Running head: Photosynthetic control of transpiration

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Bioenergetics and Photosynthesis and Whole Plant and Ecophysiology
The contribution of photosynthesis to the red light response of stomatal conductance

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ABSTRACT

To determine the contribution of photosynthesis on stomatal conductance, we contrasted the stomatal red light response of wild type tobacco (*Nicotiana tabacum* L. cv W38) with that of plants impaired in photosynthesis by antisense reductions in the content of either cytochrome b/f complex (anti-b/f plants) or Rubisco (anti-Ssu plants). Both transgenic genotypes showed a lowered content of the antisense target proteins in guard cells as well as in the mesophyll. In the anti- b/f plants CO₂ assimilation rates were proportional to leaf cytochrome b/f content, but there was little effect on stomatal conductance and rate of stomatal opening.

To compare the relationship between photosynthesis and stomatal conductance wild type plants and anti-Ssu plants were grown at 30 and 300 µmol photon m⁻² s⁻¹ irradiance (LL and ML, respectively). Growth in ML increased CO₂ assimilation rates and stomatal conductance in both genotypes. Despite the significantly lower CO₂ assimilation rate in the anti-Ssu plants, the differences in stomatal conductance between the genotypes were non-significant at either growth irradiance. Irrespective of plant genotype, stomatal density in the two leaf surfaces was two-fold higher in ML than in LL-grown plants and conductance normalized to stomatal density was unaffected by growth irradiance. We conclude that the red light response of stomatal conductance is independent of the concurrent photosynthetic rate of the guard cells or of that of the underlying mesophyll. Furthermore we suggest that the correlation of photosynthetic capacity and stomatal conductance observed under different light environments is caused by signals largely independent of photosynthesis.
INTRODUCTION

Stomata function as hydraulic valves on the surface of aerial parts of plants, with the guard cells that surround each pore rapidly adjusting their turgor to optimise photosynthetic CO₂ uptake and minimize transpirational water loss from leaves. Stomata respond to a variety of signals, either received from the environment or produced within the plant, which lead to changes in the activities of ion or solute channels regulating guard cell turgor. Stomatal opening is induced by low CO₂ concentrations, high light intensity and high humidity, and closing is promoted by high CO₂ concentrations, darkness, drought and the plant hormone abscisic acid (Outlaw, 2003).

In C₃ species stomatal opening in response to light is thought to be induced by distinct mechanisms depending on the wavelength of incident light. Blue light is perceived directly by phototropins (Kinoshita et al., 2001; Doi et al., 2004) and activates a signalling cascade that results in fast stomatal opening under background red light (Shimazaki et al., 2007). The opening response of stomata to red light requires higher irradiance than blue light and shares characteristics of photosynthesis in its action spectra in the red region (Sharkey and Raschke, 1981). Furthermore the red light response can be abolished by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a photosystem II inhibitor in whole leaf, epidermal strips and guard cell protoplasts (Sharkey and Raschke, 1981; Tominaga et al., 2001; Olsen et al., 2002; Messinger et al., 2006). Using isolated guard cell protoplasts, Tominaga et al. (2001) showed that DCMU inhibited proton pumping in red light suggesting that guard-cell chloroplasts provide ATP required for H⁺ pumping in the guard cell plasma membrane.

It has also been suggested that the guard cell response to red light is in part an indirect response to red-light-driven intercellular CO₂ uptake in the mesophyll (Roelfsema et al., 2002). For example, Roelfsema et al. (2006) have shown that chloroplast-containing guard cells in albino sections of variegated leaves do not respond to photosynthetically active radiation, but are sensitive to blue light and CO₂, bringing into question a direct role of guard cell photosynthesis on red-light mediated stomatal opening in intact leaves.

With the exception of the orchid *Paphiopedilum*, guard cells from all species studied to date contain chloroplasts. Chlorophyll fluorescence measurements (Cardon and Berry, 1992; Goh et al., 1999; Lawson et al., 2002, 2003) and biochemical and
immunolocalization experiments (Ueno, 2001; Zeiger et al., 2002) suggest that guard cell chloroplasts have the capacity for electron transport, Rubisco-mediated CO₂ assimilation and photorespiration. Guard cell photophosphorylation has been postulated as a significant energy source driving stomatal opening in red light (Tominaga et al., 2001). These results have suggested a role of guard cell photosynthesis in the red light response of stomata; however, the mechanism underpinning this link has remained elusive.

Across species and under a variety of growth conditions plants regulate their transpiration and photosynthetic rates in parallel, maintaining a balance between the stomata-mediated supply of CO₂ to the mesophyll chloroplasts and their photosynthetic demand for CO₂. This results in the conservation of the ratio of intercellular (Cᵢ) to ambient (Cₐ) CO₂ partial pressures (pCO₂) within the leaf (Wong et al., 1979, 1985; Hetherington and Woodward, 2003). This empirical direct correlation between photosynthesis and stomatal conductance was central to initial models of stomatal control of photosynthesis (Farquhar and Wong, 1984; Ball et al., 1987) and has been carried over to more recent models (Jarvis and Davies, 1998; Dewar, 2002; Buckley et al., 2003). However, the underlying regulatory mechanism is still unclear. It has been proposed that guard cells sense the metabolic status of the mesophyll via a diffusible factor that is a product of photosynthetic activity in the mesophyll (Wong et al., 1979; Lee and Bowling, 1992) and that stomatal aperture would be inversely proportional to the pool size of such metabolites (Farquhar and Wong, 1984). Possible metabolites include ATP, NADPH or RuBP, the concentration of which depends strongly on the balance between chloroplast electron transport and the carboxylation reaction catalysed by Rubisco. However, exogenous ATP added to isolated epidermis did not stimulate opening (Lee and Bowling, 1992). Alternatively, guard cells could respond to photosynthetic demand by direct sensing of Cᵢ (Mott, 1988; Roelfsema et al., 2002).

Contrary to the predictions of the above mentioned models, transgenic plants with impairments in different steps of the photosynthetic process can maintain normal stomatal conductances, resulting in elevated Cᵢ’s casting some doubt on the extent of the control of stomatal movements by Cᵢ (Hudson et al., 1992; Lauerer et al., 1993; Stitt and Schulze, 1994). In antisense plants that accumulate only 10-15 % of the wild type levels of Rubisco, and show a proportional decrease in CO₂ assimilation rate, chlorophyll fluorescence measurements have shown that the antisense reduction of the
target gene was effective in guard cells as well as in the mesophyll. However, those plants also maintained wild type values of conductance under ambient CO$_2$ concentrations and a light source with a mix of red and blue light. (von Caemmerer et al., 2004). Most of the gas exchange measurements made on transgenic plants with impaired photosynthesis have so far been made under white light and this raises the question of whether stomatal conductance would be affected when red light is the only source of illumination.

This report examines the contribution of photosynthetic activity to the stomatal response to red light in intact plants. We contrasted the red light response of stomata of wild type tobacco with that of antisense plants impaired in photosynthetic CO$_2$ assimilation either by a decrease in chloroplast electron transport rate and ATP synthesis or by a decrease in Rubisco activity and ATP consumption and find that these impairments do not affect stomatal conductance. To further explore the relationship between photosynthesis and stomatal conductance we also examine the stomatal response of wild type and antisense Rubisco plants to growth irradiance. Remarkably, despite the large difference in photosynthetic rates, the transpiration machinery of wild type and anti-Ssu plants responded in the same manner to the different light growth conditions.

RESULTS

**Cytochrome f and Rubisco content in the epidermis of wild type and transgenic tobacco**

We used three different phenotypes of tobacco, the wild type and two lines with low photosynthetic CO$_2$ assimilation rates, generated by antisense technology: anti-blf plants, which carry an antisense construct directed against the Rieske iron-sulphur subunit of the chloroplast cytochrome $b_6f$ complex (Price et al., 1998), and homozygous anti-Ssu plants with 10-15% of the wild type content of Rubisco (Ruuska et al., 1998). To minimize developmental effects of the transgenes, our plants were grown under conditions that provided similar rates of growth for the wild type and transgenic genotypes (see Materials and Methods). Figure 1 shows that the known leaf phenotype of the transgenic plants is also expressed in the epidermal tissue. As reported previously (Price et al., 1998), the segregating population of anti-blf plants exhibited a variety of cytochrome $b_6f$ content, as estimated from leaf content of cytochrome $f$, ranging from 100% to less than 10% of wild type levels. Three representative examples of plants with leaf cytochrome $f$ content that was 51, 28 and
7% of wild type, respectively, are shown in Figure 1. Anti-Ssu plants, which are homozygous for a single insertion of the transgene (Hudson et al., 1992) showed the previously observed drastic reduction in Rubisco levels, with only 17% of the wild type Rubisco small subunit content when quantified by immunoblotting (Fig. 1). The antisense transgenes were driven by the CaMV 35S promoter, thus it was expected that both transgenic genotypes would show reduced levels of the target genes in all green cell types, including guard cells. Epidermal tissue prepared from anti-b/f plants with low leaf cytochrome b$_{6f}$ content showed a corresponding decrease in the level of cytochrome f, indicating that the antisense effect of the transgene was also operational in guard cells (Fig. 1). As with the anti-b/f plants, the effect of the anti-Ssu transgene was also present in the epidermis, where Rubisco content was 10% of that present in guard cells of the wild type (Fig. 1). Compared to wild type, anti-b/f plants showed a slight decrease (15% on average) in the amount of Rubisco in whole leaves and the whole leaf cytochrome f content of anti-Ssu plants was also decreased (Fig. 1). This is consistent with published data (Jiang and Rodermel, 1995).

**Photosynthetic rates and stomatal conductances under red measuring light in wild type and anti-b/f plants**

We used red light in our gas exchange experiments to induce photosynthesis independently of any stimulation of the blue light response of stomata, which is mediated by phototropins (Shimazaki et al., 2007). Attached leaves were equilibrated to ambient $p$CO$_2$ (362 µbar) in the gas exchange chamber in the dark for at least 20 min before red light of 1000 µmol photons m$^{-2}$ s$^{-1}$ was turned on. Wild type plants gradually attained an average steady state net rate of CO$_2$ assimilation of 7.5 µmol photons m$^{-2}$ s$^{-1}$, whereas anti-b/f plants exhibited net rates of CO$_2$ assimilation ranging from wild type values to only 0.8% of the wild type rate (Fig. 2A). There was a direct correlation between the steady state photosynthetic rates under red light and cytochrome b$_{6f}$ content in all plants (Fig. 3A). Both the wild type and anti-b/f plants showed variability in the rate of stomatal opening and the maximal stomatal conductance in red light (Fig. 2B). We found that stomatal conductance in both wild type and transgenics did not always reach a complete steady state under red light and therefore reported the maximal conductance. There was little effect of low photosynthetic rate on stomatal parameters, and even the anti-b/f plant with the lowest
photosynthetic rate in the range (0.8% of wild type) showed a maximal conductance that was 50% of the average wild type value (Fig 2A and 2B).

Except for plants with wild-type rates of CO₂ assimilation, the anti-b/f plants maintained a ratio of intercellular to ambient CO₂ concentration (Cᵢ/Cₐ) higher than in the wild type (Fig. 2C). There was a marked proportionality of Cᵢ/Cₐ and cytochrome b₆f content, with the anti-b/f plant with the lowest photosynthetic rate showing a Cᵢ/Cₐ ratio close to 1 (Fig. 3C). Figure 4 shows the maximal stomatal conductance and halftimes of stomatal opening of wild type and a large number of individual anti-b/f plants plotted against their steady state CO₂ assimilation rate in red light. Although the presence of the antisense Rieske Fe-S protein transgene reduced photosynthetic rates in the anti-b/f plants, their maximal stomatal conductance and rate of stomatal opening remained remarkably similar to that of the wild type when measured with light that stimulates photosynthesis but not the blue light photoreceptors that mediate stomatal opening.

**Photosynthetic rates and stomatal conductances under red measuring light in wild type and anti-Ssu plants and the effect of growth irradiance**

To gain further insight on the relationship between stomatal conductance and photosynthesis, we contrasted the red light response of stomata in wild type and anti-Ssu plants which contained between 10-15% of wild type Rubisco content. These transgenic plants do not show the light sensitivity and phenotypic instability of the anti-b/f plants and can be grown under a broader range of irradiances. To compare with our results on anti-b/f plants, we grew the anti-Ssu plants at the same low irradiance (25-35 µmol photons m⁻² s⁻¹, LL) and also chose a growth light intensity (300 µmol photons m⁻² s⁻¹, ML). This allowed us to contrast the effect of growth irradiance on stomatal conductance in wild type and photosynthetically impaired plants. Figure 5 shows the kinetics of gas exchange in leaves of wild type and anti-Ssu plants at ambient pCO₂ (362 µbar) during a transition from darkness to 1000 µmol photons m⁻² s⁻¹ of red light. Attached leaves were equilibrated to ambient pCO₂ (362 µbar) in the gas exchange chamber in the dark for at least 20 min before red light of 1000 µmol photons m⁻² s⁻¹ was turned on. Wild type plants grown in LL conditions gradually attained a steady state rate of net CO₂ assimilation of 8.9±0.19 µmol m⁻² s⁻¹, whereas anti-Ssu plants grown under the same conditions reached a steady state CO₂ assimilation rate of only 1.64±0.3 µmol m⁻² s⁻¹ (Fig. 5A). A ten-fold increase in
irradiance during growth increased CO$_2$ assimilation rate in the wild type, to 19.1±1.3 μmol m$^{-2}$ s$^{-1}$ in ML-grown plants. The anti-SSu plants reached a CO$_2$ assimilation rate of 6.8±0.2 μmol m$^{-2}$ s$^{-1}$ when grown in ML. Stomatal conductance in both sets of plants reached steady state values within 50 min of the onset of red light illumination (Fig. 5B). The maximal stomatal conductance in ML-grown plants was 0.34±0.05 and 0.288±0.03 mol m$^{-2}$ s$^{-1}$ for wild type and anti-Ssu, respectively, and was higher than that of LL-grown plants, which had conductances of 0.16±0.06 and 0.13±0.02 mol m$^{-2}$ s$^{-1}$ for wild type and anti-Ssu respectively. However, the differences in stomatal conductance between wild type and anti-Ssu plants at either light intensity were non-significant (P=0.05). The low CO$_2$ assimilation rates and relatively unchanged stomatal conductances in anti-SSu plants resulted in higher C$_i$/C$_a$ ratios for the transgenic plants than for wild type (Fig. 5C). The initial transient lowering of C$_i$/C$_a$ results from the fact that CO$_2$ assimilation rate increases more rapidly with irradiance than stomatal conductance.

Effect of growth irradiance on stomatal density and index in wild type and anti-Ssu plants

The drastic increase in stomatal conductance in plants grown at ML compared to LL was caused by increases of stomatal density by a factor of approximately 2 in both the abaxial and the adaxial surfaces of the leaf, irrespective of plant genotype (Fig. 6A). In the abaxial leaf surface, the stomatal index rose by 33 % and 25 % in wild type and anti-SSu plants, respectively, when grown at ML, whereas the top side of the leaves showed an increase in stomatal index of 50% in both genotypes when grown at ML (Fig. 6A). Compared to LL growth conditions, both types of plants showed a slight decrease in the size of pavement cells when grown in ML. Stomatal dimensions did not vary with different growth light intensity or genotype (data not shown). There was no significant effect of genotype on the stomatal conductance, calculated relative to the combined number of stomata on both leaf surfaces (Fig. 6B).

Relationship between photosynthetic rate and stomatal conductance in wild type and transgenic tobacco

As it has been shown that there can be a strong correlation between CO$_2$ assimilation rates and stomatal conductance over a range of growth conditions and leaf ages (Wong et al., 1979), reviewed in (Hetherington and Woodward, 2003)), we were interested to see how our data on transgenic tobacco would fit with the expected
linear trend. Figure 7 (filled circles) shows the co-variation of stomatal conductance and CO₂ assimilation rates for young wild type tobacco plants grown in environmental cabinet conditions, at CO₂ concentration of 1000 µmol mol⁻¹. Differences in growth light intensity and plant-to-plant variation produced a range of wild type net rates of CO₂ assimilation between approximately 7 and 20 µmol m⁻² s⁻¹. In these plants stomatal conductance was directly proportional to net CO₂ assimilation rates. Transgenic plants with reduced photosynthetic rates, caused either by decreased Rubisco content (Fig. 7, open circle, square and diamond) or by low cytochrome b₆f complex (Fig. 7, triangles), maintain stomatal conductances higher than expected from their low CO₂ assimilation rates and thus break the linear relationship of conductance and photosynthetic rate observed for the wild type.

**Light response of CO₂ assimilation rate and stomatal conductance**

We also examined the fluency response of CO₂ assimilation rate and stomatal conductance to varying intensities of red light in wild type and anti-SSu plants (Fig. 8). Leaves from ML-grown plants were acclimated in the dark for a minimum of 20 minutes before the red light was turned on for 30 min at each irradiance. Stomatal opening continued even after 50 min in the light and we decided to make measurements at defined time interval of 30 min. CO₂ assimilation rate was similar for wild type and anti-SSu plants at low light but saturated for the anti-SSu plants at a low rate around 300 µmol quanta m⁻² s⁻¹ whereas it continued to increase for wild type leaves (Fig. 8A). Stomatal conductance on the other hand was similar for wild type and anti-SSu plants (Fig. 8B). The largest increase in conductance occurred in the first step from dark to 50 µmol quanta m⁻² s⁻¹; however conductance continued to increase up to 1500 µmol quanta m⁻² s⁻¹ in both genotypes and the response was distinctly biphasic. The different response of CO₂ assimilation rate and stomatal conductance to irradiance in the anti-SSu plants resulted in greater ratios of Cᵢ/Cₐ compared to wild type (Fig. 8C). The humidity of the chamber was not controlled after the initial adjustment and led to a decrease in leaf to air vapour pressure difference which was however similar in wild-type and anti-SSu plants (Fig. 8D).
DISCUSSION

The red light response of stomata in transgenic tobacco with impaired photosynthesis

We have used transgenic tobacco with low capacity for either chloroplast electron transport (anti-\(b/f\) plants) or \(CO_2\) fixation capacity (anti-Ssu plants) to probe the contribution of photosynthetic capacity to stomatal opening in red light. In both types of transgenic plants there was an effective decrease in the the amount of the proteins targeted by antisense technology in the guard cells, as shown by immunoblotting (Fig. 1). Thus it is expected that guard cells from these transgenic plants will share at least some of the deficiencies in photosynthetic performance that have been described before for whole leaves. In fact, (von Caemmerer et al., 2004) have observed a strong correlation of photosynthetic performance in guard cells and mesophyll cells of anti-Ssu plants comparing measurements of guard cell chlorophyll fluorescence with that of the underlying mesophyll.

The two transgenic genotypes have contrasting phenotypes. The antisense RNA decrease in Rubisco content has been shown to cause an imbalance between the capacity of the photosynthetic carbon reduction cycle to fix \(CO_2\) and the chloroplast’s capacity for electron transport, resulting in an increase in the pool size of RuBP and ATP (Quick et al., 1991a; Hudson et al., 1992), and zeaxanthin (Ruuska et al., 2000a). Conversely, in the anti-\(b/f\) plants, low cytochrome \(b/f\) content and hence low electron transport rates cause a decrease in RuBP content, altered redox state (Price et al., 1998; Ruuska et al., 2000a) together with a lowered capacity for zeaxanthin formation via the xanthophyll cycle (Hurry et al., 1996). Despite the differences in photosynthetic properties and rates, we observed no difference in steady state stomatal conductance and stomatal opening in red light between wild type and the transgenics plants. Our results clearly indicate that in intact, attached leaves, the response of stomata to a dark to light transition utilizing red light as irradiance under ambient \(pCO_2\) is independent of the concurrent photosynthetic rate of the guard cells or of the underlying mesophyll (Figs. 2-5).

The opening response of stomata to red light has frequently been linked to photosynthesis because the stomatal response saturates at similar irradiance to photosynthesis and can be abolished by photosystem II inhibitors. Our results confirm that stomatal conductance continues to increase with increasing red irradiance in both wild type and anti-SSu plants (Fig. 8). The fact that stomatal conductance
continued to respond to increasing irradiance in the anti-SSu plants although CO₂
assimilation rate was saturated a low irradiance also suggests that the stomatal
response to red light is not linked to the response of CO₂ assimilation rate to red light.

A reduction in the b/f content in our transgenic line leads to a near linear
decrease in CO₂ assimilation rate (Fig. 3) as has been previously observed (Price et al.,
1998). It is thus surprising that this reduction of chloroplast electron transport
mediated by the reduction of the cytochrome b/f content does not have a proportional
effect on the stomatal red light response and strongly suggests that the red light
response of stomata is not quantitatively linked with chloroplast electron transport of
guard cells or the mesophyll. All our transgenic plants by necessity have some
chloroplast electron transport as they can be grown autotrophically. Thus, we can’t
exclude the possibility that a complete inhibition of guard cell chloroplast electron
transport is required to decrease the extent of the stomatal red light response.

The lack of a stomatal phenotype in the anti b/f plants suggests that routes
other than photophosphorylation can provide the energy required for stomatal opening.
The importance of guard cell respiration as an energy source to drive opening has
been pointed out (Parvathi and Raghavendra, 1995). Recent experiments on plants
with reduced tricarboxylic acid cycle activity but normal chloroplast electron
transport rates support the suggestion that mitochondrial function is necessary to
maintain optimal stomatal opening and transpiration rates (Nunes-Nesi et al., 2007).

**Is there a link between photosynthetic processes and the red light
response of stomata?**

The fact that the transgenic plants used in this study maintain normal
conductances but low photosynthetic rates results in higher than wild type C₅ values
for a given ambient CO₂ partial pressure (Fig. 3). The lack of sensitivity of guard cells
to C₅ has been observed in transgenic plants with low Rubisco or cytochrome b₅f
content before (Quick et al., 1991a; Price et al., 1998; von Caemmerer et al., 2004).
However, because those experiments were performed under white light or a red/blue
light source, they did not rule out the possibility of an equal, direct blue light
stimulation of opening in wild type and transgenic plants, which could be independent
of photosynthesis. To our knowledge, this is the first report to use red light to address
this question in intact plants. The nature of the red light response mechanism remains
unresolved. The experiments in this paper would argue that the effect of red light
absorption and utilisation by photosynthesis on conductance is not as direct as previously thought. Perhaps another, so far unidentified, photoreceptor is involved. However, the opening response to red light intensity (Fig. 8) clearly shows that no matter how light perception is achieved, stomatal conductance does respond to high light flux levels in a manner not dissimilar to photosynthesis, although there is a distinctly biphasic nature to it with both low and high light response regions. Clearly, more research needs to be done to find alternative mechanisms to explain the red light response of stomata.

Recent mathematical models that attempt to link guard cell photosynthesis with stomatal function hypothesize that the response of stomatal conductance is controlled by the balance between electron transport capacity and Rubisco capacity, and Zeaxanthin and ATP have been proposed as possible metabolic links (Zhu et al., 1998; Buckley et al., 2003). In our transgenic lines the balance between electron transport and Rubisco capacity has been perturbed in opposite directions and our results suggest that the pool size of either metabolite is not the main determinant of stomatal opening under red light. Due to their low electron transport rates relative Rubisco capacity, anti-RLF plants have a substantially decreased zeaxanthin pool (Hurry et al., 1996), however, their maximal stomatal conductance and stomatal opening rate are similar to those of the wild type (Fig. 4). Conversely, anti-Ssu plants have a decreased Rubisco relative to electron transport capacity and increased levels of zeaxanthin (Ruuska et al., 2000b); nevertheless, stomatal conductance is similar to wild type. A logical interpretation of our results is that in general they do not support the hypothesis of a direct link between the stomatal response to CO2 and the photosynthetic process.

Sucrose, either synthesized inside the guard cell or imported from the apoplast, has been proposed to play a key role as an osmoregulatory solute in stomatal movements (Talbott and Zeiger, 1998; Outlaw, 2003). Low leaf sugar content has been reported in anti-Ssu plants (Quick et al., 1991b), even under elevated CO2 growth conditions (Masle et al., 1993). We don’t know whether these transgenic plants can maintain their apoplastic sucrose concentrations despite the lowered bulk leaf concentrations, however the lack of a stomatal phenotype in anti-Ssu plants would suggest that metabolites other than sucrose can act as osmoregulators during stomatal opening.
**Correlation between photosynthetic capacity and stomatal conductance**

We used growth light intensity as the environmental variable with which to investigate the effect of low photosynthetic rate in the commonly observed co-modulation of stomatal conductance and photosynthesis by environmental conditions (Hetherington and Woodward, 2003). Wild type and anti-Ssu plants responded to an increase in growth irradiance from LL to ML by doubling their CO₂ assimilation rates (Fig. 5A) and their stomatal conductance (Fig. 5B) and density (Fig. 6). For wild type plants this resulted in a strong linear correlation between CO₂ assimilation rates and conductance, as expected (Fig. 7). Although the response to growth irradiance was similar in wild type and anti-Ssu plants, the latter maintained a high stomatal conductance relative to their decreased CO₂ assimilation rates under the two light conditions, showing that stomatal conductance and photosynthetic rate can be uncoupled by genetic manipulation of Rubisco content (Fig. 7). These results are in agreement with previous findings on transgenic plants with impairments in photosynthesis due to antisense decreases in the levels of Rubisco and other PCR cycle enzymes (Hudson et al., 1992; Lauerer et al., 1993; Haake et al., 1998; Muschak et al., 1999). In contrast with these observations in plants with low photosynthetic rates, the correlation between stomatal conductance and photosynthetic rate was apparently maintained in transgenic plants with decreased mitochondrial respiration (Nunes-Nesi et al., 2007).

**Developmental implications**

The density of stomata on the leaf epidermis is controlled by the environmental conditions prevailing during leaf expansion, and once determined, it remains unchanged for the lifetime of the leaf. Stomatal densities are higher in plants grown in full sun light or at high light intensities than in plants grown in shade (Willmer and Fricker, 1996). The stomatal index of dicot plants has also been shown to increase with light intensity (Schoch et al., 1980). Advances have been made recently in elucidating the genetic pathway controlling stomatal development (Bergmann, 2006). It is thought that mature leaves sense the environment and produce a systemic signal that determines stomatal density in expanding leaves (Coupe et al., 2006). Our results suggest that this systemic developmental signal is not directly linked to photosynthetic capacity: we show that, when anti-Ssu plants are grown under elevated CO₂, which prevents a limitation of the carbon fixation

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reactions, the stomatal developmental program is able to sense light intensities during growths and responds by increasing the stomatal density and index in the same manner as the wild type (Fig. 6). It has been shown previously that a decrease in Rubisco content does not interfere with the acclimation of stomatal conductance to environmental conditions such as growth light intensity under ambient $p$CO$_2$ (Lauerer et al., 1993), at elevated $p$CO$_2$ (Masle et al., 1993; Sicher et al., 1994), and under different nitrogen nutrition regimes (Quick et al., 1992), but differences in stomatal densities under different growth conditions have not been reported before in these plants. Taken together, these data suggest that the strong photosynthetic impairment of anti-Ssu plants does not significantly affect their ability to acclimate their transpirational machinery to the prevailing growth conditions. Correlation between CO$_2$ assimilation rate and stomatal conductance is also observed throughout the lifespan of a leaf. Jiang and Rodermel (1995) showed that stomatal conductance followed similar developmental changes with leaf age in anti-Ssu and wild type plants despite their different photosynthetic rates. This is an example where the changes in stomatal conductance are not linked to variation in stomatal numbers.

**Conclusion**

Our study illustrates the power of the transgenic approach in unravelling correlative links to reveal mechanistic connections. The results show that the red light response of stomata may not be linked to photosynthesis and that further work is required to discover the nature of the red light receptor. Furthermore, we have shown that the environmentally induced correlation between stomatal conductance and photosynthetic capacity so frequently observed must be caused by signals not directly related to photosynthesis. The results have major implications for our understanding of stomatal function and demonstrate that photosynthetic metabolism can be manipulated with minimal coupling to stomatal function and aperture. This means that if plants can be genetically engineered for improved photosynthesis this should also lead to improved plant water use efficiency.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

Wild type and transgenic tobacco plants (*Nicotiana tabacum* cv. W38) were grown in 0.25 L pots in seed raising soil containing ~2g/l of a slow release fertilizer.
(Osmocote; 15/4.8/10.8/1.2 N/P/K/Mg + trace elements: B, Cu, Fe, Mn, Mo, Zn, Scotts Australia Pty Ltd., Castle Hill, Australia) and bottom-watered daily. Two types of transgenic tobacco were used; a) anti-bf plants, which exhibit a range of phenotypes with respect to cytochrome bf content and CO2 assimilation rates (Price et al., 1998), carry an antisense construct directed against the Rieske iron-sulfur protein of the chloroplast cytochrome bf complex and were raised from seed of selfed T3 plants of the line B6F-2.2-513-16 (Price et al., 1995). b) anti-Ssu plants, which contain 10-15% of the wild-type Rubisco content and carry two copies of an antisense construct directed against the small subunit of Rubisco (Ruuska et al., 1998). Plants were grown in environmentally controlled cabinets under a pCO2 of 953 µbar, with a 20 h photoperiod and a constant temperature and relative humidity of 23°C and 65 %, respectively. The light intensity at the top of the plant was kept at 25±10 µmol photons m⁻² s⁻¹ for the anti-bf plants, and 30±5 µmol photons m⁻² s⁻¹ or 300±20 µmol photons m⁻² s⁻¹ for the anti-SSu plants, with their corresponding wild type controls grown under the same irradiance. The low irradiance and long photoperiod minimized the instability of the anti-bf phenotype and the high pCO2 ensured that wild type and transgenic plants had similar growth rates. Plants were assayed 4-5 weeks after germination (six leaf stage), and the uppermost fully expanded leaves were used in the experiments. Transgenic and wild type plants had similar leaf sizes and number of leaves at the time of measurement.

**Preparation of epidermal fragments, protein extraction and immunoblotting**

A fraction enriched in epidermal tissue was prepared by adapting the method of (Kopka et al., 1997). A young expanding leaf was picked, the major veins were removed and discarded and the rest was blended with 250 ml of chilled distilled water with a Sorvall Omni Mixer blender at maximum speed, with four pulses of 30 sec each and waiting 30 sec between pulses. The resulting epidermal fragments were rinsed with 300 ml of chilled distilled water on a 100-149 µm Nytal mesh to rid them of contaminating mesophyll cells. The epidermal fragments were drained of excess water, disrupted by grinding with mortar and pestle in liquid nitrogen for 3 min and stored at -80°C until later use. The resulting fraction was highly enriched in epidermis compared to mesophyll cells (less than 1 mesophyll cell per 200 stomata was routinely observed under the compound microscope).
Total proteins from 1.28 cm$^2$ leaf disks or 100-mg of epidermal fragments were extracted at room temperature in 0.5 ml of buffer containing 100 mM Tris-HCL, pH 7.8, 20 mM EDTA, 25 mM NaCl, 10 mM dithiothreitol, 2 % (w/v) sodium dodecyl sulphate and 2 % (v/v) of protease inhibitor cocktail (Sigma, St Louis, Missouri) using a 2-ml glass homogenizer. Samples were heated to 65°C in a heat block for 10 min and centrifuged at room temperature in a microcentrifuge at maximum speed for 10 min. Protein concentration in the samples was determined with the bicinchoninic acid method (BCA Protein Assay kit, Pierce, Rockford, Illinois). Samples were prepared for gel loading by adding 0.25 vol of BioRad XT sample buffer (Bio-Rad, Hercules, California). Proteins were separated by electrophoresis on NuPAGE Bis-Tris pre-cast gels (4-12 % acrylamide concentration, Novex, San Diego, California), using the manufacturer-specified buffer system and blotted onto nitrocellulose membranes. Blots were probed with polyclonal antibodies raised against the spinach Rubisco holoenzyme or spinach cytochrome $f$. Anti-Ig G horseradish peroxidase conjugate (Pierce, Norfolk, Il) was used as secondary antibody. Blots were developed using the enhanced chemiluminescence SuperSignal West Pico substrate system (Pierce). Because of the close correlation of accumulation of cytochrome $b_{6}f$ holocomplex and its cytochrome $f$ subunit (Bruce and Malkin, 1991; Price et al., 1998), anti-cytochrome $f$ antibody was used instead of a less specific Rieske Fe-S protein antibody to quantify content of cytochrome $b_{6}f$. Protein bands were quantified using Image J software (http://rsb.info.nih.gov/ij/).

**Measurement of leaf gas exchange**

Gas exchange measurements were made with a LI-6400 portable gas-exchange system (LI-COR, Lincoln, NE, USA), equipped with a red LED light source with a maximum emission peak centred at 670nm (LI-6400-02, LI-COR). This light source was fitted on the standard 6 cm$^2$ clamp on leaf chamber. Sample $p$CO$_2$, flow rate and temperature were kept constant at 362 µbar, 500 µmol s$^{-1}$, and 25°C, respectively. Leaves were equilibrated in the gas-exchange leaf chamber in darkness, at an initial humidity of 19 mbar for a minimum of 20 min before measurements. For light response curves, the conditions were the same, and after the initial 20 min of dark adaptation the intensity of red light was increased in steps of 30 min duration. The average atmospheric pressure was 950 mbar. Gas exchange parameters were calculated using the equation derived by von Caemmerer and Farquhar (1981).
**Determination of stomatal numbers**

Stomatal numbers were determined from the same or similar leaves as used for gas exchange measurements, from impressions taken from both sides of the leaves with dental silicone (Optosil-Xantopren, Heraeus Kulzer, Hanau, Germany). Stomata and epidermal cells were counted from positives made from the impressions with nail polish, in ten different fields of view per leaf, with a compound microscope using a magnification of 200-fold. Digital photographs of each field were taken and cells were counted and measured with Image J software (http://rsb.info.nih.gov/ij/).

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**FIGURE LEGENDS**

**Figure 1.**
Cytochrome $b_{6}f$ and Rubisco content in leaf and epidermis of wild type and transgenic tobacco plants determined by immunoblotting. Total protein extracts of leaf disks and epidermal fragments are compared for content of cytochrome $f$ (cyt $f$) and the large and small subunits of Rubisco (Lsu and Ssu, respectively). Plants were grown under low (LL) or medium light intensity (ML) for comparison of wild type with anti-$b/f$ and anti-SSu leaves, respectively. Three different anti-$b/f$ plants, labelled a-bf1, a-bf2 and a-bf3, and having CO$_2$ assimilation rates that were 43 %, 36 % and 17% of wild type values, respectively, are shown as representatives of anti-$b/f$ plants with low photosynthetic rates. For whole leaf samples, gel lanes were loaded on an equal leaf area basis, and samples from ML-grown plants were diluted 5-fold relative to samples from LL-grown plants. Equal total protein amounts (20µg) were loaded when comparing epidermal samples. wt = wild type plant; a-Ssu = anti-Ssu plant.

**Figure 2.**
Kinetics of (A) CO$_2$ assimilation rate, (B) leaf conductance and (C) the ratio of intercellular to ambient CO$_2$ ($C_{i}/C_{a}$) in wild type plants (filled symbols) and a range of anti-$b/f$ plants with different cytochrome $b_{6}f$ contents (open symbols), during illumination of dark acclimated leaves at an irradiance of 1000 µmol photons m$^{-2}$ s$^{-1}$.
of red light. Symbols of different shape (open or closed) represent measurements made on different plants. Light was turned on at time = 0. Measurements were conducted at 362 µmol mol\(^{-1}\) CO\(_2\), a leaf temperature of 25 °C and a leaf chamber humidity of 19 mbar. Leaves were acclimated in the dark for a minimum of 20 min before the measurements. Stomatal conductance, \(g_s\) was normalized by subtracting the conductance values at time zero (\(g_0\)), which ranged between 0.006 to 0.035 mol m\(^{-2}\) s\(^{-1}\).

**Figure 3.**
Leaf gas exchange parameters as a function of cytochrome \(b_{6f}\) complex content in wild type (filled circles) and anti-\(b_{6f}\) (open circles) tobacco. A) CO\(_2\) assimilation rate. B) stomatal conductance. C) The ratio of intercellular to ambient CO\(_2\) (\(C_i/C_a\)). Leaf cytochrome \(b_{6f}\) complex content was determined from immunoblots as the one shown in Fig. 1 and is expressed as a fraction of the maximum wild type value. Gas exchange experiments were performed as described in Figure 2. Each point corresponds to a leaf from a different plant.

**Figure 4.**
Relationship between maximal stomatal conductance and CO\(_2\) assimilation rate in wild type and anti-\(b_{6f}\) tobacco measured under red light. A) Stomatal conductance. B) Halftimes of stomatal opening. Experimental conditions were as for Figure 2. Each point corresponds to a different plant. Filled circles: wild type plants; open circles: anti-\(b_{6f}\) plants. Half times were calculated as the time taken to reach half the maximal conductance from the time the light was turned on.

**Figure 5.**
Kinetics of (A) CO\(_2\) assimilation rate, (B) leaf conductance and (C) the ratio of intercellular to ambient CO\(_2\) (\(C_i/C_a\)) in wild type and anti-Ssu plants with 10-15% of wild type Rubisco, during illumination of dark acclimated leaves at an irradiance of 1000 µmol photons m\(^{-2}\) s\(^{-1}\) of red light. Plants were grown under an irradiance of 30 µmol photons m\(^{-2}\) s\(^{-1}\) (LL, squares) or 300 µmol photons m\(^{-2}\) s\(^{-1}\) (ML, circles). During gas exchange measurements, light was turned on at time = 0. Leaves were acclimated in the dark for a minimum of 20 min before the measurements. Experimental conditions were as for Figure 2. Data are the means of measurements on four different
plants, error bars represent standard error and are not shown if smaller than the symbols. Filled symbols: wild type plants; open symbols: anti-Ssu plants.

**Figure 6.**
Number of stomata on the leaf surface of wild type (dark bars) and anti-Ssu (light grey bars) tobacco plants as a function of growth irradiance and stomatal conductance normalised by stomatal numbers. Plants were grown under an irradiance of 30 µmol photons m\(^{-2}\) s\(^{-1}\) (LL) or 300 µmol photons m\(^{-2}\) s\(^{-1}\) (ML). A) Stomatal density. B) Stomatal index. C) Stomatal conductance normalised by stomatal numbers. Data represent mean values ± SE from four different plants.

**Figure 7.**
Relationship between stomatal conductance and CO\(_2\) assimilation rate in wild type and transgenic tobacco plants impaired in photosynthesis either by a decrease in electron transport rates (anti-b/f plants) or in Rubisco function (anti-SSu plants). Plants were grown under elevated CO\(_2\) in environmentally controlled chambers and conductance and photosynthesis measurements were performed under ambient CO\(_2\). Filled circles, wild type; open triangles, anti-b/f plants; open diamond, mean ± SE (n=4) from LL-grown anti-Ssu plants; open square, mean ± SE (n=4) of ML-grown anti-Ssu plants, open circle, mean ± SE (n=5) from ML-grown anti-Ssu plants assayed in red/blue light (from von Caemmerer et al., 2004). Arrows link data from anti-Ssu plants with the mean ± SE of 4-5 wild type plants grown and assayed under identical conditions at the same time. The solid and dashed line represent linear regression fit of all wild type data (y=0.0217 (±0.00069)*x, R=0.90), and LL-grown wild type and anti-b/f data as shown in Fig. 4A (y=0.1209 (±0.0159)+0.00514(±0.0 29)*x, R=0.34), respectively. Each data point not showing error bars corresponds to an individual plant. Error bars represent SE.

**Figure 8**
Red light response of (A) CO\(_2\) assimilation rate, (B) leaf conductance, (C) the ratio of intercellular to ambient CO\(_2\) (C\(_i\)/C\(_a\)) and (D) leaf to air vapour pressure difference in wild type and anti-Ssu plants. Measurements were conducted at 362 µbar CO\(_2\), a leaf temperature of 25°C and an initial leaf chamber humidity of 19 mbar. Leaves from ML-grown plants were acclimated in the dark for a minimum of 20 min before the red light was turned on and light intensity increased stepwise at 30 min intervals. Data are
the means of measurements on three different plants, error bars represent standard error and are not shown if smaller than the symbols. Filled circles: wild type plants; open circles: anti-Ssu plants.

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|        | whole leaf | epidermis |
|--------|------------|-----------|
|        | LL         | ML        | LL         | ML        |
| wt     |            |           | wt         |           |
| a-bf1  |            |           | a-bf1      |           |
| a-bf2  |            |           | a-bf2      |           |
| a-bf3  |            |           | a-bf3      |           |
| a-Ssu  |            |           | a-Ssu      |           |

|        | Lsu | cyt f | Ssu |
|--------|-----|-------|-----|
| whole leaf | ![Image] | ![Image] | ![Image] |
| epidermis | ![Image] | ![Image] | ![Image] |

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CO₂ assimilation rate ($\mu$mol m$^{-2}$ s$^{-1}$)

Stomatal conductance ($g_s - g_{min}$) (mmol m$^{-2}$ s$^{-1}$)

$C_i/C_a$

Time (min)
A) CO₂ assimilation rate (µmol m⁻² s⁻¹)

B) Stomatal conductance (mol m⁻² s⁻¹)

C) Cytochrome b₆f content (relative units)

Cytochrome b₆f content (relative units)
Halftime of stomatal opening (min)

Stomatal conductance (mol m\(^{-2}\) s\(^{-1}\))

CO\(_2\) assimilation rate (µmol m\(^{-2}\) s\(^{-1}\))
CO₂ assimilation rate (µmol m⁻² s⁻¹)

Stomatal conductance (mol m⁻² s⁻¹)

C₄/C₅

Time (min)
Stomatal density (mm$^{-2}$)

Conductance/stoma (mmol s$^{-1}$)

Stomatal index (stoma/pavement cell)

A

B

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Stomatal conductance (mol m$^{-2}$ s$^{-1}$)

CO$_2$ assimilation rate (µmol m$^{-2}$ s$^{-1}$)
Figures A, B, C, and D show the relationships between various physiological parameters and irradiance. In Figure A, the CO₂ assimilation rate (µmol m⁻² s⁻¹) increases with irradiance (µmol quanta m⁻² s⁻¹). Figure B illustrates the stomatal conductance (mol m⁻² s⁻¹) as a function of irradiance. Figure C depicts the ratio of Ci/Ca (where Ci is the intercellular CO₂ concentration and Ca is the atmospheric CO₂ concentration) over a range of irradiance values. Lastly, Figure D presents the variation in VPD (mbar) with increasing irradiance.

*CO₂ assimilation rate (µmol m⁻² s⁻¹) A
Stomatal conductance (mol m⁻² s⁻¹) B
Ci/Ca
VPD (mbar) D*