the CO_2 entering in the photosynthetic flux ends up at the carboxylation sites inside chloroplast stroma, while the CO_2 released in photorespiration and other respiration processes arises mainly from the mitochondria), the value of g_m retrieved using Fick's first law cannot be considered a purely diffusive conductance (or simple Fick's law proportionality factor), but rather a flux-weighted quantity. It has been demonstrated that, because of this, high rates of (photo)respiration could significantly bias g_m estimates, resulting in an apparent obligatory correlation between stomatal conductance and g_m (Tholen et al. 2012). Despite of this, there are two arguments favoring the general validity of the data

presented. First, Tholen et al. (2012) stated that the g_m estimate is significantly affected by (photo)respiration when g_s drops below 0.1 $\text{mol m}^{-2} \text{s}^{-1}$, while most (but not all) data presented here are above this value (see Fig. 1a and Online Resource 1). Second, the estimated values of g_m can be mimicked by a model consisting of measuring actual CO_2 traveling distances across organelles in mesophyll preparations under a microscope and applying known diffusion coefficients to each pathway component (Peguero-Pina et al. 2012; Tosens et al. 2012; Tomás et al. 2013). The large degree of agreement between the two fully independent estimates strongly supports the idea that the g_m estimates largely reflect a truly diffusive conductance. Overall, these arguments support our use of the g_m estimates from the literature in the present analysis and its treatment as a diffusive conductance, although it has to be kept in mind that the actual values may be to some extent biased by the issue raised by Tholen et al. (2012).

Results and discussion

The relationships between A_N , g_s , g_m and WUE across multiple species

When analyzing the dataset consisting of multiple species (Online Resource 1), under non-stressed conditions, A_N ranges from 1.9 $\mu\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *Castanea sativa* var. Giresun (Lauteri et al. 1997) to 35.1 $\mu\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *Lavatera maritima* (Galmés et al. 2007). g_s ranges from 0.014 $\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *Cicer aurantium* (Loreto et al. 1992) to 0.639 $\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *L. maritima* (Galmés et al. 2007) and g_m ranges from 0.017 $\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *C. sativa* Giresun (Lauteri et al. 1997) to 0.638 $\text{mol CO}_2 \text{mol m}^{-2} \text{s}^{-1}$ in *Triticum* spp. (Loreto et al. 1992). That is, the highest reported A_N is in the species with the highest g_s , and the lowest in the species with the lowest g_m . Under stress conditions, lower values are found for all three parameters. Pooling all data, a positive relationship is observed between A_N and both g_s (Fig. 1a) and g_m (Fig. 1b). In contrast, the correlation between g_s and g_m is poorer (Fig. 1c). These results imply that A_N is strongly influenced by both g_s and g_m , as already suggested (Flexas et al. 2004, 2008, 2012; Warren 2008a). However, which of the two conductances plays the most important role may depend on species-specific anatomical controls on g_m , since g_s and g_m are not strongly correlated when pooling all data together. There is on average a decline in the CO_2 drawdown from the atmosphere to the sub-stomatal cavities ($C_a - C_i$; Fig. 2a) with increasing g_s , although the analogous result with g_m is clearer, with decreasing g_m resulting in a roughly exponential increase of the CO_2

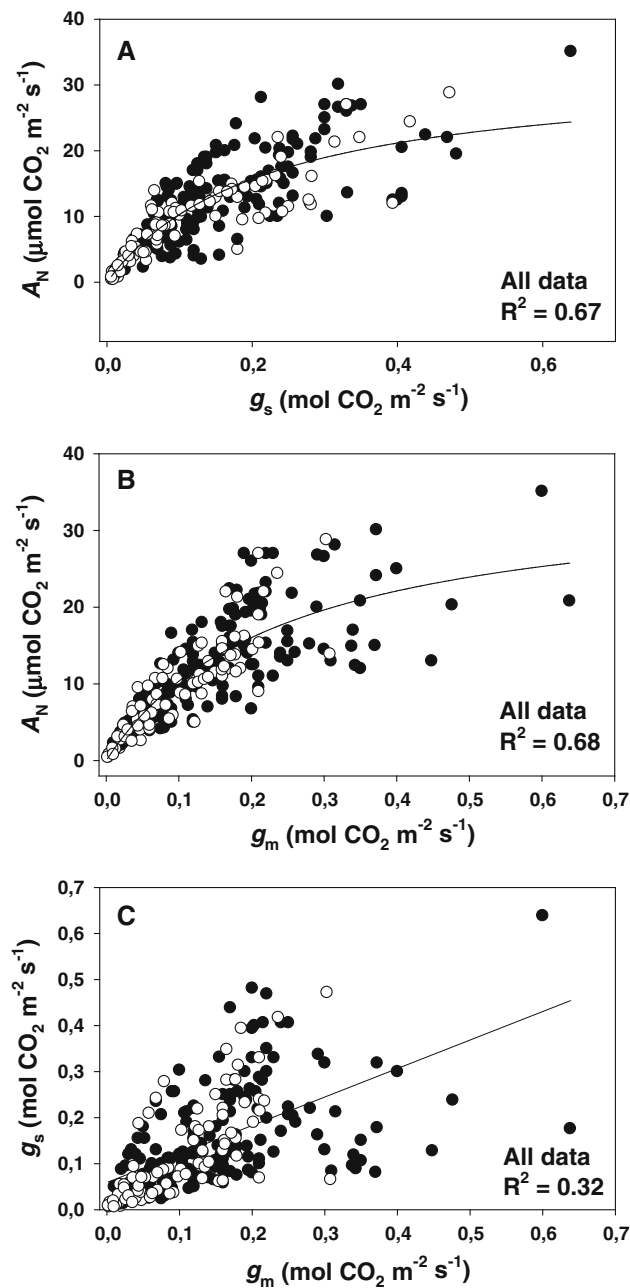


Fig. 1 The relationship between net CO₂ assimilation (A_N) and **a** stomatal conductance (g_s) and **b** mesophyll conductance (g_m) and between the two conductances **c** in a multi-species dataset (Online Resource 1). Data are pooled for different species under irrigation (filled symbols) or water stress (empty symbols). Data are from the following references: Lloyd et al. (1992), Syvertsen et al. (1995), Lauteri et al. (1997), Hanba et al. (1999), Kogami et al. (2001), Flexas et al. (2002, 2006a, 2007, 2010), Piel et al. (2002), De Lucia et al. (2003), Grassi and Magnani (2005), Niinemets et al. (2005, 2006), Priault et al. (2006), Shi et al. (2006), Diaz-Espejo et al. (2007), Juszczuk et al. (2007), and Warren (2008a, b). Regressions were selected as linear or simple hyperbolic adjustments based on the best fit

drawdown from the sub-stomatal cavities to the chloroplasts ($C_i - C_c$; Fig. 2b). Considering only the benefits of increased supply of CO₂, these CO₂ drawdowns have been

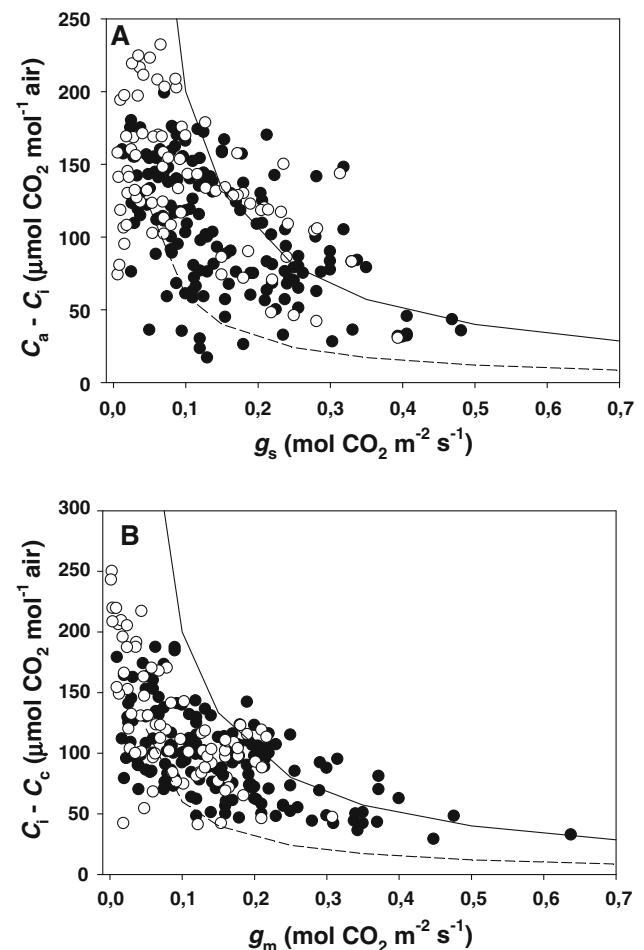


Fig. 2 The relationship between **a** CO₂ drawdown from atmosphere to sub-stomatal cavities ($C_a - C_i$) and stomatal conductance (g_s) and **b** CO₂ drawdown from sub-stomatal cavities to chloroplasts ($C_i - C_c$) and mesophyll conductance (g_m) in a multi-species dataset. Data and symbols as in Fig. 1. Line plots show modeled relationships for two values of constant A_N , $6 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (dashed line) and $20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (solid line). These relationships were estimated from $A_N = g_s (C_a - C_i) = g_m (C_i - C_c)$, fixing A_N and estimating CO₂ drawdowns for any g_s value within the range of observed values

used as proxies for the stomatal and mesophyll conductance limitations to photosynthesis (Warren 2008a; Niinemets et al. 2009a). Therefore, a large variation in CO₂ drawdowns with varying conductances shows that these strongly limit photosynthesis in a pool of species. However, modeled line plots in Fig. 2 show that different species can potentially have a similar A_N with different conductances and CO₂ drawdowns, which may reflect differences in WUE and NUE (Farquhar et al. 2002; Wright et al. 2003). For instance, at common A_N , species with high g_s and low $C_a - C_i$ would have low WUE and NUE, and species with low g_s and high $C_a - C_i$ would have high WUE and NUE.

A_N is strongly correlated with g_m (Fig. 1b) and A_N/g_s correlates with g_m/g_s (Fig. 3a). However, these two figures

do not exhibit the same shape since the relationships between A_N and g_m do not clearly saturate at high g_m . This is partly because by dividing both terms by g_s , the variation along both graph axes is dramatically reduced. That is, while A_N varies 18-fold and g_m 37-fold, there is only a 3.5-fold variation in A_N/g_s and 16-fold variation in g_m/g_s . In addition, a particular species does not necessarily occupy the same position along the A_N – g_m axis as in the A_N/g_s – g_m/g_s axis. Just to mention one example, *L. maritima* with A_N of $35 \mu\text{mol CO}_2 \text{ mol}^{-2} \text{ s}^{-1}$ and g_m of $0.6 \text{ mol CO}_2 \text{ mol}^{-2} \text{ s}^{-1}$ occupies the right-up extreme in the A_N – g_m relationship, but the left-bottom one in the A_N/g_s – g_m/g_s plot, with A_N/g_s of $55 \mu\text{mol mol}^{-1}$ and g_m/g_s of 0.94 (Online Resource 1).

On the other hand, increasing g_m does not result in any clear trend in g_m/g_s (Online Resource 4); that is, increasing g_m alone does not necessarily result in increased intrinsic WUE, A_N/g_s . Importantly, no clear dependency is observed between A_N/g_s and A_N when pooling all data together

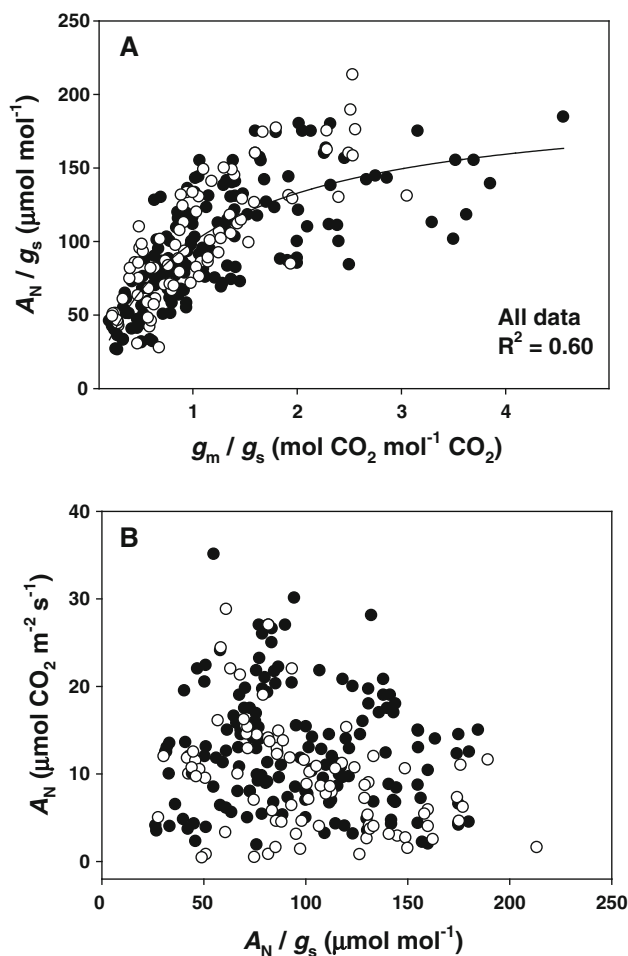


Fig. 3 The relationships between **a** A_N/g_s and g_m/g_s and **b** A_N/g_s and A_N in a multi-species dataset (Online resource 4). Data and symbols as in Fig. 1. A_N/g_s versus g_m/g_s plots are fitted by Eq. [5]

(Fig. 3b), suggesting that photosynthetic capacity and WUE do not co-scale within the plant kingdom. For instance, species with similar maximum A_N around $8.5 \mu\text{mol CO}_2 \text{ mol}^{-2} \text{ s}^{-1}$ under non-stress conditions display a disparity of A_N/g_s values, from $55 \mu\text{mol mol}^{-1}$ in *Acer palmatum* to 145 in *Pseudotsuga menziesii* (Online Resource 1). Similarly, among the three species with the highest maximum A_N in the dataset, *L. maritima* shows A_N/g_s as low as $55 \mu\text{mol mol}^{-1}$, *Prunus persica* shows an intermediate value ($94 \mu\text{mol mol}^{-1}$), and *Limonium gilbertii* shows a value as high as $132 \mu\text{mol mol}^{-1}$ (Online Resource 1).

Variability in the relationships between A_N , g_s , g_m and WUE within selected species

To assess the occurrence of intraspecific variations, we have analyzed how the relationship holds when comparing varieties, land-races, or wild native populations within a single species occupying large geographic areas (Fig. 4). Data were found in the literature for up to five species or species groups: *V. vinifera* L., *O. sativa* L., *H. vulgare* L. and *Hordeum bulbosum* L., *P. balsamifera* L., and *S. lycopersicum*. In four of the species, substantial variability was found in A_N , g_s , and g_m , and these parameters correlated with each other, although to different extents (Fig. 4a–l). In grapevine, tomato, and rice, A_N strongly correlated with both g_m and g_s (Fig. 4a, b, d, e, g, h), while the correlation between the two conductances was significant but smaller than between either of them and A_N (Fig. 4c, f, i). However, based on the ratio g_m/g_s (not shown), the most limiting of the conductances is suggested to depend on the species and treatment. Hence, in grapevine genotypes, most genotypes under irrigation are most limited by g_m (i.e., $g_m/g_s < 1$), while under water stress, they are most limited by g_s (i.e., $g_m/g_s > 1$). In contrast, in tomato, all genotypes are mostly limited by g_s , while in rice, most data lie close to co-limitation (i.e., $g_m/g_s \approx 1$) with slight differences among treatments. In barley, A_N shows only a moderate correlation with either conductance (Fig. 4j–k), but the two conductances correlate with each other more strongly (Fig. 4l). Based on g_m/g_s , g_s appears to be the most limiting in all genotypes. Finally, in poplar, the variability of A_N and g_s is relatively small, although g_m is largely variable. Hence, A_N correlates better with g_m than with g_s , and the two conductances do not correlate with each other (Fig. 4m–o). Still, based on g_m/g_s , it appears that Northern genotypes tend to be more limited by g_s , while Southern genotypes tend to be more limited by g_m . These interspecific and treatment-dependent differences in the most limiting conductance are intriguing and deserve attention in further studies.

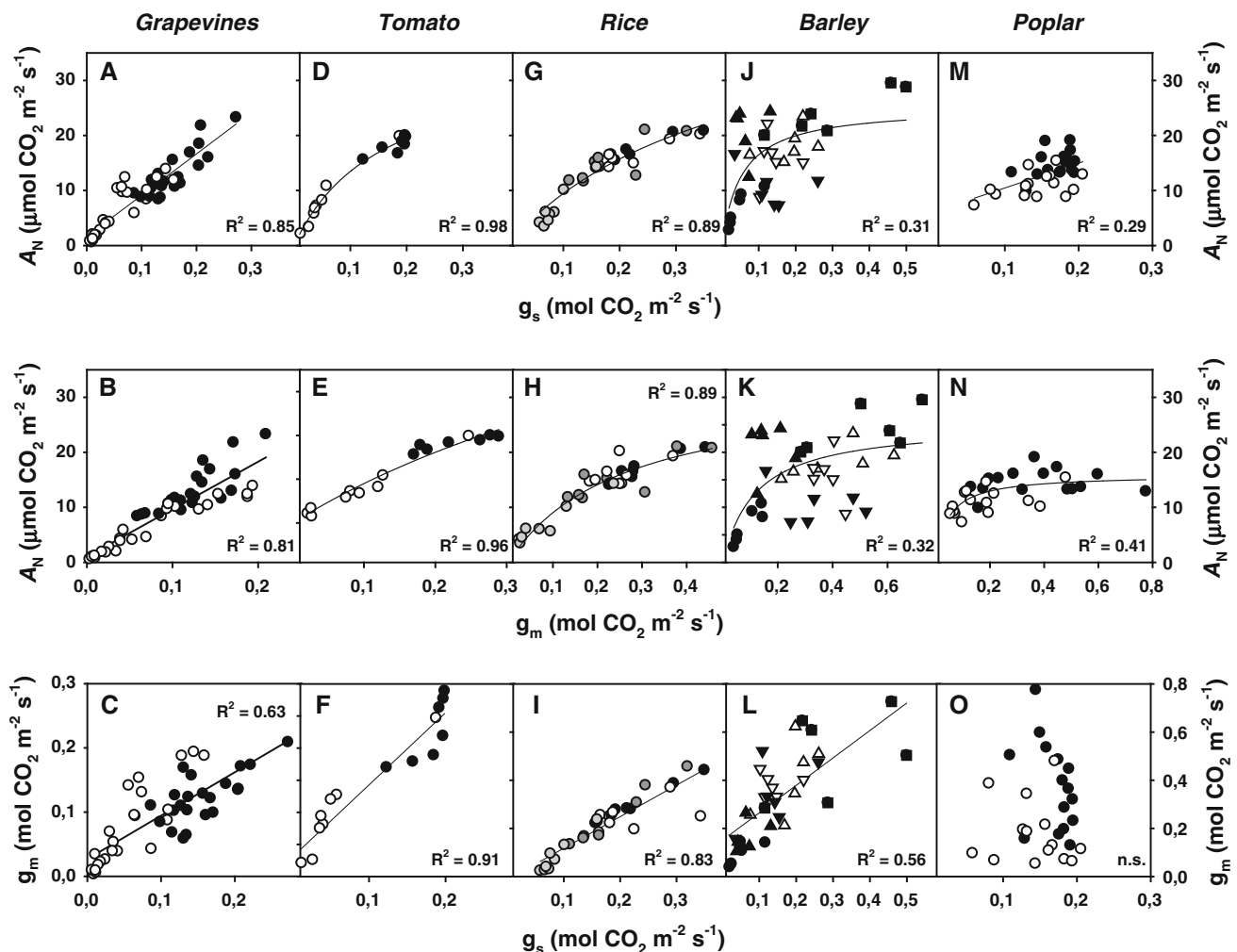


Fig. 4 The inter-relationships between A_N , g_s , and g_m for several genotypes of single species. **a–c** Grapevine genotypes (*Vitis vinifera*), including Aleluya, Argamussa, Batista, Boal, Cabernet Sauvignon, Callet, Calop Blanc, Chardonnay, Escursach, Esperó de Gall, Fogoneu, Gargollasa, Giró, Grenache, Grumiere, Malvasia de Banyalbufar, Manto Negro, Monastrell, Quigat, Sabater, Syrah, and Tempranillo, and the rootstock Richter-110 (*V. berlandieri* × *V. rupestris*), either irrigated (filled symbols) or under water stress (empty symbols). Data from Flexas et al. (2010) and Tomàs (unpublished). **d–f** Tomato genotypes (*Solanum lycopersicum*) including the Ohio OH8245 accession, a Minorcan “cherry” landrace accession, and six “Tomàtiga de Ramellet” landrace genotypes, three of them displaying the potato-leaf morphology and three the tomato-leaf morphology. Data from Galmés et al. (2011). **g–i** Rice genotypes (*Oryza sativa*) including five upland cultivars (Apo, IR71525-19-1-1, IR5541904, Moroberekan, and Vandana) and three lowland cultivars (IR64, DK98, and PSBRc80), either irrigated (filled symbols), or under mild (dark gray symbols) or severe water stress (pale gray symbols), or re-watered (empty symbols). Data from Centritto et al.

(2009). **j–l** Barley genotypes; four *Hordeum vulgare* (“Dash,” a dryland grain feed cultivar, black circles; “Omaka,” a forage cultivar, black squares; tetraploid “Golden Promise,” a salt-tolerant mutant, white upward triangles; and diploid “Golden Promise,” black upward triangles) and two *Hordeum bulbosum* (an uncultivated barley species, both tetraploid, white downward triangles, and diploid, black downward triangles). All plants were irrigated. Data from Barbour et al. (2010). **m–o** Balsam poplar (*Populus balsamifera*) genotypes from 6 populations, comprising Northern (filled symbols) and Southern (empty symbols) provenances sampled throughout species’ natural range and grown in a common garden, under irrigation. All data are averages for 4–8 replicates per genotype, from Soolanayakanahally et al. (2009) and unpublished. Notice that each plot has an independent X-axis range. As for Y-axes, those for A_N are common for all five species. To improve clarity for the g_m in the Y-axis, scale is set 0–0.3 mol CO₂ m^{−2} s^{−1} in grapevines and tomato and 0–0.8 mol CO₂ m^{−2} s^{−1} in the other three species. Regressions were selected as linear or simple hyperbolic adjustments based on the best fit

These differences among species extend to the degrees of correlation between g_m/g_s and A_N/g_s , being strongly correlated in grapevines, tomato, and poplar; somewhat correlated in barley; and totally uncorrelated in rice (not shown). It should be noted that even in the cases where g_m/g_s and A_N/g_s scale together, their increase is not necessarily

accompanied by an increase in A_N . As an example, in the grapevine dataset, three varieties show similar A_N around 16–17 μmol m^{−2} s^{−1} under non-stress conditions. Of these, A_N/g_s is 79 μmol mol^{−1} in Sabater, 89 μmol mol^{−1} in Grenache, and 99 μmol mol^{−1} in Callet. Comparing these values with those of Syrah, the variety showing the

largest A_N of around $23 \mu\text{mol m}^{-2} \text{s}^{-1}$ has an intermediate A_N/g_s of $86 \mu\text{mol mol}^{-1}$. If this genotype had been selected to improve A_N , this would have occurred at the expense of decreasing WUE when compared with Callet, with no change in WUE when compared with Grenache, or concomitantly with improved WUE if Sabater were the cultivar of reference. In this sense, the inspection of the correlations between A_N and A_N/g_s in these plants reveals interesting trends. Previously, it has been shown that the relationship between these two parameters can vary widely. For instance, when the source of variation is light intensity or nutrition, a positive correlation is observed between the two (Wong et al. 1979). However, when the variation is from different species, a negative correlation has been reported between the two parameters (Franks and Farquhar 1999). In balsam poplar, the studied provenances came from natural stands spread along a latitudinal gradient (Soolanayakanahally et al. 2009). With a few outlier genotypes (encircled), a general positive relationship is observed between A_N and A_N/g_s (Fig. 5e). This is due to the fact that g_m is largely variable in this species, while g_s is much more stable when A_N is mostly dependent on g_m (Fig. 4m–o). It appears therefore that natural variations within this species have resulted in co-scaling of photosynthetic capacity and WUE, as also observed in other temperate trees such as *Abies* (Peguero-Pina et al. 2012). For the species under human selection, instead, no (grapevines, tomato, barley) or somewhat negative (rice) relationships between A_N and A_N/g_s are found under non-stress conditions (Fig. 5a–d). However, in grapevines (with a few exceptions encircled in Fig. 5a) and rice, significant positive and steep correlations are observed under water-stressed conditions (Fig. 5a, c). Grapevines are widely cultivated in temperate and semi-arid Mediterranean areas, for which a significant part of the used genotypes has been selected for their performance under water stress (Flexas et al. 2010). Interestingly, the outlying genotypes are all ancient local varieties from Mallorca (Batista, Escursach,

Monastrell, Quigat, and Sabater), for which the selection criteria were probably based mainly on harvest and disease resistance as they were developed in the pre-phyloxera era. As for the rice genotypes analyzed, these were upland and rainfed lowland genotypes indeed selected for contrasting performance under water scarcity conditions (Centritto et al. 2009). Concerning the other species, selection for high photosynthesis and high WUE may have proceeded independently, as already reported for other cultivated species (Munoz et al. 1998; Blum 2005; Rytter 2005).

These results contradict the general view that increasing A_N always occurs at the expense of decreased WUE and vice versa (Blum 2005; Parry et al. 2005) and support the idea that it might be possible to simultaneously increase photosynthesis and WUE by means of improving g_m over g_s . Specific manipulation of genes involved in the determination of both conductances and their regulation appears therefore as a promising means for improving the world's yields while saving water, but current knowledge about such genes and their inter-regulation is scarce, particularly for g_m . Despite the broadly interesting observations, we suggest that some caution is called for in drawing general conclusions. Because of limited size of the dataset in the case of intraspecific comparisons, at least part of the observed differences could be related to differences in measuring techniques and conditions, and clearly more experimental work is needed to gain conclusive insight into the potential within-species variations in dynamic controls of A_N and WUE by different diffusion conductances

The relationships between A_N , g_s , g_m and WUE in genetically modified genotypes

With the data available, the interdependence between photosynthesis, CO_2 diffusion conductances, and WUE can be analyzed only in a restricted number of genetically modified plants (Online Resource 3). Two groups of modified plants were considered: plants with specific

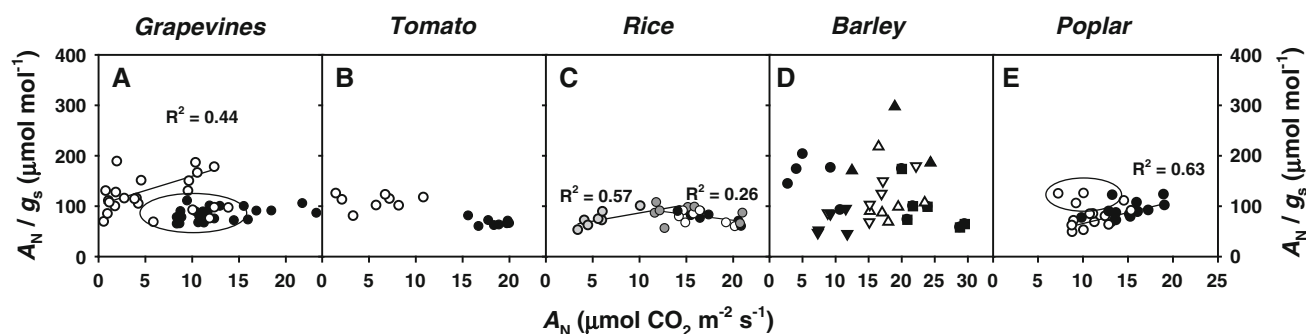


Fig. 5 The relationships between A_N/g_s and A_N for several genotypes of single species. Data and symbols as in Fig. 4. When significant, A_N/g_s versus A_N plots are fitted using linear regression. In A and E,

circles represent outliers not included in the regression. Notice the different X-scale in D

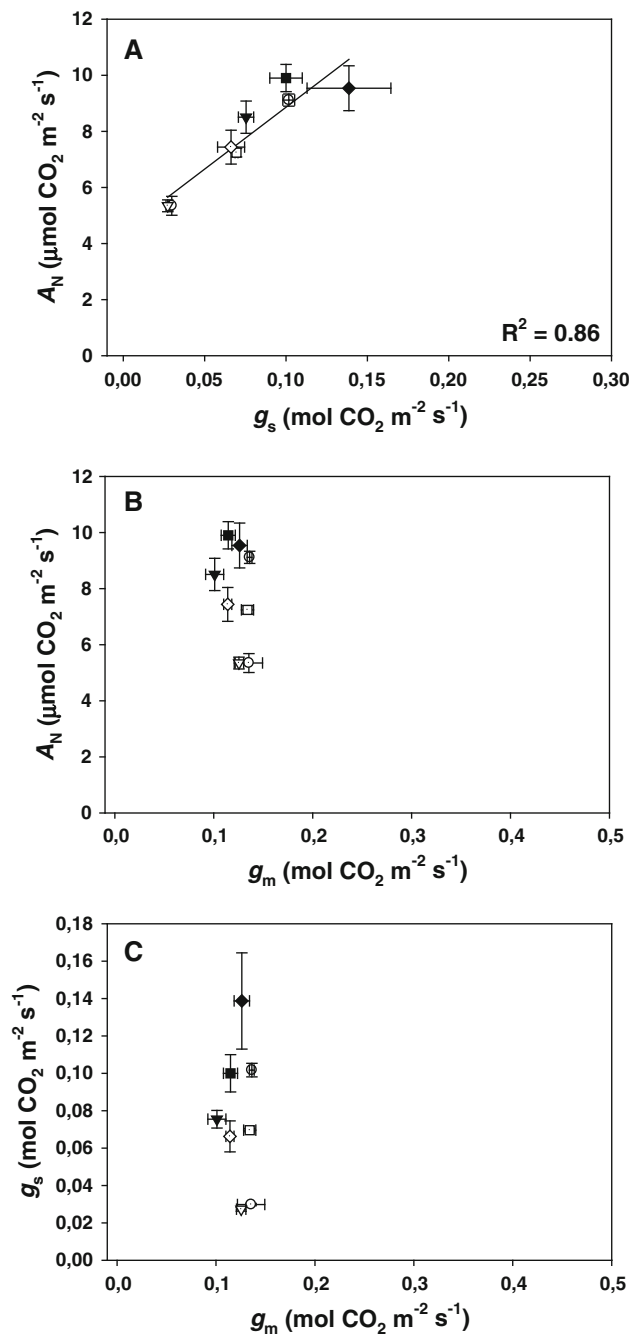


Fig. 6 The relationship between net CO₂ assimilation (A_N) and **a** stomatal conductance (g_s) and **b** mesophyll conductance (g_m) and between the two conductances **c** in several *Arabidopsis thaliana* genotypes with altered stomatal sensitivity to ABA, including ABA-insensitive *ost1-3* described in Mustilli et al. (2002) (diamonds), ABA-hypersensitive double *pp2ca-1 abil-2* described in Rubio et al. (2009) (triangles), triple *pp2ca-1 abil-2 ost1-3* mutants (squares), and WT plants (circles). All plants were grown in hydroponics solution without (black symbols) or with the addition of 0.2 mM ABA (white symbols). All data are averages for 4–8 replicates per genotype. Data in **a** were fitted by linear adjustment

alterations in g_s , consisting of several ABA-sensitive *Arabidopsis thaliana* mutants (specifically analyzed for the present study), and plants described as having a modified

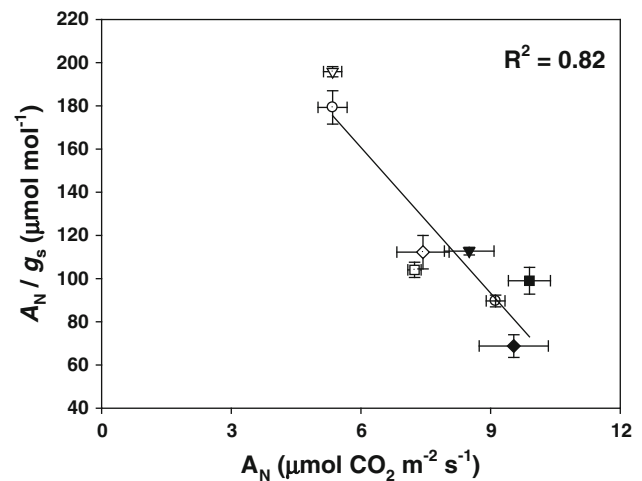
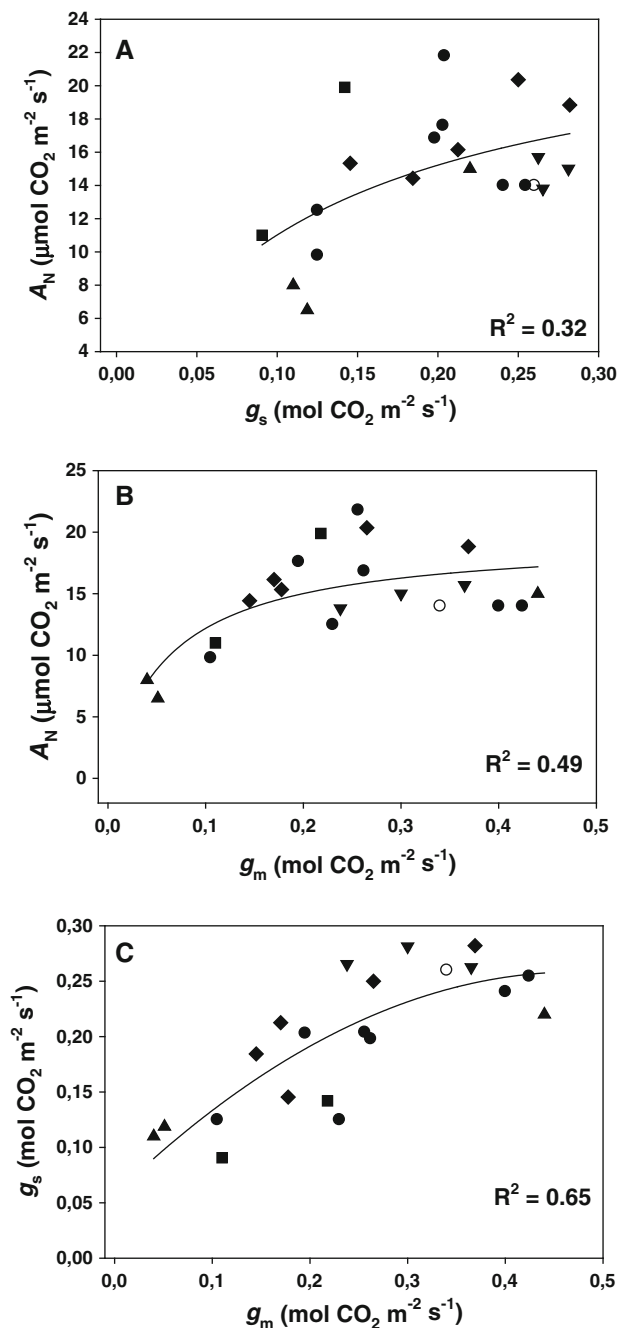


Fig. 7 The relationship between A_N/g_s and A_N in several *Arabidopsis thaliana* genotypes with altered stomatal sensitivity to ABA. Data and symbols as in Fig. 6

g_m in comparison to wild-type genotypes, including transgenic plants with an altered concentration of certain aquaporins (Hanba et al. 2004; Flexas et al. 2006a) or chloroplastic carbonic anhydrase (Price et al. 1994; Williams et al. 1996) and natural mutations with altered chloroplast disposition and movements (Sharkey et al. 1991; Tholen et al. 2008) or mitochondrial rearrangements (Priault et al. 2006; Juszczuk et al. 2007).

Although a large number of mutants and transgenic plants have been described with altered g_s and/or altered g_s responses to the environment (Wilkinson et al. 1998; Borel et al. 2001; Schroeder et al. 2001; Holbrook et al. 2002; Christmann et al. 2005; Saez et al. 2006; Merlot et al. 2007; Nilson and Assmann 2007; Zhang et al. 2008; Rubio et al. 2009) or drought tolerance and WUE (Masle et al. 2005; Nelson et al. 2007; Rivero et al. 2007), to the best of our knowledge, g_m has not been determined yet in any of them. The present report represents the first attempt to determine g_m in ABA-insensitive and ABA-hypersensitive mutants of *A. thaliana*. These genotypes presented constitutive differences in g_s even in the absence of exogenous ABA (Saez et al. 2006; Rubio et al. 2009). The ABA-insensitive *ost-1* mutants showed the largest g_s and the smallest A_N/g_s , while the ABA-hypersensitive double mutants *pp2ca-1 abil-2* presented the smallest g_s and the largest A_N/g_s , with wild-type and triple mutants (*pp2ca-1 abil-2 ost-1*) presenting intermediate values. In WT and *pp2ca-1 abil-2*, g_s decreased as expected, which happened only moderately in ABA-insensitive *ost-1* and *abil-2 pp2ca-1 ost1-3* plants. In some studies, it has been shown that g_m decreases following g_s after ABA addition (Flexas et al. 2006b; Vrábl et al. 2009), but in the present *Arabidopsis* genotypes, g_m did not differ strongly among genotypes or in response to ABA (Fig. 6). Hence, A_N correlated with g_s , but not with



g_m , and the two conductances did not correlate with each other (Fig. 6). Hence, although g_m/g_s scaled positively with A_N/g_s (not shown), the data with the highest ratios were those corresponding to the two ABA-sensitive genotypes after application of ABA, which induced stomatal closure and decreased A_N , for which a negative correlation between A_N and A_N/g_s emerged (Fig. 7). In other words, genetic manipulation of stomatal responsiveness to ABA has thus far not led to any concomitant increase of A_N and WUE.

In genotypes with altered g_m , the positive correlations between A_N and either g_s or g_m are not as clear as in

Fig. 8 The relationship between net CO_2 assimilation (A_N) and **a** stomatal conductance (g_s) and **b** mesophyll conductance (g_m) and between the two conductances **c** in mutant or transgenic genotypes with altered g_m , including transgenic tobacco (*Nicotiana tabacum*) plants with altered levels of native PIP1 aquaporin (Flexas et al. 2006b), transgenic rice plants expressing barley PIP2 aquaporin (Hanba et al. 2004), transgenic tobacco plants with reduced levels of chloroplast carbonic anhydrase (Price et al. 1994; Williams et al. 1996), tobacco mutants with altered phytochrome responses having a reduced chloroplast exposure to intercellular air spaces (Sharkey et al. 1991), *Chup1* mutants of *Arabidopsis* showing reduced chloroplast exposure to intercellular air spaces (Tholen et al. 2008), cytoplasm male sterility II (CMSII) mutants of *Nicotiana sylvestris* showing impaired mitochondrial function and g_m (Priault et al. 2006), and *Cucumis sativus* plants showing the mitochondrial rearrangement mosaic mutant MSC16 (Juszczuk et al. 2007). All plants were under irrigation. Aquaporin mutants are represented by diamonds, carbonic anhydrase mutants by downward triangles, respiratory mutants by squares, chloroplast mutants by upward triangles, and wild-type plants by circles. Data were fitted by simple hyperbolic adjustments

selected genotypes within diverse species or in ABA-sensitive mutants, but the two conductances are again strongly and positively correlated with each other (Fig. 8). Surprisingly, in most of these genotypes, g_m/g_s and A_N/g_s are unaffected or A_N/g_s does not respond to changes, so they do not show significant relationships between g_m/g_s and A_N/g_s (not shown) or A_N and A_N/g_s (Fig. 9). In transgenic plants differing in the amounts of CO_2 -transporting aquaporins (Hanba et al. 2004; Flexas et al. 2006a), for instance, proportionally similar changes in g_m , g_s , and A_N result in g_m/g_s and A_N/g_s that do not differ significantly from wild-type genotypes. This is probably due to the fact that aquaporins are also present in guard cells and probably involved in stomatal opening and closing (Kaldenhoff et al. 2008). Nevertheless, transgenic rice plants expressing a PIP2 barley aquaporin exhibited significant leaf morphological adjustments in addition to modified g_m , which may have affected the photosynthetic capacity as well (Hanba et al. 2004), and in transgenic tobacco with modified levels of native PIP1 aquaporin, an unexplained change in maximum capacity for thylakoid electron transport has also been reported (Flexas et al. 2006a). In the case of transgenic plants deficient in chloroplast carbonic anhydrase (Price et al. 1994; Williams et al. 1996), A_N/g_s remains constant despite substantial decreases in g_m/g_s . In this case, the reason seems to be that in wild types, g_s limited photosynthesis much more than g_m (i.e., $C_i - C_c$ was much smaller than $C_a - C_i$, see Supplemental Table 2). As a result, reduced g_m in the mutants results in negligible changes in g_s , C_c , and A_N and hence A_N/g_s . In mutants showing chloroplast rearrangements or impaired movements in response to light (Sharkey et al. 1991; Tholen et al. 2008), the response seems variable (Online Resource 3). In tobacco mutants, A_N/g_s remains constant despite substantial decreases in g_m/g_s due to a large compensatory increase of $C_a - C_c$. In contrast, *Chup 1* mutants of *Arabidopsis* show

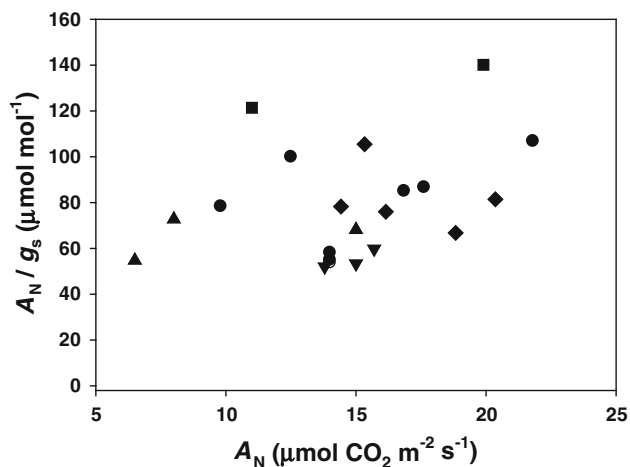


Fig. 9 The relationship between A_N/g_s and A_N in mutant or transgenic genotypes with altered g_m . Data and symbols as in Fig. 8

concomitant decreases of both g_m/g_s and A_N/g_s , i.e., the “right way” response, although this is small in magnitude. A similar situation occurs in mutants showing mitochondrial alterations (Priault et al. 2006; Juszczuk et al. 2007). In CMSII mutants of *N. sylvestris*, decreased g_m/g_s does not result in increased A_N/g_s due to compensatory increases in C_a-C_c , while in MSC16 cucumber, the “right way” response is observed although, as in *Chup 1*, small in magnitude. In summary, compensatory changes in parameters other than g_m/g_s occur so that the positive relationship between g_m/g_s and A_N/g_s is broken in these genotypes, and again a clear positive scaling of A_N and WUE has been achieved in none of them.

Final remarks

The present analysis shows that the intrinsic water-use efficiency of leaves generally increases when the ratio of mesophyll to stomatal CO_2 diffusion conductances increases. The existence of such a general relationship including plants with different growth forms, ecological habits, and evolutionary origins, as well as undergoing different water stress conditions or diverse short-term environmental and or biochemical manipulations, reinforces the idea of evolutionary trade-offs or simultaneous scaling among leaf traits irrespective of environmental conditions. There are general scaling relationships among photosynthesis and stomatal conductance (Wong et al. 1979); leaf photosynthesis and respiration with nutrient contents, leaf mass per area, and leaf life span (Wright et al. 2004); and nutrient contents and leaf mass per area with leaf size (Niklas et al. 2007) and correlations among first-order hydraulic vein density with stomatal

conductance and transpiration (Sack et al. 2008). Here, we describe another key scaling relationship of photosynthesis with mesophyll conductance and demonstrate that the balance between g_m and g_s is reflected in changes in leaf-level intrinsic water-use efficiency.

These results also suggest that there are possibilities of improving WUE by means of genetically manipulating g_m and g_s . Moreover, contrary to what is often reported (e.g., Blum 2005), improving WUE may not necessarily result in decreased A_N and productivity, at least when this is achieved by improving g_m over g_s . Therefore, manipulating g_m/g_s could potentially result in simultaneous improvements of photosynthesis and WUE. Indeed, this has been achieved during the selection of genotypes for some species under certain conditions, such as for rice and grapevine genotypes under water stress or among wild North American naturally segregating populations of *P. balsamifera*. In all of these cases, a positive correlation is observed between A_N and A_N/g_s . In genetically modified plants aimed to specifically alter either g_s or g_m , however, such positive dependence between A_N and A_N/g_s is not found due to the appearance of pleiotropic effects of diverse nature, involving co-adjustments of g_s , g_m , and/or photosynthetic capacity (see Flexas et al. 2012 for a further discussion). We suggest that future genetic engineering attempts may consider the possibility of simultaneously increasing g_m and decreasing g_s (e.g., by combining a loss-of-function clade A pp2c mutation with an overexpressing PIP1 mutation) or inducing non-constitutive changes in g_m (e.g., altering aquaporin activity instead of aquaporin expression).

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