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RESEARCH PAPER

Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and CO_2

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Abstract

Transgenic antisense tobacco plants with a range of reductions in sedoheptulose-1,7-bisphosphatase (SBPase) activity were used to investigate the role of photosynthesis in stomatal opening responses. High resolution chlorophyll *a* fluorescence imaging showed that the quantum efficiency of photosystem II electron transport (F_q'/F_m') was decreased similarly in both guard and mesophyll cells of the SBPase antisense plants compared to the wild-type plants. This demonstrated for the first time that photosynthetic operating efficiency in the guard cells responds to changes in the regeneration capacity of the Calvin cycle. The rate of stomatal opening in response to a 30 min, 10-fold step increase in red photon flux density in the leaves from the SBPase antisense plants was significantly greater than wild-type plants. Final stomatal conductance under red and mixed blue/red irradiance was greater in the antisense plants than in the wild-type control plants despite lower CO_2 assimilation rates and higher internal CO_2 concentrations. Increasing CO_2 concentration resulted in a similar stomatal closing response in wild-type and antisense plants when measured in red light. However, in the antisense plants with small reductions in SBPase activity greater stomatal conductances were observed at all C_i levels. Together, these data suggest that the primary light-induced opening or CO_2-dependent closing response of stomata is not dependent upon guard or mesophyll cell photosynthetic capacity, but that photosynthetic electron transport, or its end-products, regulate the control of stomatal responses to light and CO_2.

Key words: CO_2 concentration, guard cell photosynthesis, light response, photosynthetic electron transport, SBPase, stomata, stomatal conductance.

Introduction

Stomatal aperture controls the flux of CO_2 and H_2O between plant and atmosphere and responds to several environmental variables. Many authors have suggested that part of the mechanisms responsible for sensing these environmental variables must be located in the epidermis or the guard cells themselves because guard cells in epidermal peels respond similarly to those in intact leaf tissue (Willmer and Fricker, 1996; Frechilla *et al*., 2002). Chloroplasts are a notable feature of guard cells in almost all plant species examined, although the role they play in stomatal function and the response to CO_2 concentration remains unclear (Vavasseur and Raghavendra, 2005). However, guard cell chloroplasts are believed to be involved in several different light transduction pathways (Zeiger *et al*., 2002). The photosynthesis-independent, blue light response has been shown to saturate at low fluence rates and is often associated with rapid stomatal opening (Zeiger *et al*., 2002). Stomata also open in response to higher intensities of light within the photosynthetically active radiation (PAR) waveband (Willmer and Fricker, 1996). This response saturates at high fluence rates similar to those that saturate guard cell and mesophyll photosynthesis and is inhibited by DCMU, indicating that it is photosynthesis-dependent (Kuiper, 1964; Sharkey and Raschke, 1981; Tominaga *et al*., 2001; Zeiger *et al*., 2002; Olsen *et al*., 2002; Messinger *et al*., 2006). Under PAR illumination, guard cell chloroplasts could provide a significant energy source for H^+ extrusion and ion transport

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(Wu and Assmann, 1993; Tominaga *et al*., 2001), or they could provide sucrose through photosynthetic carbon assimilation, although the extent of this is still controversial (Talbott and Zeiger, 1998; Zeiger *et al*., 2002; Outlaw, 2003; Vavasseur and Ragavendra, 2005). Furthermore, guard cells show O_2 and CO_2 modulation of PSII electron transport, indicating Rubisco-mediated carbon assimilation (Lawson *et al*., 2002, 2003). However, Roelfsema and Hedrich (2005) have returned to the earlier suggestion that the opening response to PAR is only due to intracellular or intercellular CO_2 depletion. Thus, the involvement of guard cell photosynthesis in light- and CO_2-modulated stomatal behaviour remains to be resolved and the use of transgenic plants has offered a different approach (von Caemmerer *et al*., 2004; Baroli *et al*., 2008).

Antisense technology has been exploited extensively to study the control of photosynthetic carbon flux through the Calvin cycle (Stitt and Schulze, 1994; Raines, 2003). Analyses of transgenic plants with reduced levels of a number of individual Calvin cycle enzymes have usually shown that CO_2 assimilation rate (A) was decreased and internal CO_2 concentration (C_i) increased. Stomata usually respond to increased C_i by reducing aperture (Morison, 1987; Mott, 1990) so a reduction in stomatal conductance (g_s) would be expected in these transgenic plants. However, as most studies report no difference in g_s between wild-type (WT) and transgenic plants, this suggests that stomata do not respond to either the increase in C_i or the reduced guard cell or mesophyll photosynthesis (Muschak *et al*., 1999; Quick *et al*., 1991; Hudson *et al*., 1992). Using antisense Rubisco tobacco plants, von Caemmerer *et al*. (2004) have shown that, despite similar reductions in guard and mesophyll cell photosynthetic operating efficiency (F_q'/F_m'), stomatal responses to a step increase in mixed red and blue photon flux density (PFD) and to changes in C_i were similar to wild-type plants. These results suggested that the light-induced opening and C_i-mediated closing responses of the stomatal pores are not dependent on the photosynthetic capacity of the guard or mesophyll cells. Further support for this has come from a more recent study using antisense cytochrome b_6f complex plants that showed, despite large differences in photosynthetic electron transport rates, stomatal opening was essentially the same as wild-type (Baroli *et al*., 2008).

In this study, tobacco plants with a range of reduced levels of sedoheptulose 1,7-bisphosphatase (SBPase) have been used. There are several reasons for using these plants in comparison with the earlier work on antisense Rubisco plants (von Caemmerer *et al*., 2004). Firstly, SBPase controls the regeneration of ribulose-1,5-bisphosphate (RuBP) to drive off the Calvin cycle (regeneration capacity) in contrast to Rubisco which controls carboxylation. Secondly, the Rubisco transgenic plants used had severe reductions in enzyme activity (~80%), and therefore photosynthetic capacity, so that plants were grown in controlled environment chambers at CO_2 concentrations of 800 μmol mol^{-1}. However, growth CO_2 concentration (Lodge *et al*., 2001), water stress (Raschke, 1975), and differences between environmental chambers and greenhouse conditions have been shown to influence guard cell sensitivity to CO_2 severely (Talbott *et al*., 1996; Frechilla *et al*., 2002, 2004; Zeiger *et al*., 2002). Using the antisense SBPase tobacco plants such potential complications were avoided by growing plants in normal air under glasshouse conditions. In addition, plants could be assessed with a range of reductions in photosynthesis capacity and therefore possible dose-dependent responses could be determined. Previously it has been shown that photosynthetic carbon fixation rates are sensitive to reductions in SBPase activity (Harrison *et al*., 1998). In plants with small reductions in SBPase activity (20–35%) a reduction in the regeneration capacity of the Calvin cycle was evident, but the rate of carboxylation remained the same as in the WT plants (Harrison *et al*., 2001; Raines, 2003). Therefore, these transgenic plants provide an opportunity to determine both the impact of a range of decreases in photosynthesis on stomatal responses and also the possible contribution of regeneration capacity to guard cell function. These plants were used to investigate (i) the relationship between CO_2 assimilation rate and stomatal conductance in response to step changes in normal mixed blue/red light, and red light alone (to avoid non-photosynthetic blue light responses), and (ii) the influence of reduced Calvin cycle regeneration capacity on stomatal responses to CO_2 concentration and photon flux density.

Materials and methods

Growth of plants for physiological analysis

Wild-type and transgenic T_2 progeny tobacco (*Nicotiana tabacum* L. cv. Samsun) seeds were germinated on sterile MS medium containing 1% (w/v) sucrose (Murashige and Skoog, 1962). For transgenic seedlings the medium was supplemented with kanamycin (300 μg ml^{-1}). Three-week-old seedlings were transferred to a peat and loam-based compost (F2, Levington Horticulture Ltd., Ipswich, UK) and acclimatized in a propagator before transfer to 18 cm pots. Plants were grown in a heated glasshouse, where temperature was maintained above 20 °C at night and rarely exceeded 30 °C during the day. The plants were kept well-watered throughout. Supplementary lighting (PFD of 350 μmol m^{-2} s^{-1}) was provided from 07.00 h to 19.00 h by sodium vapour lamps.

Determination of SBPase activity

Frozen leaf discs were ground to a fine powder in liquid nitrogen using a mortar and pestle in 1.4 ml extraction buffer (50 mM HEPES, pH 8.2, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 2 mM benzamidine, 2 mM amino caprionic acid, 0.5 mM phenylmethylsulphonylfluoride; and 10 mM dithiothreitol (DTT), transferred to a prechilled 2 ml tube and spun for 1 min at 4 °C. The supernatant was desalted using a NAP-10 column (Pharmacia, Milton Keynes, UK) and equilibrated with desalting buffer (extraction buffer omitting the Triton X-100).

Proteins were eluted from the column with 1.5 ml desalting buffer and aliquots stored in liquid nitrogen. To start the reaction, 20 µl of thawed extract was added to 66 µl of assay buffer (50 mM TRIS, pH 8.2, 15 mM $MgCl_2$, 1.5 mM EDTA, 10 mM DTT, 2 mM SBP) and incubated at 25 °C for 5 min. The reaction was stopped by the addition of 50 µl of 1 M perchloric acid and stored on ice. The samples were centrifuged for 5 min and the supernatant assayed for free phosphate. Samples (30 µl) and phosphate standards (0.125–4 nM phosphate) were incubated with for 25 min at room temperature with 300 µl of Biomol Green reagent (Biomol, AK111) and the absorbance at 620 nm was measured (Leegood, 1990).

Fluorescence imaging of seedlings

Images of chlorophyll *a* fluorescence were obtained as described by Barbargallo *et al.* (2003) using a CF Imager (Technologica Ltd., Colchester, UK). Seedlings were dark-adapted for 15 min before minimal fluorescence (F_o) was measured using a weak measuring pulse. Then maximal fluorescence (F_m) was measured during an 800 ms exposure with a saturating pulse having a PFD of 4800 µmol m^{-2} s^{-1}. Plants were then exposed to an actinic PFD of 300 µmol m^{-2} s^{-1} for 15 min and steady-state F' was continuously monitored while F_m' (maximum fluorescence in the light) was measured at 5 min intervals by applying saturating light pulses. This was repeated at a photon flux density of 500 µmol m^{-2} s^{-1}. Using the images captured at F' and F_m', images of PSII quantum photosynthetic efficiency

$$F_q'/F_m' = (F_m' - F)/F_m'\Big)$$

were constructed by the imaging software. Non-photochemical quenching (*NPQ*) was determined from $(F_m/F_m') - 1$.

Single cell fluorescence imaging

High-resolution chlorophyll *a* fluorescence imaging analysis of individual cells was employed, as described previously by Lawson *et al.* (2002). A specially designed chamber attached to a gas exchange system (CIRAS 1, PP Systems, Hitchin, UK) maintained a constant CO_2 concentration of 380 µmol mol^{-1}, relative humidity of 60%, and a temperature of 25 °C around the leaf. Chlorophyll *a* fluorescence was imaged through a 680 nm bandpass filter. All images were taken from the abaxial surface of leaves using a ×40 objective, which provided images of 310×205 µm with a pixel size of 534 nm^2. Chloroplasts within guard cell pairs were isolated from images using the ends-in search and editing tools of the FluorImager computer programme (Technologica Ltd., Colchester, UK) as described in Oxborough and Baker (1997), Lawson *et al.* (2002), and Oxborough (2004).

Effect of light on assimilation rate and stomatal conductance

Gas exchange measurements were made on young plants with nine leaves with a gas exchange system (Li-Cor 6400, Lincoln, Nebraska). For the light response experiments, light was provided by red or blue/red LED light source (Li-Cor 6400-02 and 02B). Leaves were first equilibrated at a PFD of 100 µmol m^{-2} s^{-1} for 20–30 min. The PFD was then increased to 1000 µmol m^{-2} s^{-1} for 30 min and then returned to 100 µmol m^{-2} s^{-1}. During the experiment, leaf chamber CO_2 and humidity were maintained at 380 µmol mol^{-1} and 23 mmol mol^{-1} and leaf temperature at 25 °C. This resulted in a constant leaf to air vapour pressure difference of approximately 1.0 kPa.

Effect of CO_2 on assimilation rate and stomatal conductance

Measurements of gas exchange were made on young plants which had eight leaves using the same Li-Cor gas exchange system. Leaves were first equilibrated at a PFD of 1000 µmol m^{-2} s^{-1} red light, and a chamber CO_2 concentration of 400 µmol mol^{-1} for 30–50 min. The CO_2 concentration was stepwise decreased, followed by stepwise increases to cover a range of CO_2 concentrations from 50–1600 µmol mol^{-1}. At each CO_2 concentration the leaf was allowed to stabilize to steady-state conditions for >30 min. Throughout the experiment VPD was maintained at 0.85 kPa with a dew point generator (Li-Cor, Lincoln, Nebraska), PFD and temperature were maintained at 1000 µmol m^{-2} s^{-1} and at 25 °C, respectively.

Effect of red PFD on g_s whilst C_i was maintained constant

The effect of red light on photosynthesis and stomatal conductance with a constant C_i was assessed on several of the plants used for the CO_2 response curves using a CIRAS-1 gas exchange system. Leaves were first equilibrated at a PFD of 1000 µmol m^{-2} s^{-1} red light (wavelengths >650 nm) provided by using a long pass filter and cold mirror placed in front of a halogen light source. The light was stepwise decreased and the leaf allowed to stabilize to steady-state conditions for about 30 min. Internal CO_2 concentration was maintained at about 280 µmol mol^{-1} by manipulating the external CO_2 concentration. Temperature and humidity were maintained throughout the experiment at 26±2 °C and 23 mmol mol^{-1}, respectively.

Statistical analysis

Data shown in Fig. 4 are means (±se) from three to five replicates. Differences in stomatal conductance after 25 min increased light were analysed by ANOVA in Systat 11 (Systat Software Inc., California USA). The rate of stomatal opening and closing were determined using linear regression analysis (Excel 2002, Microsoft Corporation) of the increasing and decreasing slopes of stomatal responses. Differences were analysed using ANOVA (Systat 11, Systat Software Inc., California USA). Correlation analysis (Fig. 6) for all data points was performed in Excel. Data in Fig. 7 are from three or four replicates and differences between stomatal responses to CO_2 were determined using ANOVA with a repeated measures design for the different CO_2 concentrations (Systat 11).

Results

Relationship between photosynthesis and SBPase activity

Leaf net CO_2 assimilation rates (*A*) measured in different light and CO_2 conditions in 2% O_2 were used to estimate *in vivo* SBPase activity [calculated as (assimilation rate+ dark respiration rate)/3], in WT and antisense plants (Fig. 1). There was a strong positive correlation between *in vivo* and *in vitro* SBPase activity in high light and moderate or high CO_2 (Fig. 1A, C) but not when light or CO_2 fluxes were low (Fig. 1B, D). This illustrates that under conditions of high photosynthetic rates SBPase activity has a high control coefficient on mesophyll carbon assimilation (Fig. 1A, C).

Whole plant chlorophyll *a* fluorescence imaging was used to determine the influence of SBPase activity on photosynthetic electron transport, and it also provided a rapid, non-invasive screening tool to select antisense plants for further study (Barbagallo *et al.*, 2003).

Chlorophyll fluorescence imaging of seedlings showed a positive correlation between SBPase activity and F_q'/F_m' (quantum efficiency of PSII electron transport; data not shown). The images in Fig. 2 show that, in wild-type seedlings, the leaves had the same uniform photosynthetic characteristics while the antisense SBPase plants showed reduced F_q'/F_m' with decreased amounts of SBPase activity and increased non-photochemical quenching as estimated by *NPQ* (F_m/F_m') −1). The older leaves tended to have greater reduction in F_q'/F_m' and increased *NPQ* compared with the younger leaves. In antisense plants with SBPase activities reduced to 74% and 60% of WT plants, F_q'/F_m' values averaged across all leaves were 0.36 and 0.33, respectively, compared with WT values of 0.41 (reductions by 12% and 20%, respectively). *NPQ* was higher in the antisense plants, with values of 0.56 and 0.78 for plants with 74% and 60% of WT SBPase activity and 0.26 for WT plants (Fig. 2).

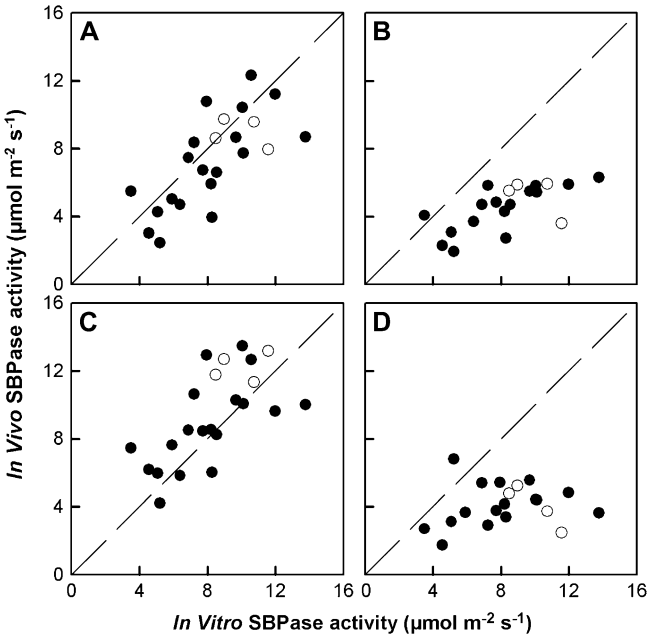

Fig. 1. Relationship between *in vitro* and estimated *in vivo* SBPase activity at (A) 1000 μmol m^{-2} s^{-1} photon flux density (PFD) and 380 μmol mol^{-1} CO_2 concentration, (B) 300 μmol m^{-2} s^{-1} PFD and 380 μmol mol^{-1} CO_2 concentration, (C) 1000 μmol m^{-2} s^{-1}PFD and 1500 μmol mol^{-1} CO_2 concentration, and (D) 1000 μmol m^{-2} s^{-1} PFD and 155 μmol mol^{-1} CO_2 concentration. Estimated *in vivo* SBPase activity was calculated as (assimilation rate+dark respiration rate)/3. Solid circles represent transgenic antisense plants and open circles WT.

Chlorophyll a fluorescence imaging of guard and mesophyll cells

High-resolution chlorophyll *a* fluorescence imaging with a microscope was used to determine the effect of reductions in SBPase activity on mesophyll and guard cell quantum efficiency of PSII photochemistry. The response of F_q'/F_m' to increasing PFD in fully expanded leaves showed that photosynthetic capacity was reduced by approximately 20% in both guard and mesophyll cells in the antisense SBPase plants with the lowest levels of SBPase activity when compared to WT plants (Fig. 3A, B). Although guard cell F_q'/F_m' was between 5–25% lower than mesophyll F_q'/F_m, similar decreases in F_q'/F_m' of both guard and mesophyll cells with decreasing SBPase activity were observed (Fig. 3C).

Red and mixed blue/red light effects on stomatal conductance

To assess the impact of reduced photosynthetic efficiency of electron transport on light-induced stomatal opening,

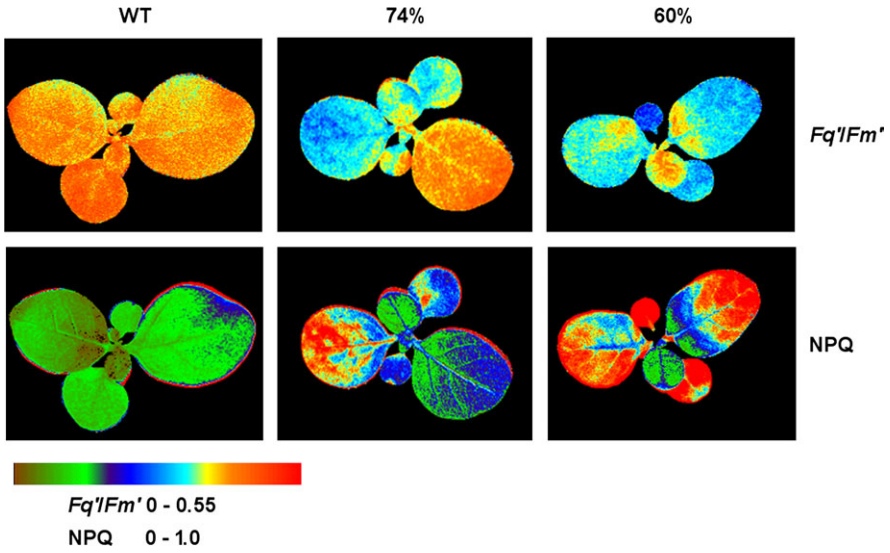

Fig. 2. Chlorophyll *a* fluorescence images of F_q'/F_m' and *NPQ* for WT and SBPase antisense tobacco seedlings at a photon flux density of 300 μmol m^{-2} s^{-1}. Images are shown for SBPase antisense plants with 74% and 60% WT SBPase activity.

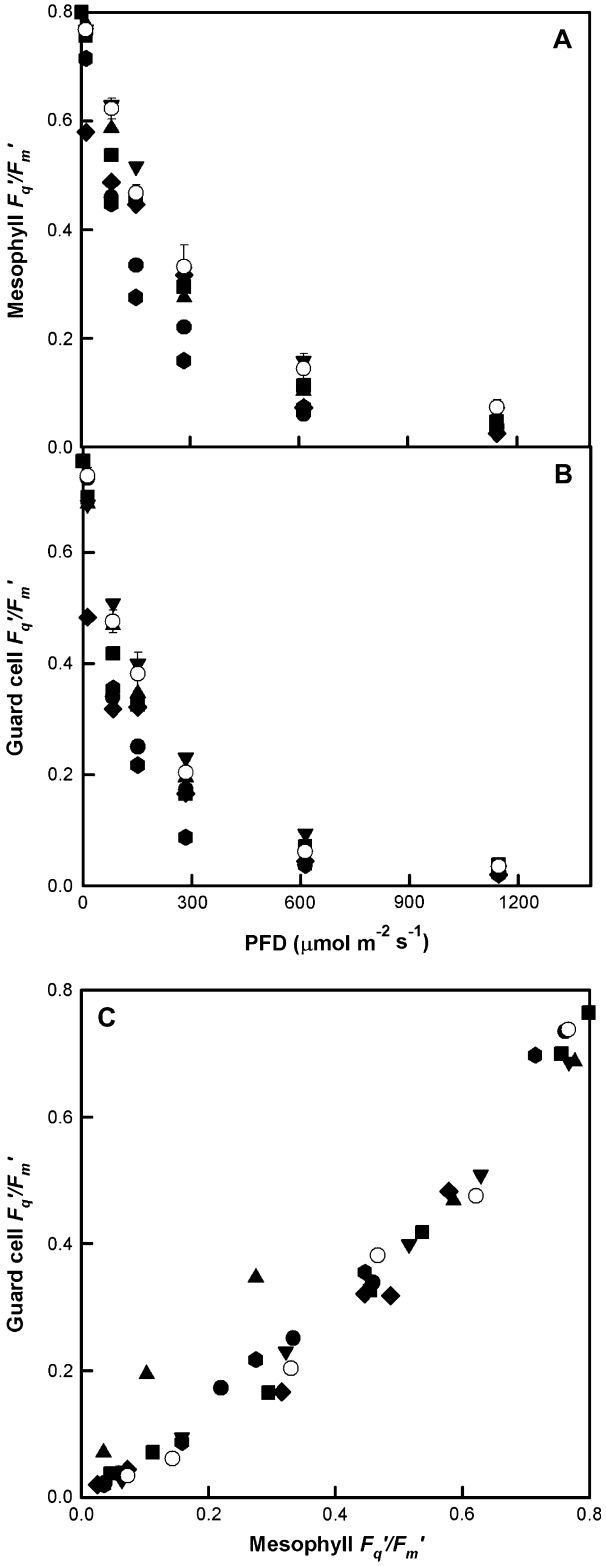

Fig. 3. Response of F_q'/F_m' to photon flux density in (A) mesophyll cells, (B) guard cells of WT (open symbols) and anti-SBPase (closed symbols) tobacco leaves. Measurements were made on the underside of leaves at 25 °C and ambient CO_2 concentration of 380 μmol mol^{-1}. Data for WT are the means of six replicate leaves ±SE. (C) Relationship between F_q'/F_m' of guard cells and adjacent mesophyll cells for measurements shown in (A) and (B).

leaves were subjected to a step-increase in PFD and the effect on net CO_2 assimilation rate (A) and stomatal conductance (g_s) was measured using gas exchange. In WT plants, A increased rapidly from about 4 μmol m^{-2} s^{-1} to 12 and 15 μmol m^{-2} s^{-1} immediately after a step-increase in either mixed blue/red or pure red PFD, then increased more slowly to a maximum of *c.* 19 μmol m^{-2} s^{-1} in mixed and 22 μmol m^{-2} s^{-1} in red PFD (Fig. 4A, B). A similar two-phase response was seen with the transgenic plants but in this case the maximum attained was clearly dependent on SBPase activity. When the PFD was returned to 100 μmol m^{-2} s^{-1}, CO_2 assimilation rate in both WT and transgenic plants returned to the original value.

Stomatal conductance (g_s) in both WT and transgenic plants increased at with a step increase in illumination

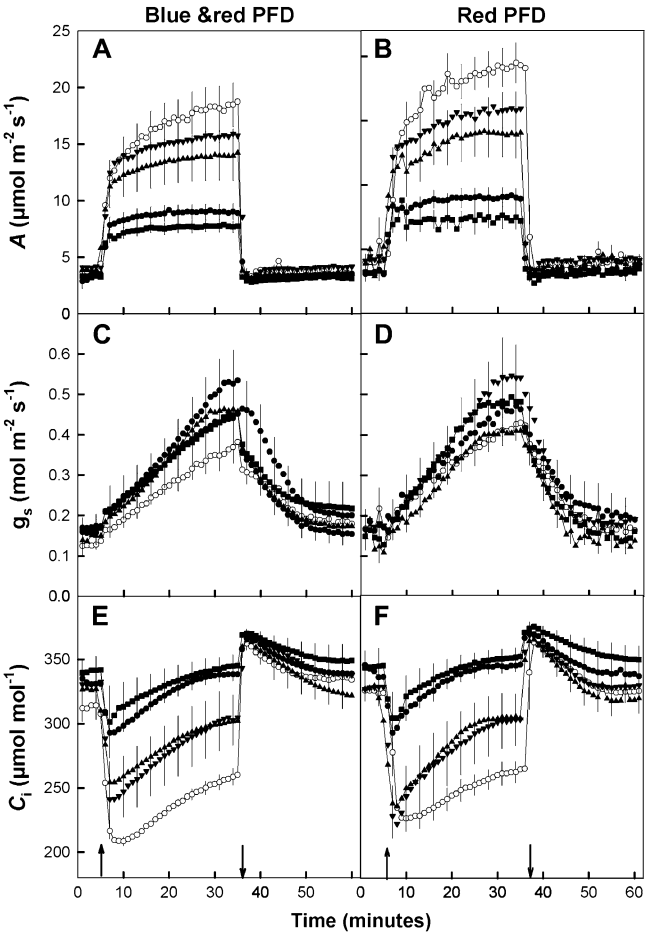

Fig. 4. Changes in (A, B) CO_2 assimilation rate, (C, D) leaf conductance, and (E, F) intercellular CO_2 with time after a step change in photon flux density (PFD) from 100 to 1000 μmol m^{-2} s^{-1} for WT and transgenic tobacco with SBPase activities of 8.9, 8.3, 7.0, 5.9, and 5.0 μmol m^{-2} s^{-1} (open circles n=6, solid inverted triangles n=3, triangles n=4, circles n=4, and squares n=3, respectively). Ambient CO_2 and water vapour were maintained at 380 μmol mol^{-1} and 23 mmol mol^{-1}. Arrows indicate when PFD was increased from 100 to 1000 μmol m^{-2} s^{-1} and returned to 100 μmol m^{-2} s^{-1}. Error bars are the standard error of the mean.

(both mixed blue/red and red alone), from values around 0.13 mol m^{-2} s^{-1} to nearly 0.4 mol m^{-2} s^{-1} for WT plants. For the antisense SBPase plants g_s rose from slightly higher values at low PFD to between 0.45–0.55 mol m^{-2} s^{-1} at high PFD. When plants were illuminated with red light alone the rate of stomatal opening was greater in the transgenic plants compared with the WT controls (P <0.05, df 16). By contrast, no statistically significant difference in the rate of opening was observed under mixed blue/red irradiance. However, in both red and blue/red the conductance after 30 min in the light was greater in the transgenic plants (P <0.05). When the PFD was returned to 100 μmol m^{-2} s^{-1}, g_s in both WT and antisense SBPase plants decreased to close to the original values (Fig. 4C, D). The response time of g_s to decreasing PFD under both pure red and blue/red illumination was greater in transgenic plants compared with the WT controls (P >0.05). Rates of stomatal opening and closure were closely correlated under pure red (r=0.72, df=16, P <0.001) and blue/red illumination (r=0.68, df=16, P <0.001) (data not shown).

At a PFD of 100 μmol m^{-2} s^{-1}, C_i values for all plants were around 330 μmol mol^{-1}, irrespective of the illumination wavebands. C_i decreased rapidly when PFD was increased to 1000 μmol m^{-2} s^{-1}, with the extent of the drop dependent on the amount of SBPase activity. In WT plants, with SBPase activity of 8.9 μmol m^{-2} s^{-1}, C_i decreased to a minimum of 215 μmol mol^{-1} and in antisense plants it decreased to 241, 254, 293, and 301 μmol mol^{-1} with SBPase activities of 8.3, 7.0, 5.9, and 5.0 μmol m^{-2} s^{-1}, respectively, under mixed blue/red illumination and 222, 238, 293, and 304 μmol mol^{-1} under red light. During the rest of the high light period, C_i increased in all plants as stomatal conductance rose, but the final C_i reached at the end of the 30 min was different depending on SBPase activity (Fig. 4E, F), but independent of the wavebands used.

Correlations between SBPase activity and photosynthetic gas exchange parameters

The steady-state values of A, g_s, and C_i at the end of the high light period and the initial rate of stomatal opening (calculated as the slope of g_s increase between 5 min and 21 min) were plotted against SBPase activity (Fig. 5). Although assimilation rate showed a pronounced decrease with reductions in SBPase activity (Fig. 5A), there was no clear relationship between g_s and SBPase activity (Fig. 5B), and therefore C_i increased with lower SBPase activity (Fig. 5C). Similarly, there was no clear relationship between the rate of stomatal opening and SBPase activity (Fig. 5D), although antisense plants in many cases had higher g_s than WT and a more rapid rate of stomatal opening which was more pronounced in plants subjected to red light alone. In general, there was a tendency for greater stomatal opening rates to result in higher steady-state conductances (Fig. 6), however the relationship between opening rates and final conductance were only statistically correlated (r=0.78, df=12, P <0.001) in the plants subjected to red light alone (Fig. 6B), but not in plants subject to mixed blue/red illumination (r=0.30, df=12) (Fig. 6A).

Effect of CO_2 concentration on stomatal and photosynthetic behaviour

The light response data in Fig. 4 showed that stomatal opening in the transgenic plants was similar to or even greater than that in WT plants despite the higher C_i. This suggests that either the stomata in the SBPase plants were less sensitive to C_i or that light was overriding this response. To test this hypothesis, g_s was determined at a constant PFD (either red or mixed blue/red) under a range of concentrations of C_i. To allow time for stomata to respond, the external CO_2 concentration was increased in steps, with a minimum of 30 min between each increment (Fig. 7). Assimilation rate increased with increasing C_i in a characteristic A/C_i response curve. Maximum assimilation rates were dependent on SBPase activity (Fig. 7A) and was significantly higher in WT plants compared with antisense SBPase plants with 78% SBPase activity (Tukey, P <0.05, df 10) and with those exhibiting 45% SBPase activity (Tukey, P <0.001, df 10). Carboxylation capacity was significantly lower in the 45% SBPase activity plants compared with WT and transgenic plants with 78% SBPase activity (See inset Table in Fig. 7A; Tukey, P <0.001). Potential electron transport rate calculated as J_{max} from the A/C_i curves also showed a significant decrease between WT and plants with 78% (Tukey, P <0.05) and 45% WT SBPase activity (Tukey, P <0.05). Changes in C_i revealed a small but significant difference between the conductances in the WT and transgenic plants (repeated measures ANOVA, P <0.001). At C_i values of >600 μmol mol^{-1}, g_s did not change in either the WT or antisense plants (Fig. 7B). When C_i was decreased to <600 μmol mol^{-1}, g_s increased significantly, reaching a value of between 0.6–0.7 mol m^{-2} s^{-1} at the lowest C_i in both the WT and transgenic plants. At C_i concentrations between 300 and 600 μmol mol^{-1}, the transgenic plants tended to have higher g_s than the WT plants and the highest conductances values were observed in the antisense plants with 78% SBPase activity. The C_i/C_a ratio was maintained between 0.6–0.7 for the WT plants over a range of C_a concentration from 150 to 1800 μmol mol^{-1} (Fig. 7C). The transgenic plants with 78% SBPase activity maintained a slightly higher C_i/C_a of between 0.8–0.9 over the same range of C_a concentrations, whilst plants with only 45% SBPase activity maintained an even higher ratio of between 0.9 and 1.0. The same results were found when these measurements

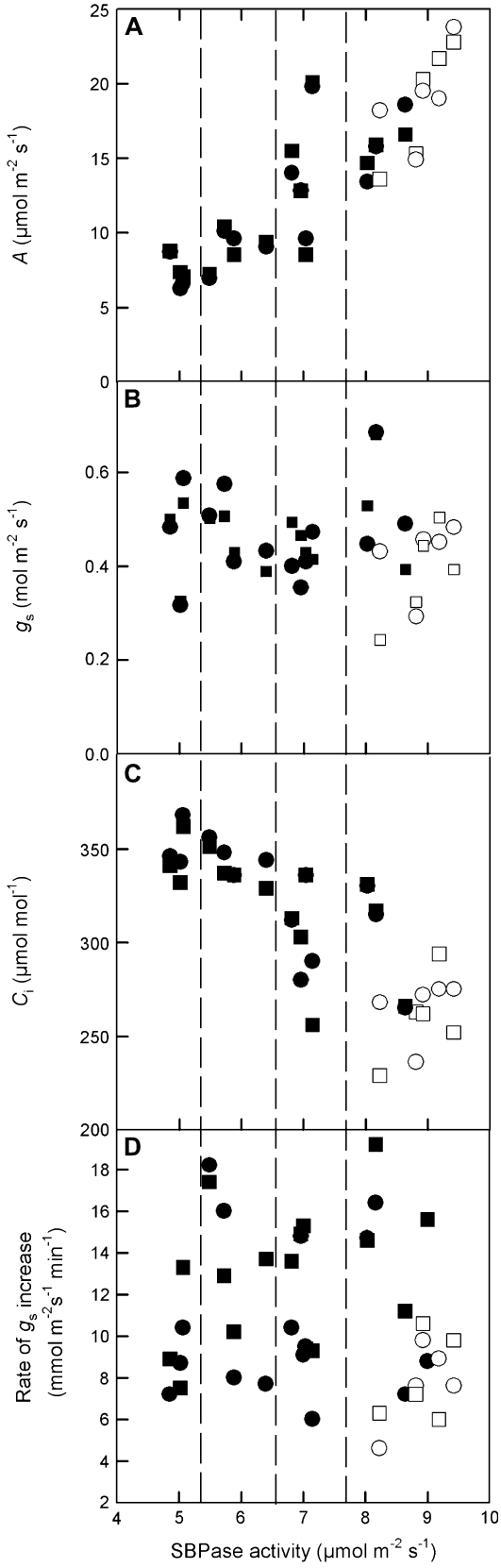

Fig. 5. Relationships between SBPase activity and (A) net CO_2 assimilation rate, (B) stomatal conductance, (C) internal CO_2 concentration, and (D) the rate of stomatal opening in WT (open symbols) and antisense SBPase transgenic tobacco (closed symbols). Measurements were taken at the end of the 30 min illumination period of 1000 μmol m^{-2} s^{-1} photon flux density of red (circles) or red and blue/red (squares) light as shown in Fig. 4. Water vapour was maintained at 23 mmol mol^{-1} and leaf temperature of 25 °C providing a leaf–air vapour pressure difference of 1.0 kPa. Dashed lines represent the SBPase grouping of Fig. 4.

were conducted under mixed blue/red PFD (data not shown).

Effect of red light intensity on stomata at a constant C_i

The red light response of stomata is considered to be associated with guard cell photosynthesis, although recent reports have suggested the link with photosynthesis is only due to photosynthetic mesophyll consumption of CO_2 and stomatal response to lowering internal CO_2 concentration (see Introduction). To eliminate possible effects of changes in C_i in the light response, the effects of increasing red light and PFD on A and g_s whilst maintaining C_i at approximately 280 μmol mol^{-1} (Fig. 8) were examined. Stomatal conductance showed a large (4–6-fold) increase with increasing red PFD in both WT and transgenic plants (Fig. 7B) even though C_i was maintained constant throughout the experiment (Fig. 8C). Although assimilation rates were much lower in the antisense plants (Fig. 8A), the stomatal responses to PFD were indistinguishable from that of the WT plants. Therefore any obligatory role for changing C_i in the observed light responses can be discounted.

Discussion

Using antisense tobacco plants with a range of SBPase activities, it has been demonstrated that reductions in SBPase activity reduced leaf net CO_2 assimilation rate substantially (Figs 1, 4–7), and that this reduction in photosynthesis was greatest in high light intensity, particularly at high CO_2 concentrations (Figs 1, 3, 7). This is because the reduced SBPase activity affects the regeneration capacity of the Calvin cycle (Fig. 7) and confirms the importance of SBPase in the Calvin cycle (Raines, 2003). For whole seedlings (Fig. 2), decreased SBPase activity reduced the quantum efficiency of PSII electron transport. The imbalance between the capacity for RuBP regeneration and CO_2 fixation in the antisense plants led to a reduced consumption of photosynthetic electron transport products (ATP and NADPH) resulting in decreased photosynthetic efficiency and increased NPQ (Fig. 2; see also von Caemmerer *et al.*, 2004). In the antisense plants the photosynthetic efficiency of guard cells was reduced to a similar extent to that in the adjacent mesophyll (Fig. 3), suggesting the same SBPase-caused changes in Calvin cycle activity occurred in the guard

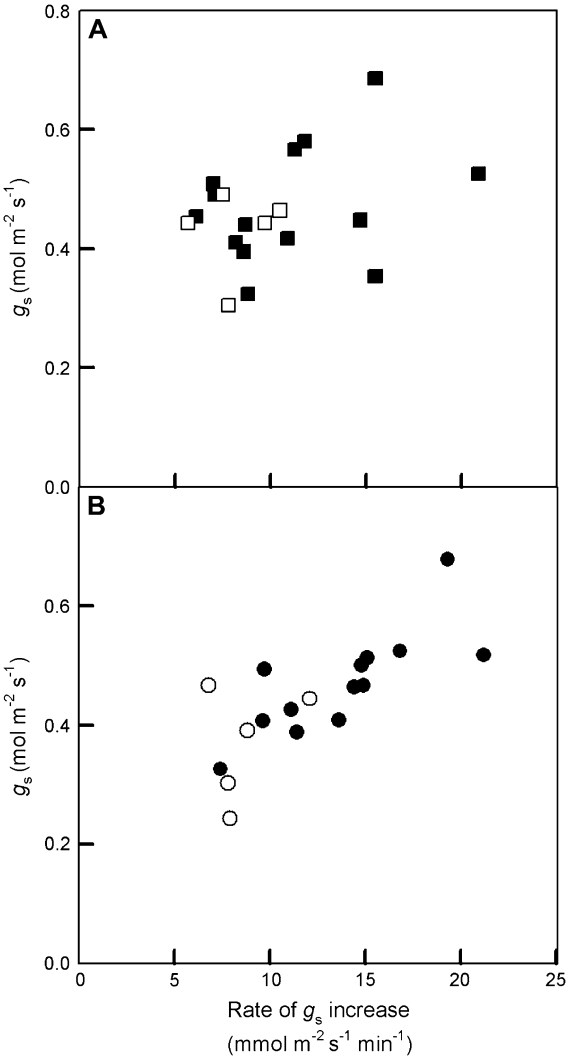

Fig. 6. Relationship between rate of stomatal opening and final stomatal conductance at the end of 30 min illumination period of 1000 μmol m^{-2} s^{-1} photon flux density of (A) mixed blue/red (squares) or (B) red (circles) light. Open symbols represent WT plants and closed symbols antisense SBPase transgenic tobacco. Water vapour was maintained at 23 mmol mol^{-1} and leaf temperature at 25 °C providing a leaf–air vapour pressure difference of 1.0 kPa.

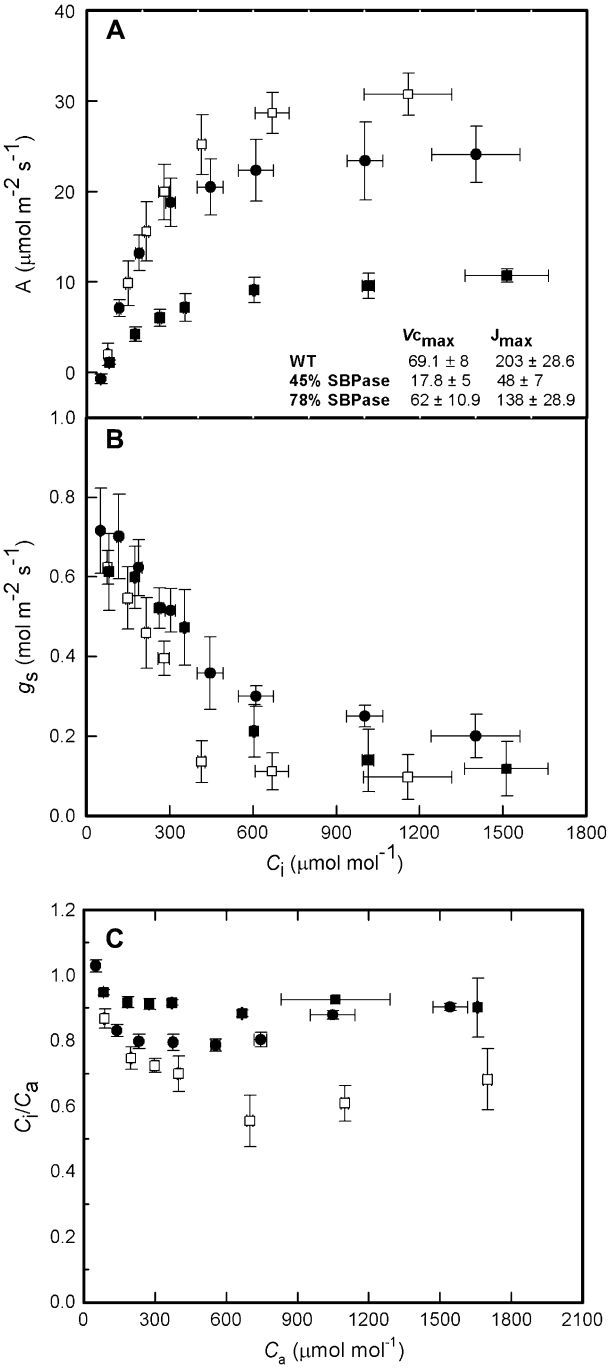

Fig. 7. (A) Net CO_2 assimilation rate and (B) stomatal conductance as a function of internal CO_2 concentration (C_i). Values were allowed to stabilize for at least 30 min at each CO_2 concentration. (C) The relationship between ambient CO_2 concentration C_a and C_i/C_a ratio. WT plants (n=6) are shown by open squares, with antisense SBPase transgenic plants represented by closed squares (78% WT activity, n=4) and closed circles (45% WT activity, n=4). Error bars are the standard error of the mean.

cells. This novel observation provides further support for the view that Calvin cycle activity in guard cells is a major sink for ATP and NADPH produced through photosynthetic electron transport (Cardon and Berry, 1992; Lawson *et al*., 2002, 2003; von Caemmerer *et al*., 2004).

Despite reductions in both guard and mesophyll cell photosynthetic electron transport and the consequent higher C_i, the stomatal opening and closing responses to either mixed blue/red or red light in the antisense SBPase plants followed a similar pattern to those in WT plants (Fig. 4). These date provide further support for the suggestion from previous antisense studies that photosynthetic CO_2 fixation in guard cells is not essential for light-induced stomatal opening in tobacco (von Caemmerer *et al*., 2004; Barioli *et al*., 2008). However, some subtle but notable differences were observed in both the rate and extent of stomatal opening in the antisense SBPase plants compared to WT. The g_s reached following a large step

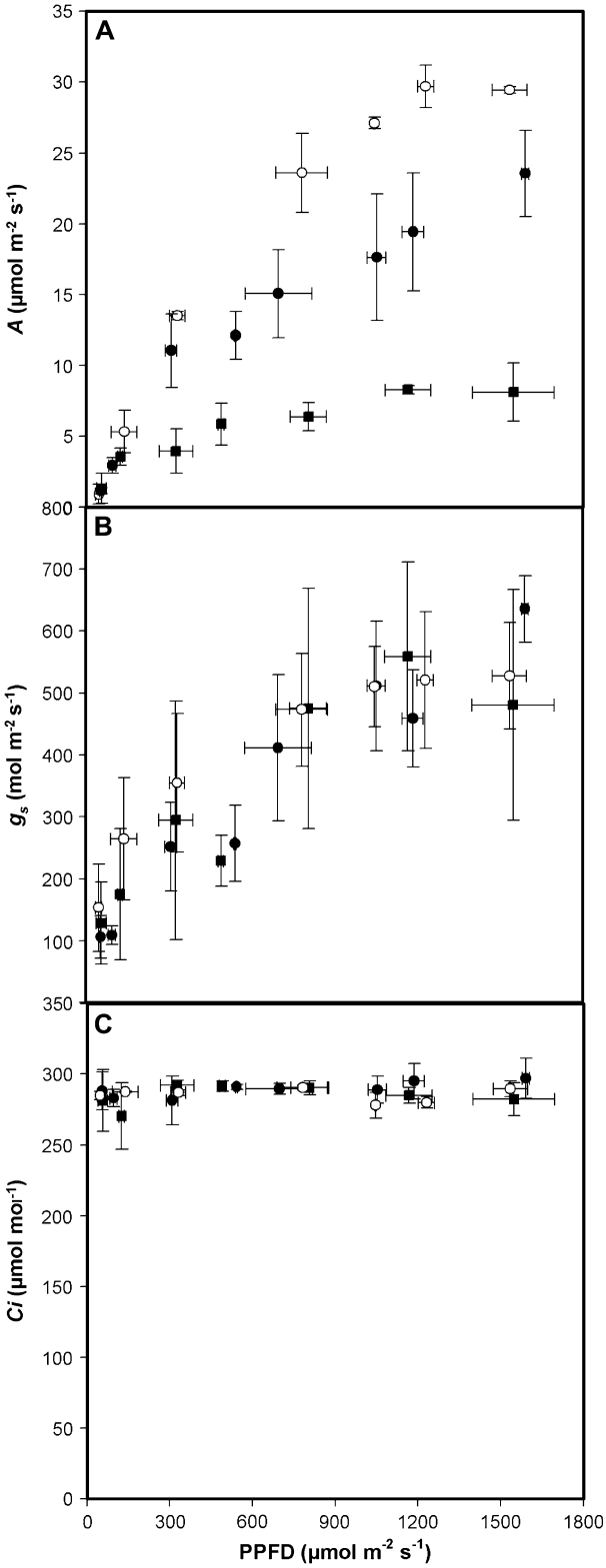

Fig. 8. (A) Net CO_2 assimilation rate and (B) stomatal conductance as a function of an increase in red photon flux density whilst maintaining (C) internal CO_2 concentration (C_i) at *c*. 300 μmol mol^{-1}. Values were allowed to stabilize for at least 30 min at each light level, whilst external CO_2 concentration was altered to maintain C_i. WT plants (n=4) are shown by open circles, with antisense SBPase transgenic plants represented by closed circles (78% WT activity, n=4) and closed squares (45% WT activity, n=4). Error bars are the standard error of the mean.

increase in either mixed blue/red or red light was higher in the antisense SBPase plants than in WT plants. Furthermore illumination with red light alone resulted in a more rapid rate of stomatal opening in the SBPase plants (Fig. 5B, D). The rate of opening under red illumination was strongly correlated with the final conductance, whilst under mixed blue/red illumination duel mechanisms probably masked this relationship (Fig. 6). The fact that no difference in opening rate was observed under mixed blue/red is possibly due to an over-riding specific blue light response (Talbott and Zeiger, 1993), which does not depend on either guard or mesophyll cell photosynthesis (see Introduction). Red-light-induced stomatal opening responses have been shown to be dependent on photosynthetic electron transport (Tominaga *et al*., 2001). Therefore the 20% decrease in electron transport rate, estimated from F_q'/F_m', in both the guard cells and the mesophyll of the antisense SBPase plants (Figs 2, 3) might be expected to reduce the light-induced stomatal opening (Fig. 8B). However, inhibition of electron transport using DCMU prevents the development of a proton gradient across the thylakoid membrane, thus reducing ATP availability for cation uptake (Tominaga *et al*., 2001). By contrast, in the antisense SBPase plants ATP levels may be increased, despite the reduced photosynthetic electron transport, because of the reduced ATP consumption by the Calvin cycle (Figs 4, 5). Baroli *et al*. (2008) reported that red-light-induced stomatal opening was unaffected in transgenic plants with reduced electron transport and reduced ATP.

Previous experiments have indicated that changes in C_i act as a signal modulating stomatal aperture, with C_i usually maintained around 230–250 μmol mol^{-1} (Mott, 1988). However, the internal CO_2 concentration (C_i) in the SBPase antisense plants was significantly higher than in WT plants, but the stomatal conductance was greater (Figs 4, 5). This result was unexpected as C_i concentrations of >250 μmol mol^{-1} in the antisense SBPase plants would have been expected to reduce g_s (Thomas *et al*., 1991, in tobacco; Wong *et al*., 1978; Morison, 1987; Mott, 1990). Despite the well-documented correlation found between C_i and g_s it is difficult to explain the results obtained from studies using antisense SBPase and Rubisco plants based on this relationship.

Although it has long been suggested that the opening of stomata in response to red light involves guard cell photosynthesis, Roelfsema *et al*. (2002) suggested that the PAR light response in intact leaves was due to CO_2 depletion by the mesophyll. Our data do not support this proposal as in both wild-type and antisense plants stomata opened in response to increasing light when C_i was kept constant (Fig. 8B), as has also been shown in previous work (Morison and Jarvis, 1983; Messinger *et al*., 2006).

An alternative explanation of these results is that guard cells respond to CO_2 concentrations nearer C_a rather than the estimated bulk mesophyll C_i (von Caemmerer *et al*., 2004). In support of this suggestion, many of the small differences between the stomatal CO_2 response curves in WT and antisense plants in Fig. 7B are removed if the abscissa is C_a not C_i (data not shown).

Recently it has been proposed that stomatal responses to light and C_i are dependent on the balance between the photosynthetic carbon reduction reactions and electron transport capacity (Messinger *et al*., 2006). In this study, a group of antisense plants was identified with small reductions in SBPase activity (20–35% activity) in which the regenerative capacity was reduced without any effects on the carboxylation efficiency. In this group of transgenic plants the closing response to increasing C_i was reduced compared with WT plants. Reduction of Calvin cycle activity, whether through reduced substrate availability (C_i) or antisense technology, could result in increased chloroplastic ATP levels (Farquhar *et al*., 1980; Farquhar and Wong, 1984; Messinger *et al*., 2006). Increases in ATP could provide either a sensory mechanism (Buckley *et al*., 2003) or be directly used for proton pumping at the plasma membrane, which is known to be activated under red light and would increase stomatal aperture (Tominaga *et al*., 2001). Several years ago, Zhu *et al*. (1998) proposed that zeaxanthin was the sensory mechanisms for C_i, demonstrating a strong positive correlation between stomatal aperture and guard cell zeaxanthin concentration. This could account for the differences observed; decreased Calvin cycle activity and reduced ATP utilization would result in an increase in the H^+ electrochemical gradient across the thylakoid membrane, which in turn would induce zeaxanthin formation as part of plants non-photochemical quenching mechanism (Niyogi *et al*., 2005). Such an increase in guard cell zeaxanthin concentration would, according to Zhu *et al*. (1998), result in greater stomatal apertures.

Leaf and guard cell apoplastic sucrose concentration have also been proposed as a mechanism linking photosynthesis and transpiration rate with stomatal movements (Outlaw and De Vlieghere-He, 2001; Kang *et al*., 2007). These data presented here would fit with the observation that lower photosynthetic rates in apoplastic phloem loaders, such as tobacco, would result in a reduced apoplastic sucrose concentration and consequently greater stomatal aperture (Kang *et al*., 2007).

Conclusion

Our data demonstrate that both guard and mesophyll photosynthetic electron transport are reduced to a similar degree in antisense plants with reduced levels of SBPase activity. It has been shown that reducing Calvin cycle regenerative capacity led to reductions in guard cell photosynthetic efficiency of electron transport. The fact that pore opening is still entirely functional and that apertures are as great as WT plants, strengthen suggestions from previous antisense studies that photosynthetic CO_2 fixation in guard cells is not specifically necessary for the primary light-induced stomatal opening response in tobacco (von Caemmerer *et al*., 2004; Barioli *et al*., 2008). However, reductions in SBPase activity resulted in more rapid stomatal opening under red light illumination and a higher steady-state stomatal conductance, suggesting a role for mesophyll or guard cell photosynthesis in the fine-tuning of stomatal responses to light and CO_2.

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References

Baroli I, Price D, Badger MR, von Caemmerer S. 2008. The contribution of photosynthesis to the red light response of stomatal conductance. *Plant Physiology* **146,** 737–747.

Barbagallo RP, Oxborough K, Pallett KE, Baker NR. 2003. Rapid, noninvasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant Physiology* **132,** 485–493.

Buckley TN, Mott KA, Farquhar GD. 2003. A hydromechanical and biochemical model of stomatal conductance. *Plant, Cell and Environment* **26,** 1767–1785.

Cardon ZG, Berry J. 1992. Effects of O_2 and CO_2 concentration on the steady-state fluorescence yield of single guard-cell pairs in intact leaf-disks of *Tradescantia albiflora*: evidence for Rubisco-mediated CO_2 fixation and photorespiration in guard cells. *Plant Physiology* **99,** 1238–1244.

Farquhar GD, von Caemmerer S, Berry JA. 1980. A biochemical model of photosynthetic CO_2 assimilation in leaves of C_3 species. *Planta* **149,** 78–90.

Farquhar GD, Wong SC. 1984. An empirical model of stomatal conductance. *Australian Journal of Plant Physiology* **11,** 191–210.

Frechilla S, Talbott LD, Zeiger E. 2002. The CO_2 response of *Vicia faba* guard cells acclimates to growth environment. *Journal of Experimental Botany* **53,** 545–550.

Frechilla S, Talbott LD, Zeiger E. 2004. The blue light-specific response of *Vicia faba* stomata acclimates to growth environment. *Plant and Cell Physiology* **45,** 1709–1714.

Harrison EP, Olcer H, Lloyd JC, Long SP, Raines CA. 2001. Small decreases in SBPase cause a linear decline in the apparent RuBP regeneration rate, but do not affect Rubisco carboxylation capacity. *Journal of Experimental Botany* **52,** 1779–1784.

Harrison EP, Willingham NM, Lloyd JC, Raines CA. 1998. Reduced sedoheptulose-1,7-bisphosphatase levels in transgenic tobacco lead to decreased photosynthetic capacity and altered carbohydrate accumulation. *Planta* **20,** 27–36.

Hudson GS, Evans JR, von Caemmerer S, Arvidsson YBC, Andrews TJ. 1992. Reduction of ribulose-1,5-*bis*phosphate carboxylase/oxygenase content by antisense RNA reduces photosynthesis in transgenic tobacco plants. *Plant Physiology* **98,** 294–302.

Kang Y, Outlaw Jr WH, Anderson PC, Fiore GB. 2007. Guard cell apoplastic sucrose concentration: a link between leaf photosynthesis and stomatal aperture size in apoplastic phloem loader *Vicia faba* L. *Plant. Cell and Environment* **30,** 551–558.

Kuiper PJC. 1964. Dependence upon wavelength of stomatal movement in epidermal tissue of *Senecio odoris*. *Plant Physiology* **39,** 952–955.

Lawson T, Oxborough K, Morison JIL, Baker NR. 2002. Responses of photosynthetic electron transport in stomatal guard cells and mesophyll cells in intact leaves to light, CO_2, and humidity. *Plant Physiology* **128,** 52–62.

Lawson T, Oxborough K, Morison JIL, Baker NR. 2003. The response of guard cell photosynthesis to CO_2, O_2, light and water stress in a range of species are similar. *Journal of Experimental Botany* **54,** 1734–1752.

Leegood RC. 1990. Enzymes of the Calvin cycle. *Methods in Plant Biochemistry* **3,** 15–37.

Lodge RJ, Dijkstra P, Drake BG, Morison JIL. 2001. Stomatal acclimation to increased CO_2 concentration in a Florida scrub oak species *Quercus myrtifolia* Willd. *Plant, Cell and Environment* **24,** 77–88.

Messinger SM, Buckley TN, Mott KA. 2006. Evidence for involvement of photosynthetic processes in the stomatal response to CO_2. *Plant Physiology* **140,** 771–778.

Morison JIL. 1987. Intercellular CO_2 concentration and stomatal response to CO_2. In: Zeiger E, Farquhar GD, Cowan IR, eds. *Stomatal function*. Stanford: Stanford University Press, 229–251.

Morison JIL, Jarvis PG. 1983. Direct and indirect effects of light on stomata. II. In *Commelina communis* L. *Plant*, *Cell and Environment* **6,** 103–109.

Mott KA. 1988. Do stomata respond to CO_2 concentrations other than intercellular? *Plant Physiology* **86,** 200–203.

Mott KA. 1990. Sensing of atmospheric CO_2 by plants. *Plant, Cell and Environment* **13,** 731–737.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiology Plantarum* **15,** 473–497.

Muschak M, Willmitzer L, Fisahn J. 1999. Gas-exchange analysis of chloroplastic fructose-1,6-*bis*phosphatase antisense potatoes at different air humidities and at elevated CO_2. *Planta* **209,** 104–111.

Niyogi KK, Li X-P, Rosenberg V, Jung H-S. 2005. Is PsbS the site of non-photochemical quenching in photosynthesis? *Journal of Experimental Botany* **56,** 375–382.

Olsen RL, Pratt RB, Gump P, Kemper A, Tallman G. 2002. Red light activates a chloroplast-dependent ion uptake mechanism for stomatal opening under reduced CO_2 concentrations in *Vicia* spp. *New Phytologist* **153,** 497–508.

Outlaw WH. 2003. Integration of cellular and physiological functions of guard cells. *Critical Reviews in Plant Science* **22,** 503–529.

Outlaw Jr WH, De Vleighere-He X. 2001. Transpiration rate: an important factor in controlling sucrose content of the guard cell apoplast of broad bean. *Plant Physiology* **126,** 1716–1724.

Oxborough K. 2004. Imaging of chlorophyll *a* fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. *Journal of Experimental Botany* **55,** 1195–1205.

Oxborough K, Baker NR. 1997. Resolving chlorophyll *a* fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components: calculation of *qP* and F_q'/F_m' without measuring F_o^1. *Photosynthesis Research* **54,** 135–142.

Quick WP, Schurr U, Fichtner K, Schulze E-D, Rodermel SR, Bogorad L, Stitt M. 1991. The impact of decreased Rubisco on photosynthesis, growth, allocation and storage in tobacco plants which have been transformed with antisense rbcS. *The Plant Journal* **1,** 51–58.

Raines CA. 2003. The Calvin cycle revisited. *Photosynthesis Research* **75,** 1–10.

Raschke K. 1975. Simultaneous requirement of carbon dioxide and abscisic acid for stomatal closing in *Xanthium strumarium* L. *Planta* **125,** 243–259.

Roelfsema MRG, Hanstein S, Fell HH, Hedrich R. 2002. CO_2 provides an intermediate link in the red light response of guard cells. *The Plant Journal* **32,** 65–75.

Roelfsema MRG, Hedrich R. 2005. In the light of stomatal opening: new insights into 'the Watergate'. *New Phytologist* **167,** 665–691.

Sharkey TD, Raschke K. 1981. Separation and measurement of direct and indirect effects of light on stomata. *Plant Physiology* **68,** 33–40.

Stitt M, Schulze D. 1994. Does Rubisco control the rate of photosynthesis and plant growth: an exercise in molecular ecophysiology. *Plant, Cell and Environment* **17,** 465–487.

Talbott LD, Srivastava A, Zeiger E. 1996. Stomata from growth-chamber grown *Vicia faba* have an enhanced sensitivity to CO_2. *Plant, Cell and Environment* **19,** 1188–1194.

Talbott LD, Zeiger E. 1993. Sugar and organic acid accumulation in guard cells of *Vicia faba* in response to red and blue light. *Plant Physiology* **102,** 1163–1169.

Talbott LD, Zeiger E. 1998. The role of sucrose in guard cell osmoregulation. *Journal of Experimental Botany* **9,** 329–337.

Thomas C, Davis SD, Tallman G. 1991. Responses of senescing and non-senescing leaves of *Nicotiana glauca* to changes in intercellular concentrations of carbon dioxide. *Plant, Cell and Environment* **14,** 971–978.

Tominaga M, Kinoshita T, Shimazaki K. 2001. Guard-cell chloroplasts provide ATP required for H^+ pumping in the plasma membrane and stomatal opening. *Plant and Cell Physiology* **42,** 795–802.

Vavasseur A, Raghavendra AS. 2005. Guard cell metabolism and CO_2 sensing. *New Phytologist* **165,** 665–682.

von Caemmerer S, Lawson T, Oxborough K, Baker NR, Andrews TJ, Raines CA. 2004. Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *Journal of Experimental Botany* **55,** 1157–1166.

Willmer C, Fricker M. 1996. *Stomata*, 2nd edn. London: Chapman and Hall.

Wong SC, Cowan IR, Farquhar GD. 1978. Leaf conductance in relation to assimilation in *Eucalyptus pauciflora* Sieb. ex Spreng. Influence of irradiance and partial pressure of carbon dioxide. *Plant Physiology* **78,** 821–825.

Wu WH, Assmann SM. 1993. Photosynthesis by guard-cell chloroplasts of *Vicia faba* L.: effects of factors associated with stomatal movement. *Plant and Cell Physiology* **34,** 1015–1022.

Zeiger E, Talbott LD, Frechilla S, Srivastava A, Zhu JX. 2002. The guard cell chloroplast: a perspective for the twenty-first century. *New Phytologist* **153,** 415–424.

Zhu J, Talbott LD, Jin X, Zeiger E. 1998. The stomatal response to CO_2 is linked to changes in guard cell zeaxanthin. *Plant, Cell and Environment* **21,** 813–820.