Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO₂ in vivo

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Summary

Leaf mesophyll conductance to CO₂ (gm) has been recognized to be finite and variable, rapidly adapting to environmental conditions. The physiological basis for fast changes in gm is poorly understood, but current reports suggest the involvement of protein-facilitated CO₂ diffusion across cell membranes. A good candidate for this could be the Nicotiana tabacum L. aquaporin NtAQP1, which was shown to increase membrane permeability to CO₂ in Xenopus oocytes. The objective of the present work was to evaluate its effect on the in vivo mesophyll conductance to CO₂, using plants either deficient in or overexpressing NtAQP1. Antisense plants deficient in NtAQP1 (AS) and NtAQP1 overexpressing tobacco plants (O) were compared with their respective wild-type (WT) genotypes (CAS and CO). Plants grown under optimum conditions showed different photosynthetic rates at saturating light, with a decrease of 13% in AS and an increase of 20% in O, compared with their respective controls. CO₂ response curves of photosynthesis also showed significant differences among genotypes. However, in vitro analysis demonstrated that these differences could not be attributed to alterations in Rubisco activity or ribulose-1,5-bisphosphate content. Analyses of chlorophyll fluorescence and on-line ¹³C discrimination indicated that the observed differences in net photosynthesis (AN) among genotypes were due to different leaf mesophyll conductances to CO₂, which was estimated to be 30% lower in AS and 20% higher in O compared with their respective WT. These results provide evidence for the in vivo involvement of aquaporin NtAQP1 in mesophyll conductance to CO₂.

Keywords: aquaporins, Nicotiana, CO₂ permeability, photosynthesis, leaf conductance.

Introduction

Photosynthesis requires the diffusion of CO₂ from the atmosphere into the leaf and then to the site of carboxylation in the chloroplast stroma. The rate of fixation can be limited by the conductance of CO₂ into the leaf through stomata (gs) and by the conductance from substomatal cavities through the leaf mesophyll to the chloroplast stroma (gm). From Fick’s law of diffusion, the net photosynthetic flux (AN) can be expressed as: AN = gm(Ça–Çi) = gm(Çi–Çc), where Ça, Çi, and Çc are the CO₂ concentrations (µmol mol⁻¹ air) in the atmosphere, the substomatal cavity and the chloroplast stroma, respectively (Long and Bernacchi, 2003).

Gas exchange studies have usually assumed that gm is large and constant, i.e. that Çc ≈ Çi (Farquhar et al., 1980). However, there is now evidence that gm may be sufficiently small so as to significantly decrease Çc relative to Çi, therefore limiting photosynthesis (von Caemmerer and Evans, 1991; Di Marco et al., 1990; Evans and von Caemmerer, 1996; Evans and Loreto, 2000; Evans et al., 1986; Harley et al., 1992; Loreto et al., 1992). Moreover, gm is not
constant, since it has been shown to acclimate during leaf development (Hanba et al., 2001; Miyazawa and Terashima, 2001) and senescence (Loreto et al., 1994), as well as to light conditions during growth (Piel et al., 2002) and CO₂ environments (Singhaas et al., 2004). There is also evidence for rapid variation of \( g_m \) in response to drought (Brugnoli et al., 1998; Flexas et al., 2002; Roupsard et al., 1996), salinity (Bongi and Loreto, 1989; Delfine et al., 1998, 1999; Loreto et al., 2003), changes in light intensity (JF and DTH, unpublished results), leaf temperature (Bernacchi et al., 2002) and CO₂ concentration (Centritto et al., 2003; Düring, 2003). With respect to CO₂ concentration, the response of \( g_m \) has been shown to be as rapid and reversible as that of \( g_s \) (Centritto et al., 2003).

Despite substantial evidence for the large variability of \( g_m \), the mechanism behind these variations remains unclear. Early literature assumed that structural properties of the leaf caused most variations in \( g_m \) (von Caemmerer and Evans, 1991; Lloyd et al., 1992; Nobel, 1999). While structural properties could be involved in adaptive and acclimation responses, they could not account for the rapid variations observed in response to varying environmental conditions. Most likely, a metabolic process is involved changes in \( g_m \) in these cases. Based on a temperature response coefficient (\( Q_{10} \)) of approximately 2.2 for \( g_m \) in tobacco leaves, Bernacchi et al. (2002) speculated that enzymatic or protein-facilitated diffusion of CO₂ controls \( g_m \). The most likely candidates for this effect would be carbonic anhydrase and aquaporins.

Some authors have suggested that the activity of carbonic anhydrase is closely associated with \( g_m \) in C₃ plants (Makino et al., 1992; Sasaki et al., 1996; Volokita et al., 1993). However, modification of carbonic anhydrase activity revealed little or no change in \( g_m \) and photosynthesis (Price et al., 1994; Williams et al., 1996). Recently, Gillon and Yakir (2000) showed that the relative contribution of carbonic anhydrase to the overall \( g_m \) is species dependent. They hypothesized that carbonic anhydrase-mediated CO₂ diffusion may be more important when \( g_m \) is low due to structural properties of the leaves, as is the case for woody species, where cell wall conductance is much lower than chloroplast conductance. In mesophyte species, such a tobacco (Nicotiana tabacum L.), the influence of carbonic anhydrase in \( g_m \) seems negligible (Gillon and Yakir, 2000).

Aquaporins are water channel integral membrane proteins that increase the permeability of membranes to water, thus playing an essential role in plant water relations (Chrispeels et al., 2001; Kaldenhoff et al., 1998; Kjellbom et al., 1999; Maurel and Chrispeels, 2001; Maurel et al., 1993, 1997; Tyerman et al., 2002). Some aquaporins also facilitate the membrane transport of other small, uncharged molecules, such as ammonia, boric acid, hydrogen peroxide, glycerol or urea, across membranes (Baiges et al., 2002; Beitz et al., 2006; Biela et al., 1999; Holm et al., 2004; Jahn et al., 2004; Liu et al., 2003; Meinild et al., 1998; Tyerman et al., 2002). The hypothesis that aquaporins could be involved in regulation of \( g_m \) was motivated by the observation that oocytes expressing mammalian aquaporin 1 (AQ1) or NtAQP1 had increased permeability of the membrane to CO₂ (Cooper and Boron, 1998; Nakhoul et al., 1998; Uehlein et al., 2003). Until recently, the possibility that CO₂ may permeate aquaporins in plants has not been examined in detail (Tyerman et al., 2002). The first indirect evidence was provided by Terashima and Ono (2002), who impaired mesophyll conductance to CO₂ using HgCl₂ (a non-specific inhibitor of some aquaporins). Hanba et al. (2004) showed that expressing the barley aquaporin HvPIP2;1, in transgenic rice leaves resulted in an increased \( g_m \), though it also produced changes in leaf morphology.

NtAQP1 is a member of the plasma membrane aquaporin 1 family (PIP1), which is mercury-insensitive and permeable to water and glycerol, but impermeable to Na⁺, K⁺ and Cl⁻ (Biela et al., 1999). While NtAQP1 is expressed preferentially in roots, it has been shown to be present in almost all organs of tobacco (Otto and Kaldenhoff, 2000). In leaves, NtAQP1 accumulates in cells of the spongy parenchyma, with the highest concentration around substomal cavities (Otto and Kaldenhoff, 2000). Using antisense plants, Siefritz et al. (2002) demonstrated in vivo that NtAQP1 is involved in root cellular transport of water, root hydraulic conductivity and response of the whole plant to water stress. More recently, Uehlein et al. (2003) demonstrated that tobacco aquaporin NtAQP1 facilitates transmembrane CO₂ transport by expression in Xenopus oocytes. Transgenic NtAQP1 plants (antisense and induced overexpression) regulated stomatal conductance upon transferring leaves from darkness to light in a manner suggesting that NtAQP1 expression could be affecting \( g_m \) (Uehlein et al., 2003). However, a direct analysis of the role of NtAQP1 in \( g_m \) is still not available. The objective of the present study was to determine \( g_m \) in planta using transgenic tobacco plants that differ in NtAQP1 expression by a combination of gas exchange, chlorophyll fluorescence and on-line ¹³C isotope discrimination techniques.

Results

Constitutive antisense (AS) plants were constructed from Samsun strains (CAS), while overexpressing plants (O) were constructed from Hō 20.20 strains (CO). NtAQP1 overexpression was under control of a tetracycline-inducible promoter. The presence of NtAQP1 was significantly reduced in AS plants compared with their control CAS, and significantly increased in O plants compared with their control CO 2 days after tetracycline application (Figure 1). These results are consistent with a previous analysis of RNA expression in the same lines (Uehlein et al., 2003).
Approximately 8 weeks after sowing, and despite having very different NtAQP1 contents, CAS, AS, CO and O plants were visually undistinguishable. Plant height, stem diameter, the number of leaves per plant and the mean leaf length were very similar (Table 1). Although there were significant differences in leaf mass area and relative water content (RWC) between plants from the Samsun line and plants from the Hö 20.20 line, no differences were observed in general between control and transgenic plants. Only RWC was significantly lower in O compared with CO, but the difference was nevertheless small. Both AS and O plants had a similar surface area of mesophyll cells ($S_{m}$) and chloroplasts ($S_{e}$) exposed to intercellular air spaces per leaf area to their respective controls (Table 1).

Despite similarities in plant and leaf morphology, significant differences were observed among plant lines in photosynthetic function, particularly at near-saturating and saturating light intensities (Figure 2). The electron transport rate ($J$) was similar among plant lines (Figure 2a,b), but net CO$_2$ assimilation ($A_N$) was lower in AS than in CAS, and higher in O than in CO (Figure 2c,d). Gas exchange and fluorescence rates were identical in CO and O plants prior to the induction of overexpression of NtAQP1 by adding tetracycline (data not shown). Therefore, the differences observed in Figure 2 originated from tetracycline-induced gene expression of NtAQP1.

Furthermore, the response of $J$ and $A_N$ to varying substomatal CO$_2$ concentration ($C_i$) differed strongly between the plant lines (Figure 3). At high light intensities, $J$ first increased in response to $C_i$ in all lines, with maximum $J$ at $C_i$ around 400–600 $\mu$mol CO$_2$ mol$^{-1}$ air (Figure 3a,b), and decreased thereafter. A similar response was described by Sharkey et al. (1998) under high light intensities, and it is thought to be caused by a feedback limitation from utilization of the end product. At any given $C_i$, $J_{PSII}$ was lower in AS than in CAS, and higher in O than in CO. $A_N$–$C_i$ curves showed similar trends (Figure 3c,d), with a clear limitation by triose-phosphate utilization (TPU) at high $C_i$ in AS and CAS, as evidenced by a small decline of $A_N$ with increasing $C_i$ (Harley and Sharkey, 1991; Long and Bernacchi, 2003) that was not apparent in O and CO. Both the saturated rate and the initial slope (insets) of these curves were significantly lower in AS than in CAS, and significantly higher in O than in CO. Prior to application of tetracycline, no significant differences had been observed between CO and O plants in their $A_N$–$C_i$ curves (Figure 4).

In total the data from both $A_N$–PFD (where PFD is the photosynthetically active photon flux density) and $A_N$–$C_i$ curves, illustrate significant differences in $A_N$ at a PFD of 1000 $\mu$mol m$^{-2}$ secure$^{-1}$ and $C_o$ of 400 $\mu$mol CO$_2$ mol$^{-1}$ among different plant lines (Table 2). The O plants displayed the highest values (21.9 $\mu$mol CO$_2$ m$^{-2}$ secure$^{-1}$) and the AS plants the lowest (17.2 $\mu$mol CO$_2$ m$^{-2}$ secure$^{-1}$), with the two control lines presenting middle values. Therefore, a difference of up to 21% was found in $A_N$ between the two extreme lines. Similar differences were observed in stomatal conductance ($g_s$), so that $A_N$/$g_s$ and $C_i$ did not differ significantly among lines. In contrast to gas exchange parameters, the maximum

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**Figure 1.** Relative NtAQP1 concentration.
(a) Equal amounts of leaf-protein from controls (CO, control overexpression; CAS, control antisense), overexpressing (O) and antisense (AS) plants were subjected to a Western analysis using an NtAQP1-specific antibody. The signal intensity at the size of the NtAQP1 monomeric protein was increased in O or decreased in AS protein preparations indicating improved and reduced expression of NtAQP1, respectively. Protein samples were obtained from pooled individual plants subjected to physiological analysis.
(b) Quantification of NtAQP1 protein from Western blot. Signal intensities from protein samples obtained from pooled CAS and CO controls (C, white column) were set as 1 and the data from NtAQP1 overexpressing lines (O, grey column) or NtAQP1 antisense lines (AS, black column) are given in relation to it. Values represent means of 30 independent plants from three lines (AS) or six plants from a single line (O). Standard deviation is indicated by bars.
quantum yield of photosystem II ($F_{V}/F_{M}$) did not differ among lines (Table 2). Likewise, no significant differences were observed in carbonic anhydrase activity, amount or total activity of Rubisco, ribulose-1,5-bisphosphate (RuBP) content or the respiration rate in the dark (Table 2).

Estimations of $g_{m}$ according to the methods of either Harley et al. (1992) or Terashima and Ono (2002) showed that, in general, the first method yielded somewhat higher values than the second (Table 2). However, both methods revealed proportionally similar important differences among plant lines. In AS plants, $g_{m}$ was less than half that in O. Again, control lines showed intermediate values. As a consequence, the estimated CO$_2$ concentration in the chloroplasts differed among lines (Table 2). Prior to application of tetracycline, and according to the method by Terashima, $g_{m}$ averaged $0.330 \pm 0.058$ in CO and $0.335 \pm 0.053$ mol m$^{-2}$ s$^{-1}$ in O.

These results were confirmed in two additional independent experiments, using two different techniques based on on-line $^{13}$C discrimination for $g_{m}$ estimations (Table 3). The plants were grown under different conditions and with different ages at the time of measurement, depending on the experiments. This resulted in different values of $A_{N}$ for each genotype (Tables 2 and 3). Despite the variation between treatments, it was clear that all methods for estimation of $g_{m}$ generate values for AS plants that are 20–40% lower than CAS and 20–50% higher for O than for CO. Therefore, the $g_{m}$ of O plants was around twice as large as that of AS. Last but not least, in all the experiments $A_{N}$ and $g_{s}$ changed proportionally to $g_{m}$.

### Table 1
Morphological plant and leaf characteristics of the studied genotypes. Values are means ± SE of six replicates from independent plants per genotype.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CAS</th>
<th>AS</th>
<th>CO</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant height (cm)</td>
<td>101 ± 3</td>
<td>95 ± 8</td>
<td>107 ± 3</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>Stem diameter (mm)</td>
<td>19 ± 1</td>
<td>16 ± 1</td>
<td>20 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>No. leaves per plant</td>
<td>24 ± 2</td>
<td>27 ± 3</td>
<td>29 ± 2</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Leaf length (cm)</td>
<td>27.0 ± 0.6</td>
<td>24.5 ± 1.4</td>
<td>23.8 ± 0.6</td>
<td>22.3 ± 0.6</td>
</tr>
<tr>
<td>LMA (g m$^{-2}$)</td>
<td>25.7 ± 2.3</td>
<td>26.5 ± 2.2</td>
<td>39.5 ± 2.2</td>
<td>39.0 ± 1.8</td>
</tr>
<tr>
<td>$S_{m}$ (m$^{-2}$ m$^{-2}$)</td>
<td>12.3 ± 1.1</td>
<td>12.4 ± 1.0</td>
<td>14.1 ± 0.6</td>
<td>13.4 ± 0.9</td>
</tr>
<tr>
<td>$S_{c}$ (m$^{-2}$ m$^{-2}$)</td>
<td>9.1 ± 0.6</td>
<td>9.8 ± 1.1</td>
<td>10.5 ± 0.8</td>
<td>10.3 ± 0.6</td>
</tr>
<tr>
<td>RWC (%)</td>
<td>79.1 ± 1.4</td>
<td>75.8 ± 1.5</td>
<td>90.5 ± 0.7</td>
<td>86.6 ± 0.9*</td>
</tr>
</tbody>
</table>

*Statistically significant ($P < 0.05$) difference between CAS and AS or between CO and O.

Figure 2. The response of the electron transport rate, $J$ (a, b) and net photosynthesis, $A_{N}$ (c, d) to incident light intensity, PFD, in (a, c) CAS (filled circles) and AS (empty circles) and (b, d) CO (filled triangles) and O (empty triangles). Values are means ± SE of six replicates from independent plants per genotype.
Having an estimate of $g_m$ allows simple conversion of $A_N$–$C_i$ curves into response curves of $A_N$ to chloroplast CO$_2$ concentration, $C_c$ (Manter and Kerrigan, 2004). Using this method we found that differences among genotypes in the initial slope of $A_N$–$C_i$ curves disappeared in $A_N$–$C_c$ (Figure 5).

We then applied the equations of Farquhar et al. (1980) to the $A_N$–$C_c$ curves shown in Figure 5 to estimate the maximum carboxylation capacities ($V_{\text{max}}$) which were identical (145 μmol m$^{-2}$ sec$^{-1}$) in CAS, AS and CO plants, and somewhat higher (160 μmol m$^{-2}$ sec$^{-1}$) in O plants. In contrast, the observed differences in light-saturated and CO$_2$-saturated rates persisted even in $A_N$–$C_c$ curves (Figure 5). The maximum electron transport capacity ($J_{\text{max}}$) was also higher in CAS (183 μmol m$^{-2}$ sec$^{-1}$) than AS (157 μmol m$^{-2}$ sec$^{-1}$) plants, and in O (181 μmol m$^{-2}$ sec$^{-1}$) than CO (160 μmol m$^{-2}$ sec$^{-1}$) plants.

On-line $\delta^{13}$C and fluorescence-based estimations of $g_m$ reflect instantaneous measurements, and were thus not representative of the complete plant life cycle. A continuous difference in $g_m$ will be reflected in the carbon isotope composition of the leaf dry matter (Evans et al., 1986). Significant differences were found among plant lines in $\delta^{13}$C of dry matter of leaves that developed during the 2 weeks subsequent to the first tetracycline application (Table 3), with the lowest values ($-29.91\%$) in O and the highest ($-28.44\%$) in AS plants. These differences in $\delta^{13}$C were not correlated with the $C_i$/$C_a$ ratio, but rather with the $C_c$/$C_i$ ratio.

**Discussion**

The involvement of aquaporins in the regulation of $g_m$ has been inferred from indirect evidence, like: (i) the prediction
of a protein-facilitated diffusion process based on the temperature response of \( g_m \) (Bernacchi et al., 2002), (ii) a decrease in \( g_m \) after exogenous application of HgCl2, a non-specific inhibitor of several aquaporins (Terashima and Ono, 2002), and (iii) evidence that the tobacco aquaporin NtAQP1 facilitates CO2 membrane transport when inserted in Xenopus oocytes (Uehlein et al., 2003). Our results clearly support a role for the aquaporin NtAQP1 in the regulation of \( g_m \) in vivo, under normal photosynthetic conditions.

In the present study we demonstrated that variation of NtAQP1 expression caused significant differences in \( g_m \). Differences between CAS and AS or between CO and O were not accompanied by any considerable change in any of the morphological (Table 1) or physiological (Table 2) traits analysed. Only \( A_n \) and \( g_s \), in addition to \( g_m \), differed substantially among lines. Because these three parameters are usually co-regulated (Evans et al., 1994; Flexas et al., 2004), it is possible – but unlikely – that altering the

### Table 2: Photosynthetic characteristics of the studied genotypes (as determined at light saturation, 25°C and 400 \( \mu \)mol m\(^{-2}\) CO\(_2\)). Values are means ± SE of 12 \( (A_n, g_s, A_n'g_s, F_v'/F_m, g_m, \delta^{13}C) \), six [carbonic anhydrase (CA) activity, RuBP content, respiration rate] or four (Rubisco activity and amount) replicates from independent plants per genotype.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CAS</th>
<th>AS</th>
<th>CO</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_n ) ((\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>19.7 ± 0.7</td>
<td>17.2 ± 0.9*</td>
<td>18.5 ± 0.6</td>
<td>21.9 ± 0.8*</td>
</tr>
<tr>
<td>( g_s ) ((\text{mol H}_2\text{O m}^{-2} \text{ sec}^{-1}))</td>
<td>0.241 ± 0.013</td>
<td>0.201 ± 0.019*</td>
<td>0.208 ± 0.012</td>
<td>0.271 ± 0.021*</td>
</tr>
<tr>
<td>( A_n/g_s ) ((\mu\text{mol CO}_2 \text{ mol H}_2\text{O}^{-1}))</td>
<td>83 ± 3</td>
<td>89 ± 5</td>
<td>92 ± 5</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>( C_r ) ((\mu\text{mol } \text{ mol}^{-1}))</td>
<td>240.6 ± 5.0</td>
<td>234.4 ± 8.8</td>
<td>228.6 ± 8.6</td>
<td>240.8 ± 10.0</td>
</tr>
<tr>
<td>( C_i ) ((\mu\text{mol } \text{ mol}^{-1}))</td>
<td>151.8 ± 6.4</td>
<td>138.3 ± 7.7*</td>
<td>189.3 ± 8.9</td>
<td>211.1 ± 11.6*</td>
</tr>
<tr>
<td>( F_v'/F_m )</td>
<td>0.834 ± 0.001</td>
<td>0.826 ± 0.001*</td>
<td>0.837 ± 0.002</td>
<td>0.837 ± 0.002</td>
</tr>
<tr>
<td>Total Rubisco activity ((\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>70 ± 5</td>
<td>75 ± 4</td>
<td>83 ± 10</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>Rubisco holoenzyme ((\mu\text{mol m}^{-2}))</td>
<td>2.7 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>3.4 ± 0.4</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>CA activity ((\times 10^3) ((\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>1062 ± 188</td>
<td>1247 ± 198</td>
<td>2112 ± 130</td>
<td>1979 ± 161</td>
</tr>
<tr>
<td>RuBP content ((\mu\text{mol m}^{-2}))</td>
<td>133 ± 17</td>
<td>121 ± 12</td>
<td>85 ± 16</td>
<td>70 ± 5</td>
</tr>
<tr>
<td>Respiration rate ((\mu\text{mol O}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>-0.6 ± 0.1</td>
<td>-0.6 ± 0.1</td>
<td>-0.7 ± 0.1</td>
<td>-0.7 ± 0.1</td>
</tr>
<tr>
<td>( \delta^{13}C ) in leaf dry matter ((%_c))</td>
<td>-28.87 ± 0.11</td>
<td>-28.44 ± 0.12*</td>
<td>-29.26 ± 0.09</td>
<td>-29.91 ± 0.11*</td>
</tr>
</tbody>
</table>

*Statistically significant \((P < 0.05)\) differences between CAS and AS or between CO and O.

### Table 3: \( A_n \), \( g_s \), \( C_r \) and \( g_m \) values for the studied plant genotypes in two additional experiments performed in 2005 at (a) University of New Mexico and (b) University of New Mexico.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CAS</th>
<th>AS</th>
<th>CO</th>
<th>O</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_n ) ((\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>15.2 ± 1.1</td>
<td>13.5 ± 1.2*</td>
<td>13.9 ± 1.3</td>
<td>16.2 ± 0.6*</td>
</tr>
<tr>
<td>( g_s ) ((\text{mol H}_2\text{O m}^{-2} \text{ sec}^{-1}))</td>
<td>0.351 ± 0.078</td>
<td>0.257 ± 0.071*</td>
<td>0.341 ± 0.078</td>
<td>0.523 ± 0.083*</td>
</tr>
<tr>
<td>( C_r ) ((\mu\text{mol } \text{ mol}^{-1}))</td>
<td>289 ± 9</td>
<td>245 ± 11</td>
<td>270 ± 9</td>
<td>289 ± 6*</td>
</tr>
<tr>
<td>( C_i/C_r )</td>
<td>0.68 ± 0.02</td>
<td>0.61 ± 0.03*</td>
<td>0.67 ± 0.02</td>
<td>0.72 ± 0.01*</td>
</tr>
<tr>
<td>( \Delta^{13}C )</td>
<td>18.0 ± 0.5</td>
<td>14.2 ± 2.0*</td>
<td>17.9 ± 0.8</td>
<td>19.4 ± 0.7*</td>
</tr>
<tr>
<td>( g_m ) ((\mu\text{mol CO}_2 \text{ m}^{-2} \text{ sec}^{-1}))</td>
<td>0.339 ± 0.041</td>
<td>0.231 ± 0.049*</td>
<td>0.323 ± 0.048</td>
<td>0.451 ± 0.079*</td>
</tr>
</tbody>
</table>

*Statistically significant \((P < 0.05)\) differences between CAS and AS or between CO and O. 
\( ^a \) \( g_m \) determined using on-line \(^{13}C \) discrimination measurements with a dual-inlet mass spectrometer. Measurements were done at 400 \( \mu \)mol \( \text{ mol}^{-1} \) \( \text{CO}_2 \), 1500 \( \mu \)mol photon \( \text{ m}^{-2} \text{ sec}^{-1} \), and 25°C. Values are means ± SE of six replicates from independent plants per genotype.

\( ^b \) \( g_m \) determined using on-line discrimination with tunable diode laser absorption spectroscopy. Measurements are reported for 380 \( \mu \)mol \( \text{ mol}^{-1} \) \( \text{CO}_2 \), 25°C, and saturating light except the slope-based \( g_m \) calculation which used light intensities from 400 to 2000 \( \mu \)mol photon \( \text{ m}^{-2} \text{ sec}^{-1} \). CO and O were not significantly different from each other for any parameter prior to tetracycline treatment (data not shown). Values are means ± SE of three replicates from independent plants for CO, four for O and five for AS and CAS.
expression of NtAQP1 resulted in a direct effect on $A_N$ or $g_s$, and that $g_m$ subsequently adjusted to these changes. The fact that NtAQP1 has been demonstrated to change CO$_2$ permeability of biological membranes (Uehlein et al., 2003) strongly suggests that changing the amounts of NtAQP1 resulted in modified $g_m$. This, in turn, means an induced adjustment of $A_N$ and $g_s$. Alteration of $g_m$ by changing NtAQP1 content without substantially modifying any other leaf trait analysed is in contrast with the results by Hanba et al. (2004). They found that transgenic rice plants overexpressing barley aquaporin HvPIP2;1 not only produced differences in $g_m$, but also in important anatomical ($S_m$, $S_c$, mesophyll porosity, stomatal density, stomatal size) and physiological (level of Rubisco) differences. This discrepancy could arise from the fact that in the study by Hanba et al. (2004) a foreign protein was expressed in transgenic plants while in the present study the levels of a native protein were modified. It is also evident that HvPIP2;1 and NtAQP1 belong to different aquaporin subfamilies, i.e. PIP2 and PIP1, respectively, which show functional differences. In addition, rice and tobacco may have a different sensitivity of their acclimation responses. Despite these differences between the two studies, both suggest an important role for aquaporins in the regulation of $g_m$.

The involvement of aquaporins in the regulation of $g_m$ provides a physiological basis for the observation that the response of $g_m$ to drought, salinity or varying CO$_2$ concentration can be as strong and rapid as that of $g_s$ (Centritto et al., 2003; Flexas et al., 2002, 2004). Although the mechanisms that regulate aquaporin activity in the short term are not fully understood, several mechanisms have been proposed, including direct phosphorylation of aquaporins (Kjellbom et al., 1999), an osmotically driven cohesion/tension mechanism (Ye et al., 2004), pH-dependent gating of aquaporins (Tournaire-Roux et al., 2003) and transcriptional regulation and protein stability (Eckert et al., 1999). Rapid regulation of $g_m$ in response to environmental stresses may be an important mechanism for the observed photosynthetic downregulation.

Indeed, the 20–50% increase in $g_m$ in O plants compared with CO plants was accompanied by a 15–20% increase in $A_N$ at saturating light, depending on the experiment, which agrees with the observations of Aharon et al. (2003) and Hanba et al. (2004), who compared plants overexpressing Arabidopsis aquaporinPIP1;2 in transgenic tobacco and barley aquaporin HvPIP2;1 in rice, respectively. Therefore, the present results show that modifying the expression of a native aquaporin results in changes in $g_m$ and photosynthesis. Not only did overexpression increase $g_m$ and $A_N$, but it also reduced the expression of NtAQP1 in AS plants, resulting in a 20–40% decrease in $g_m$ compared with CAS, and a 10–15% decrease in $A_N$. In contrast to saturating light conditions, at low light non-significant differences in $A_N$ were found among genotypes (Figure 2). Most likely, this effect may be due to the fact that at subsaturating light photosynthesis is limited by electron transport and not by the availability of CO$_2$.

In addition to a modified $g_m$, different NtAQP1 levels resulted in light-saturated and CO$_2$-saturated photosynthesis, i.e. photosynthetic capacity ($J_{max}$) that differed by about 12% between CAS and AS and between CO and O. Also $V_{cmax}$ was about 14% higher in O than in CO plants, but no differences were observed between AS and CAS plants. These results were unexpected, since photosynthetic capacity is not related to CO$_2$ diffusion but rather to the capacity of the photochemical and biochemical (Calvin cycle) machinery (Long and Bernacchi, 2003). This difference was clearly associated with NtAQP1 expression, since the differences between CO and O plants did not appear prior to the application of tetracycline (see Figure 4). We do not know at present the reason for such co-regulation.

Figure 5. The response of net photosynthesis ($A_N$) to chloroplast CO$_2$ concentration ($C_c$) in CAS and AS (a) and CO and O (b). Values are means ± SE of six replicates from independent plants per genotype. Symbols as in Figure 2.
between $g_m$ and the photosynthetic capacity. However, because altered $g_m$ modified CO$_2$ concentrations in the chloroplast (and, presumably, the cytosol; Table 2), and CO$_2$ is a well-known regulator of the expression of several photosynthetic genes (Deng et al., 2003; Ludewig and Sonnewald, 2000; Van Oosten et al., 1994), we hypothesize that modified intercellular CO$_2$ concentrations trigger differences in the development of leaf photosynthetic capacity. This would also explain why $g_m$ usually scales with photosynthetic capacity, as observed by broad comparisons between different species (Evans and Loreto, 2000; Evans et al., 1994).

Finally, carbon isotope discrimination models (Evans et al., 1986; Farquhar et al., 1982) include a term for $g_m$ that is often neglected. These models assume that most of the leaf discrimination against $^{13}$C is due to discrimination by Rubisco and phosphoenolpyruvate carboxylase, with another minor fractionation due to CO$_2$ diffusion in air through stomata. As a result, $^{13}$C discrimination ($\Delta$) is proportional to the $C_i/C_a$ ratio, and $\delta^{13}$C in leaf dry matter is interpreted as resulting from the ‘mean leaf-life’ $C_i/C_a$ ratio, which can be related to intrinsic efficiency of water use ($A/W/g_m$). Therefore, $\delta^{13}$C in leaf dry matter has been used as a long-term estimation to compare efficiency of water use between species or genotypes. The present results demonstrate that discrimination during CO$_2$ diffusion within the leaf should not be neglected. Substantial differences were found in leaf $\delta^{13}$C between different lines with only small changes in their $C_i/C_a$ ratio, which weakens the correlation between $\delta^{13}$C and efficiency of water use. However, differences in $\delta^{13}$C were strongly correlated with $C_i/C_a$ ratio and $g_m$ (not shown) as suggested by Le Roux et al. (2001). These experimental data match well with theoretical calculations by Warren and Adams (2006) that showed that differences in $g_m$ could induce a difference of up to 2–4 in leaf $\delta^{13}$C without any difference in the efficiency of water use.

In summary, the present results provide evidence that NtAQP1 is involved in $g_m$ regulation in vivo. They also strengthen the need to incorporate a term that considers $g_m$ in current photosynthesis models, such as that by Farquhar et al. (1980), as already suggested by Bernacchi et al. (2002) and Ethier and Livingston (2004). Clearly, the response of $A_N$ to $C_i$ significantly differed between lines (Figure 3). According to the Farquhar et al. model, this may be interpreted in terms of differences in the maximum carboxylation reaction ($V_{cmax}$) and the maximum electron transport rate ($J_{cmax}$). However, once $g_m$ is used to assess the response of $A_N$ to $C_i$ the results are rather different, showing that $V_{cmax}$ does not differ (CAS versus AS) or differs to a lesser extent (CO versus O), but $J_{cmax}$ still differs substantially among genotypes. Therefore, including a $g_m$ term would improve the predictive accuracy of the photosynthesis models.

### Experimental procedures

#### Plant material

Antisense and overexpressing tobacco plants (*Nicotiana tabacum*) were obtained from different lines: var. Samsun for the antisense (AS) lines (Siefritz et al., 2002) and line H6 20.20 for the overexpressing (O) lines (Uehlein et al., 2003). Plants of each line with normal NtAQP1 expression were used as controls (CAS and CO).

For initial characterization, three independently transformed antisense lines were selected. Plants from individual lines were self-fertilized and the T$_1$ was subjected to further analysis. Each line was transformed with a single NtAQP1 antisense construct, as confirmed by Southern hybridization, and showed a reduction of NtAQP1 expression of between 90% and 95% at the mRNA level. The three lines were chosen for data assessments and the data for all measurements were pooled. For characterization of NtAQP1 overexpressing lines three independently transformed lines were initially characterized. All showed an increase in NtAQP1 expression after treatment with tetracycline. A representative line was chosen for further experiments.

In AS lines NtAQP1 expression was inhibited by a 3SSCaMV promoter-driven antisense construct, while in O lines the NtAQP1 coding region was under the control of a tetracycline-inducible promoter (Uehlein et al., 2003). For the initial experiment, six to eight plants per line were germinated and grown in a growth chamber in 5-l pots containing a mixture of perlite, horticultural substrate and clay. The environmental conditions were set to a 12-h photoperiod (25°C day/20°C night), 40–60% relative humidity and a photon flux density at plant height of about 800–1000 μmol m$^{-2}$·sec$^{-1}$ (halogen lamps). Plants were irrigated daily at pot capacity with 100% Hoagland’s solution during the entire experiment. All plants were 7 weeks old at the beginning and 14 weeks old at the end of the experiments.

For induction of NtAQP1 expression in O plants, pots were watered with 10 mg l$^{-1}$ tetracycline once a day, and photosynthetic measurements were performed 2 or 3 days after tetracycline treatment. The CO plants were also irrigated with tetracycline to compensate for any possible indirect effect of tetracycline besides NtAQP1 expression. These plants continued being irrigated with tetracycline until the end of the experiments, about 3 weeks later, and they developed between three and six new leaves during that time. These most recently developed leaves were sampled for carbon isotope analysis at the end of the experiment.

Two additional experiments were performed using the same four lines described to confirm the results, one at the Universitat de les Illes Balears, Spain (UIB) and one jointly at the University of New Mexico (UNM) and the Los Alamos National Laboratory (LANL), USA. At UIB, seeds were germinated in early August, and the plants were grown under ambient conditions during autumn in a greenhouse in 10-l pots containing a mixture of perlite, horticultural substrate and clay. Plants were irrigated daily at pot capacity with 50% Hoagland’s solution during the entire experiment, and were 14 weeks old when measured. At UNM, seeds were germinated in late September and plants were grown under greenhouse conditions (25/20°C day/night) for 6 weeks and then transported to a greenhouse in LANL. Plants were allowed to acclimate to the new conditions (25/16°C day/night) for at least 2 weeks prior to measurement with supplementary light to maintain a 14-h photoperiod. Plants were grown in 10-l pots containing a commercial soil-less peat mix (Metro-mix 360, W.R. Grace and Company, Cambridge, MA, USA), watered daily to field capacity with a half-strength commercial fertilizer (Jack’s 20-20-20, J.R. Peters, Inc., Allentown, PA, USA) and supplemented every 2 weeks (0.15 ml l$^{-1}$ with a
commercial micronutrient mix (Microl Total 5-0-0, Growth Products, Ltd, White Plains, NY, USA). To induce overexpression in O plants, application of tetracycline was as described for the previous experiment and measurements were conducted 4–6 days after initiation of treatment.

**Plant water status, leaf mass area and plant size determinations**

The leaf relative water content (RWC) was determined as follows: 
\[
\text{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100
\]

To determine the turgid weight, leaves were kept in distilled water in dark at 4°C to minimize respiration losses until they reached a constant weight (full turgor, typically after 12 h). Their dry weight was obtained after 48 h at 70°C in an oven. Leaf mass area (LMA) was calculated from leaf dry weight and leaf area as LMA = dry weight/leaf area.

Plant height, basal stem diameter, leaf length and total number of leaves per plant were measured at the end of the experiments. Plant height, leaf length and basal shoot diameter were also measured.

**Light microscopy and leaf mesophyll traits**

Small pieces of leaf (2 mm²) were sampled after gas exchange measurements and fixed in 2.5% glutaraldehyde in 25 mM sodium cacodylate buffer (pH 7.2) at 4°C for 2 days. The samples were post-fixed in 2% osmium tetroxide for 3-5 h, dehydrated in acetone and propylene series, and embedded in Spurr’s resin. For light microscopy, sections were cut at 0.8 µm thick with an ultramicrotome and stained with toluidine blue. Sections were photographed at magnification of 100× and 500× (Miyazawa and Terashima, 2001). The photographs were quantitatively analysed using the ArcView 3.2 GIS software package (ESRI, Redlands, CA, USA). Following Syversten et al. (1995), the areas of mesophyll (Sm) and chloroplast (Sₚ) surfaces directly exposed to the intercellular air spaces on a leaf area basis were determined. Cells were assumed to be cylindrical, and a curvature factor of 1.38 was applied after Syversten et al. (1995).

**Carbon isotope composition in leaf dry matter**

At the end of the experiments, the most recently developed leaves were sampled from each line, dried for 48 h at 70°C, ground into powder and subsampled for analysis of the C isotope ratios. Samples were combusted in an elemental analyser (Carlo-Erba, Rodano, Italy), CO₂ was separated by chromatography and directly injected into a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus, Bremen, Germany). Peach leaf (NIST 1547) standards were run every six samples. The standard deviation of the analysis was below 0.1‰. The calculation of the isotope ratio (δ¹³C) was done as: 
\[
\delta^{13}C_{\text{sample}}/R_{\text{standard}} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000
\]

where PFD = photosynthetic photon flux density, and δ¹³C is a term which includes the product of leaf absorption and the partitioning of absorbed quanta between photosystems I and II. δ¹³C was previously determined for each line as the slope of the relationship between φPSII and φCO₂ obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing 1% O₂ (Valentini et al., 1995). These two relationships passed through the origin. δ¹³C was 0.408 for CAS and AS plants and 0.364 for CO and O plants.

**Dark respiration rates**

Dark respiration rates were determined at 25°C in leaf discs using a liquid-phase O₂ electrode (OXY042A, Rank Brothers, Cambridge, UK), to avoid any possible interference of the CO₂ released with the partial pressure of oxygen in a closed cuvette (Davey et al., 2004).

**Enzyme activities and metabolite contents**

For measurements of Rubisco content, six discs (5.3 cm²) per line of fully illuminated leaves at ambient CO₂ were freeze clamped into liquid nitrogen and stored at −70°C until assay. Extraction and assays were performed following Giménez et al. (1992).

Assays of Rubisco activity were performed on leaf punches (1.56 cm²) that were immediately frozen in liquid N₂ after gas exchange measurements and stored at −70°C until analysis. Extraction and assay of total Rubisco activity was performed as in Whitney et al. (1999) and Sharkey et al. (1991) respectively. Total Rubisco content was also determined from activated aliquots via stoichiometric binding of ¹⁴C-carboxyarabinitol bisphosphate (¹⁴CABP) and gel filtration (Buitz and Sharkey, 1988; Ruuska et al., 1998).

**Measurements of gas exchange and chlorophyll fluorescence**

Leaf gas exchange was determined simultaneously with measurements of chlorophyll fluorescence using the open gas exchange system Li-6400 (Li-Cor Inc., Lincoln, NE, USA) with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.). Measurements were made on the youngest fully expanded leaf before stem elongation. In dark-adapted leaves (i.e. pre-dawn), the maximum photochemical efficiency of photosystem II (Fₚ/Fₘ) was determined as 
\[
Fₚ/Fₘ = (Fₘ - F₁)/Fₘ
\]

and maximum fluorescence (Fₚ) during a light-saturating pulse of about 8000 µmol m⁻² sec⁻¹ (Fₚ). In light-adapted leaves, the actual photochemical efficiency of photosystem II (φPSII) was determined by measuring steady-state fluorescence (Fₛ) and maximum fluorescence (Fₚ) during a light-saturating pulse of about 8000 µmol m⁻² sec⁻¹ (φPSII), following Genty et al. (1989):

\[
\phi_{PSII} = (Fₚ - Fₛ)/Fₘ
\]

The electron transport rate (J) was then calculated as:

\[
J = \phi_{PSII} \times PFD \times x
\]

where PFD is the photosynthetically active photon flux density and x is a term which includes the product of leaf absorption and the partitioning of absorbed quanta between photosystems I and II. x was previously determined for each line as the slope of the relationship between φPSII and φCO₂ obtained by varying light intensity under non-photorespiratory conditions in an atmosphere containing 1% O₂ (Valentini et al., 1995). These two relationships passed through the origin. x was 0.408 for CAS and AS plants and 0.364 for CO and O plants.

In light-adapted leaves, photosynthesis was induced in saturating light (1000 µmol m⁻² sec⁻¹) and 400 µmol mol⁻¹ CO₂ surrounding the leaf (Cₑ). The amount of blue light was set to 15% PFD to optimize stomatal aperture. Leaf temperature was maintained at 25°C and the leaf-to-air vapour pressure deficit was kept between 1 and 2 kPa during all measurements. Once steady state was reached (usually between 30 and 60 min after clamping the leaf), either a light-response curve or a CO₂-response curve was measured. Six light-response curves and six CO₂-response curves were obtained for each plant line. Leakage of CO₂ into and out of the empty chamber was determined for the range of CO₂ concentrations used in this study and used to correct measured leaf fluxes (Bernacchi et al., 2002; Long and Bernacchi, 2003)
Estimation of $g_m$ by gas exchange and chlorophyll fluorescence

Two different methods using simultaneous gas exchange and chlorophyll fluorescence measurements were used to estimate $g_m$, as described by Terashima and Ono (2002) and Harley et al. (1992), respectively.

The method of Terashima and Ono (2002) consists of a comparison of the initial slopes of the $A_C$-$C_i$ and the $A_{N}$-$C_i$ curves. $C_i$ was calculated with the usual gas exchange procedures (Long and Bernacchi, 2003). $C_i$ was estimated according to procedures described by Epron et al. (1995) as:

$$C_i = (O/S)(J + 8(A + R))/(2J - 8(A + R))$$

where $O$ is the $O_2$ concentration in the chloroplast (assumed to be 0.21 mol mol$^{-1}$), $S$ is the specificity factor of Rubisco and $R_i$ is the rate of mitochondrial respiration in the light. The value of $S$ was considered to be 2459 mol mol$^{-1}$, corresponding to a CO$_2$ compensation point in the absence of respiration ($I^*$) of 42.75 mol mol$^{-1}$ (Bernacchi et al., 2002). $R_i$ was calculated using a linear regression line of the relationship between $A_C$ and $C_i$ for the range of $C_i$ below 150 mol mol$^{-1}$. $A_C$ at $I^*$ was assumed to be equal to $R_i$ (Brooks and Farquhar, 1985). $g_m$ was obtained as:

$$g_m = (m_m)/(m_t - m)$$

where $m_i$ and $m_t$ are the slopes of the $A_{N}$-$C_i$ and the $A_{C}$-$C_i$ curves, respectively, at $C_i$ below 200 mol mol$^{-1}$ and $C_t$ below 150 mol mol$^{-1}$.

The method by Harley et al. (1992) uses $A_C$ and $R_i$ measured from gas exchange and $J$ estimated from fluorescence to calculate $g_m$ as follows:

$$g_m = A_C/(C_i - (I^* + 8(A_C + R_i))/(J - 4(A_C + R_i)))$$

$I^*$ and $R_i$ were estimated as in the previous method. For the Harley method, estimations of $g_m$ were done at $C_i = 400$ mol mol$^{-1}$.

The primary difference between the two methods is that the first integrates a $g_m$ value for the whole CO$_2$-limited region of the CO$_2$-response curve, while the second estimates a $g_m$ value for each separate point of the curve.

Estimation of $g_m$ by carbon isotope discrimination

Two additional methods based on $^{13}$C discrimination were used for estimations of $g_m$ in additional experiments, one at the UIB and one jointly at the UNM and LANL.

At UIB, gas exchange parameters were measured as described with a LI-6400 system under steady-state conditions for a minimum of 45 min. For instantaneous carbon isotope discrimination, the air exiting the cuvette through the match valve system was passed through a magnesium perchlorate water trap and collected in a hand-made 100 ml glass flask at a flow rate of 150 ml min$^{-1}$. Once the steady state was reached, the stopcocks of the flasks were closed. Two sets of flasks were collected, one in the presence of the leaf (sample) and one in the absence of the leaf (reference). CO$_2$ from the collected air was concentrated in a sample loop under liquid nitrogen and introduced to the corresponding dual-inlet bellow. Carbon isotope discrimination was measured in a dual-inlet isotope ratio mass spectrometer (Thermo, Delta XPlus, Bremen, Germany). The dual-inlet analysis of the isotope ratio compared the CO$_2$ from the sample gas with the CO$_2$ from the reference gas. Carbon isotope discrimination was calculated as described by Evans et al. (1986), as:

$$\Delta^{13}C_{obs} = \xi(\delta^{13}C_o - \delta^{13}C_e)/[1000 + \delta^{13}C_o - \xi(\delta^{13}C_o - \delta^{13}C_e)]$$

where $\xi = C_i/(C_e - C_i)$, and $C_o$ and $C_e$ are the CO$_2$ concentrations entering and leaving the gas-exchange cuvette, respectively. Gas-exchange parameters $A_o$, $C_o$, and $C_e$ were determined as described with the LI-6400, and $\delta^{13}C_o$ equalled 0 and $\delta^{13}C_e$ was the measured discrimination value. Mesophyll conductance was determined by comparison of the expected discrimination, where $C_e$ is equal to $C_i$, and the measured discrimination, as described by Evans et al. (1986).

At UNM, gas exchange parameters were measured using a LI-6400 system in conjunction with a tuneable diode laser absorbance spectrometer (TDLAS) (TGA100A, Campbell Scientific, Logan, UT, USA) which measures absolute concentrations of $^{13}$CO$_2$ and $^{12}$CO$_2$ in air (Bowling et al., 2003). The TDLAS was adapted from atmospheric sampling to leaf-level measurement of on-line, real-time carbon isotope discrimination by subsampling air entering the LI-6400 reference IRGA and the air leaving the leaf cuvette through the match valve port (DTH, unpublished). Both air streams were dried with a Nafion dryer prior to analysis (PD625 dual configuration, Campbell Scientific, Logan, UT, USA). The TDLAS samples at an approximate flow rate of 190 ml min$^{-1}$ and LI-6400 flows were maintained slightly above this to ensure the room air was not sampled. A ‘T’ junction with one side open to the room was placed between the LI-6400 and the TDLAS to avoid pressure changes related to TDLAS subsampling. The empty, closed leaf cuvette was also measured before and after each leaf to correct for slight variation caused by changes in gasket leakiness between samples.

Upon clamping each leaf in the cuvette leaves were exposed to 2000 mol photons m$^{-2}$ sec$^{-1}$ light, 380 mol CO$_2$ mol$^{-1}$ air, flow of 220 mol sec$^{-1}$, and relative humidity around 50%. When steady-state was achieved the leaf was sampled for another 6 min (three 2-min cycles). The light intensity was decreased stepwise to 400 mol photons m$^{-2}$ sec$^{-1}$ and measurements were continued for at least 6 min after steady state at each light intensity.

Carbon isotope discrimination was calculated according to Evans et al. (1986) as described above. Mesophyll conductance values were determined by comparing predicted discrimination ($\Delta \lambda$) with $\Delta^{13}C_{obs}$ across the entire light-response curve using the slope of $(\Delta \lambda - \Delta^{13}C_{obs})/p_o$ versus $A_o/p_o$, where $p_i$ and $p_o$ are the intercellular and ambient partial pressures of CO$_2$ respectively (Equation 5 in von Caemmerer and Evans, 1991).

Protein extraction, separation and Western blotting

At the end of the experiments, fresh leaf material (50–100 mg per sample) was collected from several plants of each genotype, and ground in 1 ml buffer containing 5 mM EDTA, 5 mM EGTA, 10 mM KPO$_4$, pH 7.8 supplemented with Protease-Inhibitors from Sigma (St. Louis, MO, USA), followed by a 5-min centrifugation at 800 g in a tabletop-centrifuge. The supernatant was transferred to a new tube and mixed with a 4× concentrated Laemmli buffer (100 mM Tris pH 6.8, 200 mM DTT, 40% glycerol, 4% SDS, 0.2% bромphenol blue). Equal amounts (5 µg) of protein per lane were loaded on a PAGE gel and run at 30 mA.

Western blotting was then performed as described by Santoni et al. (2003), with some modifications. Proteins were transferred to Protran B83 (Whatman, Middlesex, UK) in 10% methanol, 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11 for 16 h at 35 V. The membrane was blocked for 1 h in phosphate-buffered saline containing 0.1% (v/v) Tween 20 and 1% BSA and subsequently incubated with the primary antibody (1:5000 dilution) for 2 h at room temperature. After washing (2× 10 min) in the phosphate buffer described above, the blot was incubated for 1 h...
with a phosphatase-labelled secondary antibody. After washing (2×10 min) in phosphate-buffered saline, a chemiluminescent signal using CDP Star (Applied Biosystems, Foster City, CA, USA) substrate was visualized on an autoradiography film (Hyperfilm, GE Healthcare, Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

One-way ANOVA was applied to assess the differences for each parameter between each separate control and its derive line. Differences among means were established using a Duncan test (P<0.05). The data were analysed applying the SPSS 10.0 programme for Windows. At UNM comparisons between control and derivative lines were analysed using pairwise t-tests calculated with Microsoft Excel 2002.

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Aquaporin involvement in mesophyll conductance to CO₂


