

RESEARCH PAPER

# Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and CO<sub>2</sub>

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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<sub>2</sub> assimilation rates and higher internal CO<sub>2</sub> concentrations. Increasing CO<sub>2</sub> 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<sub>i</sub> levels. Together, these data suggest that the primary light-induced opening or CO<sub>2</sub>-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<sub>2</sub>.

Key words: CO<sub>2</sub> concentration, guard cell photosynthesis, light response, photosynthetic electron transport, SBPase, stomata, stomatal conductance.

## Introduction

Stomatal aperture controls the flux of CO<sub>2</sub> and H<sub>2</sub>O 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<sub>2</sub> 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<sup>+</sup> 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 (Talbot and Zeiger, 1998; Zeiger *et al.*, 2002; Outlaw, 2003; Vavasseur and Ragavendra, 2005). Furthermore, guard cells show O<sub>2</sub> and CO<sub>2</sub> 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<sub>2</sub> depletion. Thus, the involvement of guard cell photosynthesis in light- and CO<sub>2</sub>-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<sub>2</sub> assimilation rate (*A*) was decreased and internal CO<sub>2</sub> concentration (*C*<sub>i</sub>) increased. Stomata usually respond to increased *C*<sub>i</sub> by reducing aperture (Morison, 1987; Mott, 1990) so a reduction in stomatal conductance (*g*<sub>s</sub>) would be expected in these transgenic plants. However, as most studies report no difference in *g*<sub>s</sub> between wild-type (WT) and transgenic plants, this suggests that stomata do not respond to either the increase in *C*<sub>i</sub> 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*<sub>i</sub> were similar to wild-type plants. These results suggested that the light-induced opening and *C*<sub>i</sub>-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<sub>6</sub>f* 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<sub>2</sub> concentrations of 800 μmol mol<sup>-1</sup>. However, growth CO<sub>2</sub> 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<sub>2</sub> severely (Talbot *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<sub>2</sub> 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<sub>2</sub> concentration and photon flux density.

## Materials and methods

### *Growth of plants for physiological analysis*

Wild-type and transgenic T<sub>2</sub> 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<sup>-1</sup>). 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<sup>-2</sup> s<sup>-1</sup>) 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<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 2 mM benzamidine, 2 mM amino capronic acid, 0.5 mM phenylmethylsulphonyl fluoride; 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<sub>2</sub>, 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<sup>-2</sup> s<sup>-1</sup>. Plants were then exposed to an actinic PFD of 300 µmol m<sup>-2</sup> s<sup>-1</sup> 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<sup>-2</sup> s<sup>-1</sup>. Using the images captured at  $F'$  and  $F_m'$ , images of PSII quantum photosynthetic efficiency

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

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<sub>2</sub> concentration of 380 µmol mol<sup>-1</sup>, 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<sup>2</sup>. 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<sup>-2</sup> s<sup>-1</sup> for 20–30 min. The PFD was then increased to 1000 µmol m<sup>-2</sup> s<sup>-1</sup> for 30 min and then returned to 100 µmol m<sup>-2</sup> s<sup>-1</sup>. During the experiment, leaf chamber CO<sub>2</sub> and humidity were maintained at 380 µmol mol<sup>-1</sup> and 23 mmol mol<sup>-1</sup> 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<sub>2</sub> 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<sup>-2</sup> s<sup>-1</sup> red light, and a chamber CO<sub>2</sub> concentration of 400 µmol mol<sup>-1</sup> for 30–50 min. The CO<sub>2</sub> concentration was stepwise decreased, followed by stepwise increases to cover a range of CO<sub>2</sub> concentrations from 50–1600 µmol mol<sup>-1</sup>. At each CO<sub>2</sub> 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<sup>-2</sup> s<sup>-1</sup> 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<sub>2</sub> response curves using a CIRAS-1 gas exchange system. Leaves were first equilibrated at a PFD of 1000 µmol m<sup>-2</sup> s<sup>-1</sup> 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<sub>2</sub> concentration was maintained at about 280 µmol mol<sup>-1</sup> by manipulating the external CO<sub>2</sub> concentration. Temperature and humidity were maintained throughout the experiment at 26±2 °C and 23 mmol mol<sup>-1</sup>, 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<sub>2</sub> were determined using ANOVA with a repeated measures design for the different CO<sub>2</sub> concentrations (Systat 11).

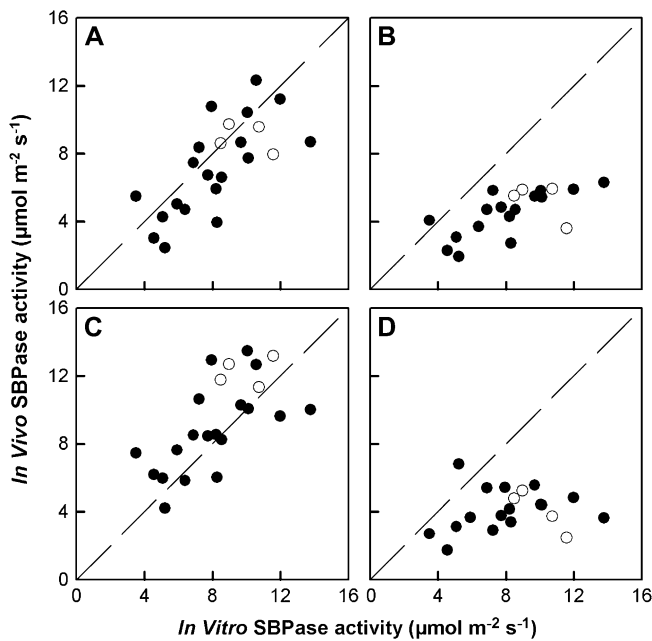
## Results

#### Relationship between photosynthesis and SBPase activity

Leaf net CO<sub>2</sub> assimilation rates ( $A$ ) measured in different light and CO<sub>2</sub> conditions in 2% O<sub>2</sub> 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<sub>2</sub> (Fig. 1A, C) but not when light or CO<sub>2</sub> 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 (Barbargallo *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



**Fig. 1.** Relationship between *in vitro* and estimated *in vivo* SBPase activity at (A)  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density (PFD) and  $380 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  concentration, (B)  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  PFD and  $380 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  concentration, (C)  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PFD and  $1500 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  concentration, and (D)  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PFD and  $155 \mu\text{mol mol}^{-1}$   $\text{CO}_2$  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.

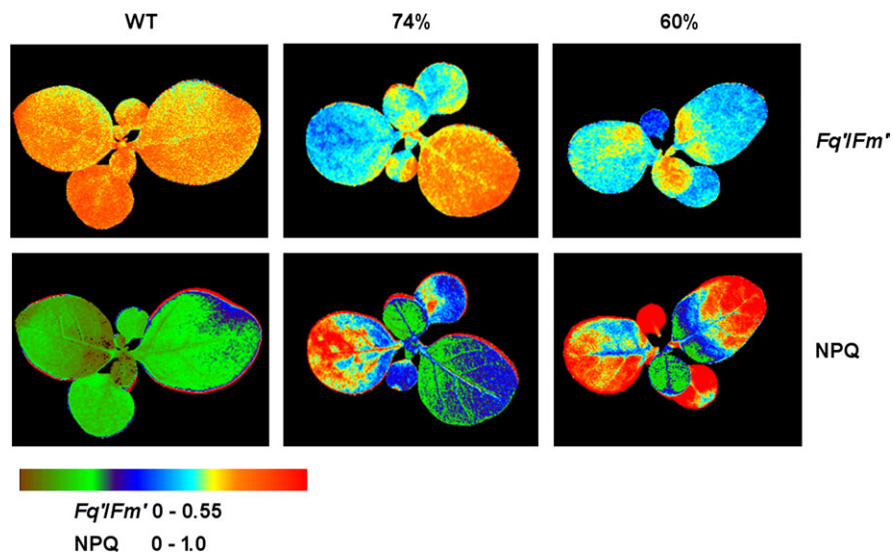
reduced  $F_q'/F_m'$  with decreased amounts of SBPase activity and increased non-photochemical quenching as estimated by  $NPQ (F_m/F_m' - 1)$ . The older leaves tended to have greater reduction in  $F_q'/F_m'$  and increased  $NPQ$  compared with the younger leaves. In antisense plants with SBPase 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).

#### Chlorophyll *a* fluorescence imaging of guard and mesophyll cells

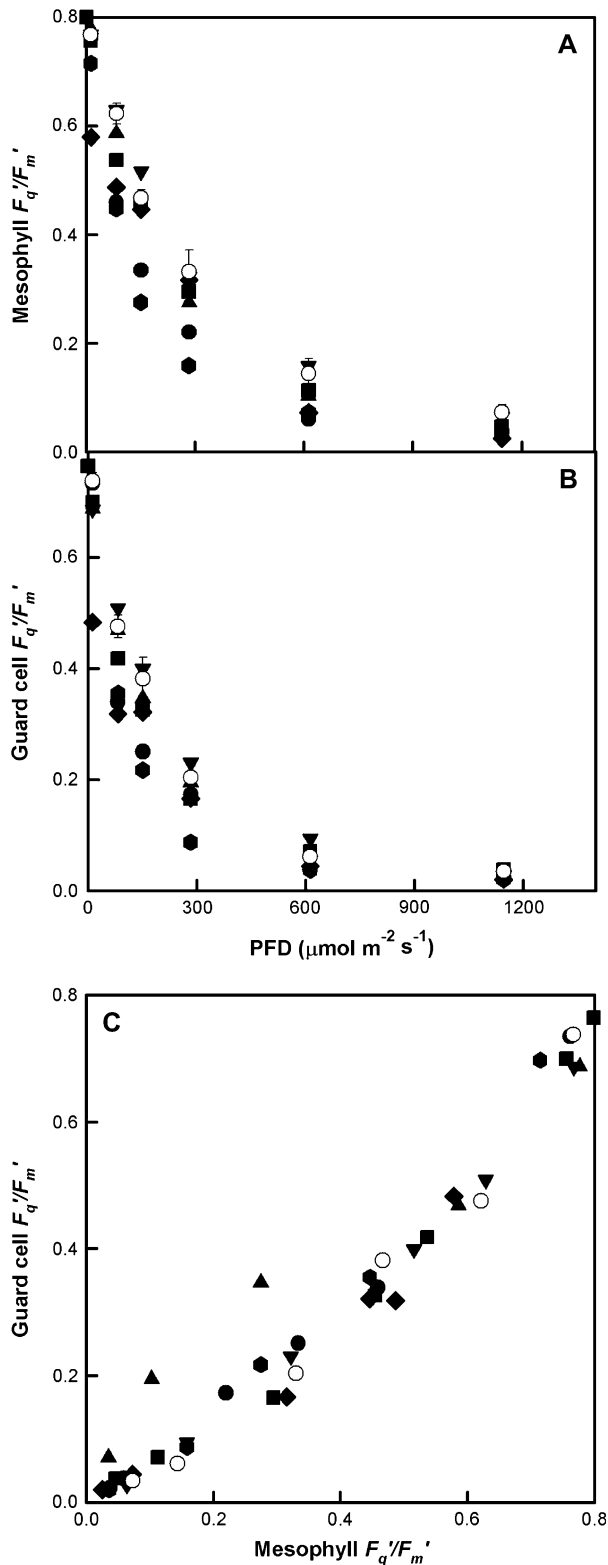
High-resolution chlorophyll *a* fluorescence imaging with a microscope was used to determine the effect of reductions in SBPase activity on mesophyll and guard cell quantum efficiency of PSII photochemistry. The response of  $F_q'/F_m'$  to increasing PFD in fully expanded leaves showed that photosynthetic 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,



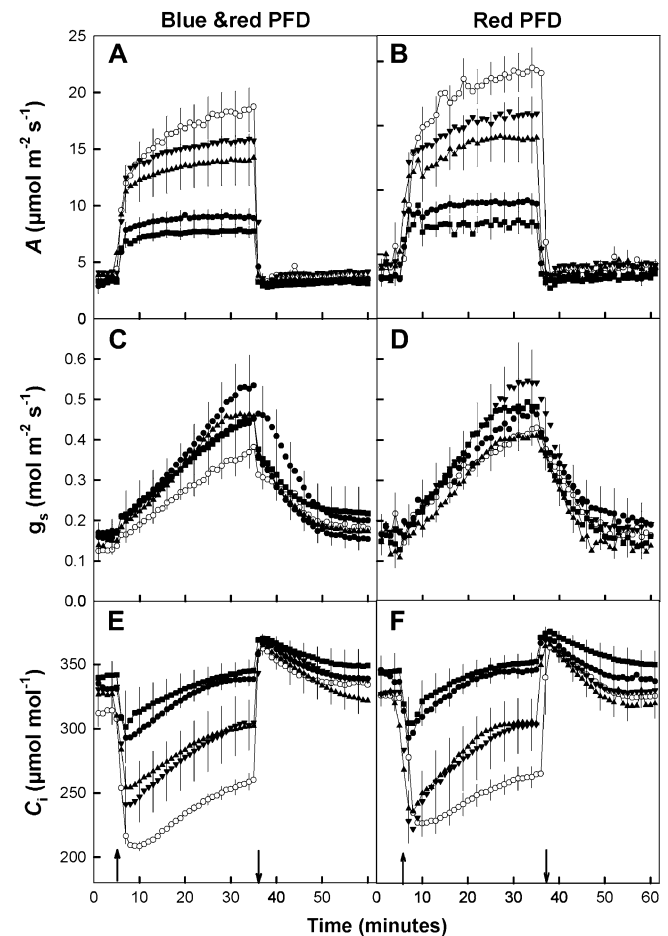
**Fig. 2.** Chlorophyll *a* fluorescence images of  $F_q'/F_m'$  and  $NPQ$  for WT and SBPase antisense tobacco seedlings at a photon flux density of  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Images are shown for SBPase antisense plants with 74% and 60% WT SBPase activity.



**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  $\text{CO}_2$  concentration of  $380 \mu\text{mol mol}^{-1}$ . Data for WT are the means of six replicate leaves  $\pm$  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  $\text{CO}_2$  assimilation rate (A) and stomatal conductance ( $g_s$ ) was measured using gas exchange. In WT plants, A increased rapidly from about  $4 \mu\text{mol m}^{-2} \text{s}^{-1}$  to 12 and  $15 \mu\text{mol m}^{-2} \text{s}^{-1}$  immediately after a step-increase in either mixed blue/red or pure red PFD, then increased more slowly to a maximum of *c.*  $19 \mu\text{mol m}^{-2} \text{s}^{-1}$  in mixed and  $22 \mu\text{mol m}^{-2} \text{s}^{-1}$  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  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ ,  $\text{CO}_2$  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)  $\text{CO}_2$  assimilation rate, (C, D) leaf conductance, and (E, F) intercellular  $\text{CO}_2$  with time after a step change in photon flux density (PFD) from  $100$  to  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  for WT and transgenic tobacco with SBPase activities of 8.9, 8.3, 7.0, 5.9, and  $5.0 \mu\text{mol m}^{-2} \text{s}^{-1}$  (open circles  $n=6$ , solid inverted triangles  $n=3$ , triangles  $n=4$ , circles  $n=4$ , and squares  $n=3$ , respectively). Ambient  $\text{CO}_2$  and water vapour were maintained at  $380 \mu\text{mol mol}^{-1}$  and  $23 \text{ mmol mol}^{-1}$ . Arrows indicate when PFD was increased from  $100$  to  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  and returned to  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Error bars are the standard error of the mean.

(both mixed blue/red and red alone), from values around  $0.13 \text{ mol m}^{-2} \text{ s}^{-1}$  to nearly  $0.4 \text{ mol m}^{-2} \text{ s}^{-1}$  for WT plants. For the antisense SBPase plants  $g_s$  rose from slightly higher values at low PFD to between  $0.45\text{--}0.55 \text{ mol m}^{-2} \text{ s}^{-1}$  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  $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ,  $g_s$  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  $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ,  $C_i$  values for all plants were around  $330 \mu\text{mol mol}^{-1}$ , irrespective of the illumination wavebands.  $C_i$  decreased rapidly when PFD was increased to  $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , with the extent of the drop dependent on the amount of SBPase activity. In WT plants, with SBPase activity of  $8.9 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ,  $C_i$  decreased to a minimum of  $215 \mu\text{mol mol}^{-1}$  and in antisense plants it decreased to 241, 254, 293, and  $301 \mu\text{mol mol}^{-1}$  with SBPase activities of 8.3, 7.0, 5.9, and  $5.0 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , respectively, under mixed blue/red illumination and 222, 238, 293, and  $304 \mu\text{mol mol}^{-1}$  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.

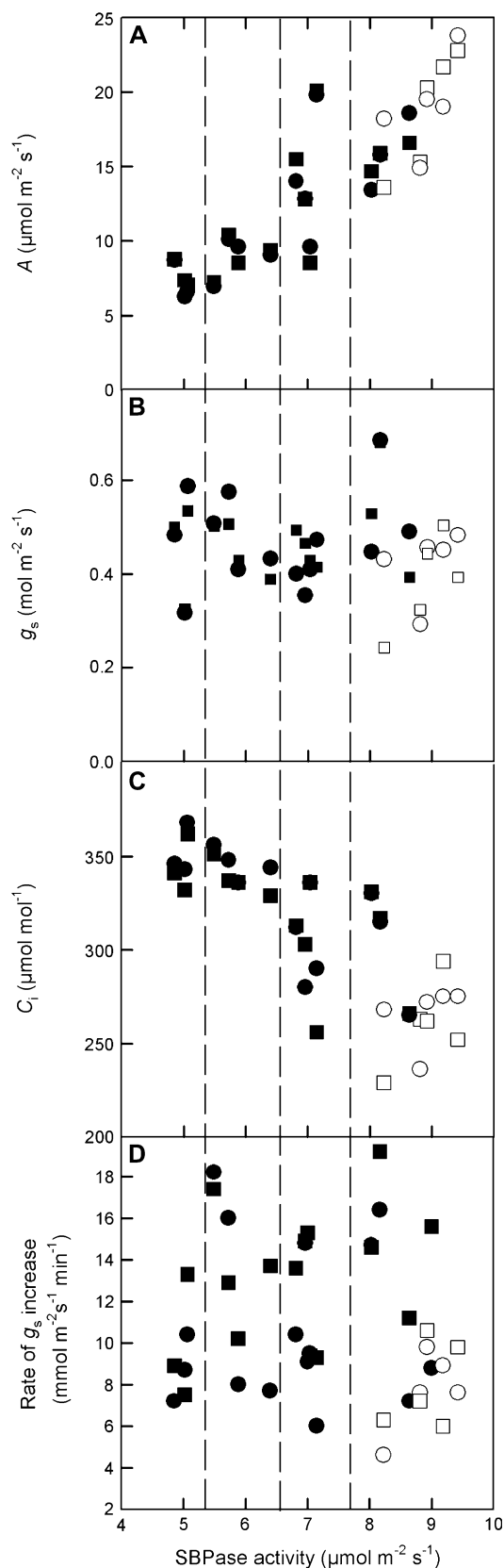
#### *Correlations between SBPase activity and photosynthetic gas exchange parameters*

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 $\text{CO}_2$ 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  $\text{CO}_2$  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  $J_{\text{max}}$  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  $>600 \mu\text{mol mol}^{-1}$ ,  $g_s$  did not change in either the WT or antisense plants (Fig. 7B). When  $C_i$  was decreased to  $<600 \mu\text{mol mol}^{-1}$ ,  $g_s$  increased significantly, reaching a value of between  $0.6\text{--}0.7 \text{ mol m}^{-2} \text{ s}^{-1}$  at the lowest  $C_i$  in both the WT and transgenic plants. At  $C_i$  concentrations between 300 and  $600 \mu\text{mol mol}^{-1}$ , 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  $1800 \mu\text{mol mol}^{-1}$  (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  $\text{CO}_2$  assimilation rate, (B) stomatal conductance, (C) internal  $\text{CO}_2$  concentration, and (D) the rate of stomatal opening in WT (open symbols) and

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

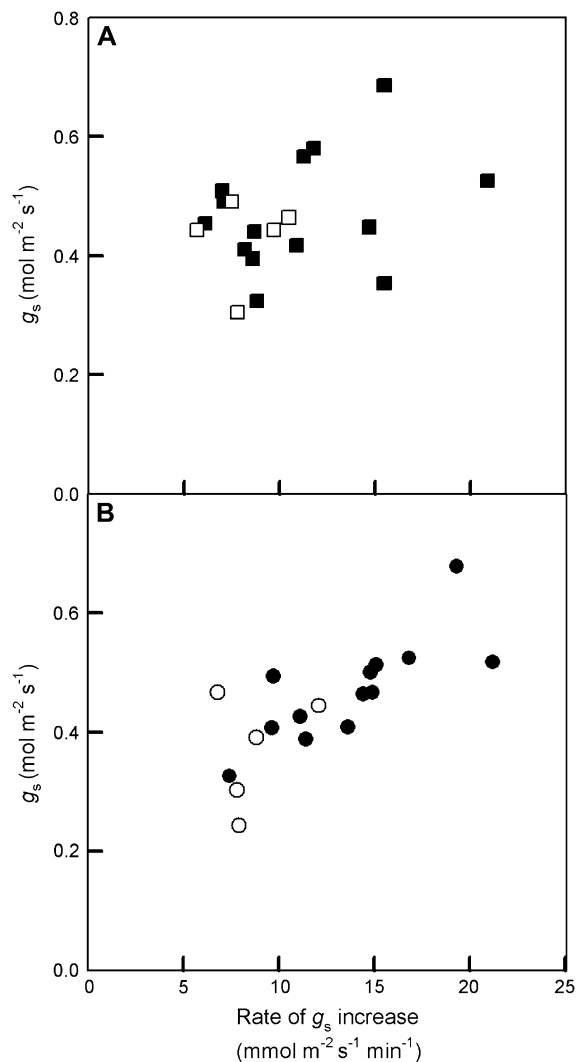
#### Effect of red light intensity on stomata at a constant $C_i$

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  $\text{CO}_2$  and stomatal response to lowering internal  $\text{CO}_2$  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  $280 \mu\text{mol mol}^{-1}$  (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  $\text{CO}_2$  assimilation rate substantially (Figs 1, 4–7), and that this reduction in photosynthesis was greatest in high light intensity, particularly at high  $\text{CO}_2$  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  $\text{CO}_2$  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

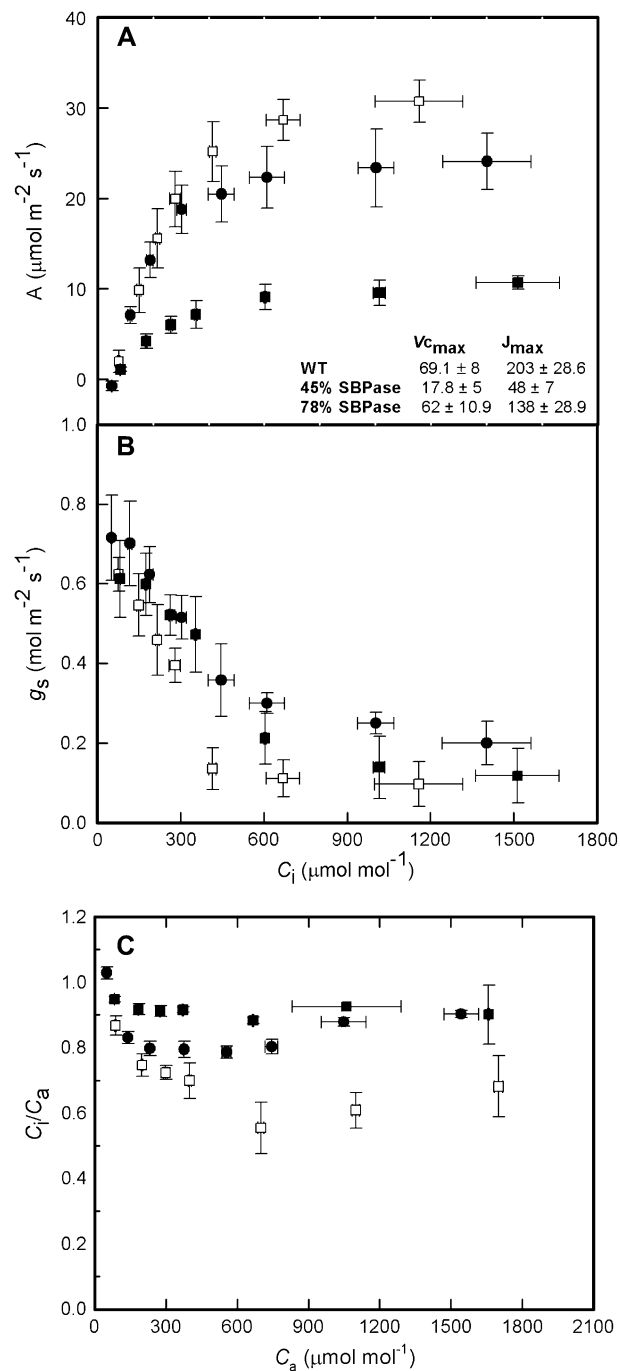
antisense SBPase transgenic tobacco (closed symbols). Measurements were taken at the end of the 30 min illumination period of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density of red (circles) or red and blue/red (squares) light as shown in Fig. 4. Water vapour was maintained at  $23 \text{ mmol mol}^{-1}$  and leaf temperature of  $25 \text{ }^\circ\text{C}$  providing a leaf–air vapour pressure difference of  $1.0 \text{ kPa}$ . Dashed lines represent the SBPase grouping of Fig. 4.



**Fig. 6.** Relationship between rate of stomatal opening and final stomatal conductance at the end of 30 min illumination period of  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  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  $23 \text{ mmol mol}^{-1}$  and leaf temperature at  $25^\circ \text{C}$  providing a leaf–air vapour pressure difference of  $1.0 \text{ kPa}$ .

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).

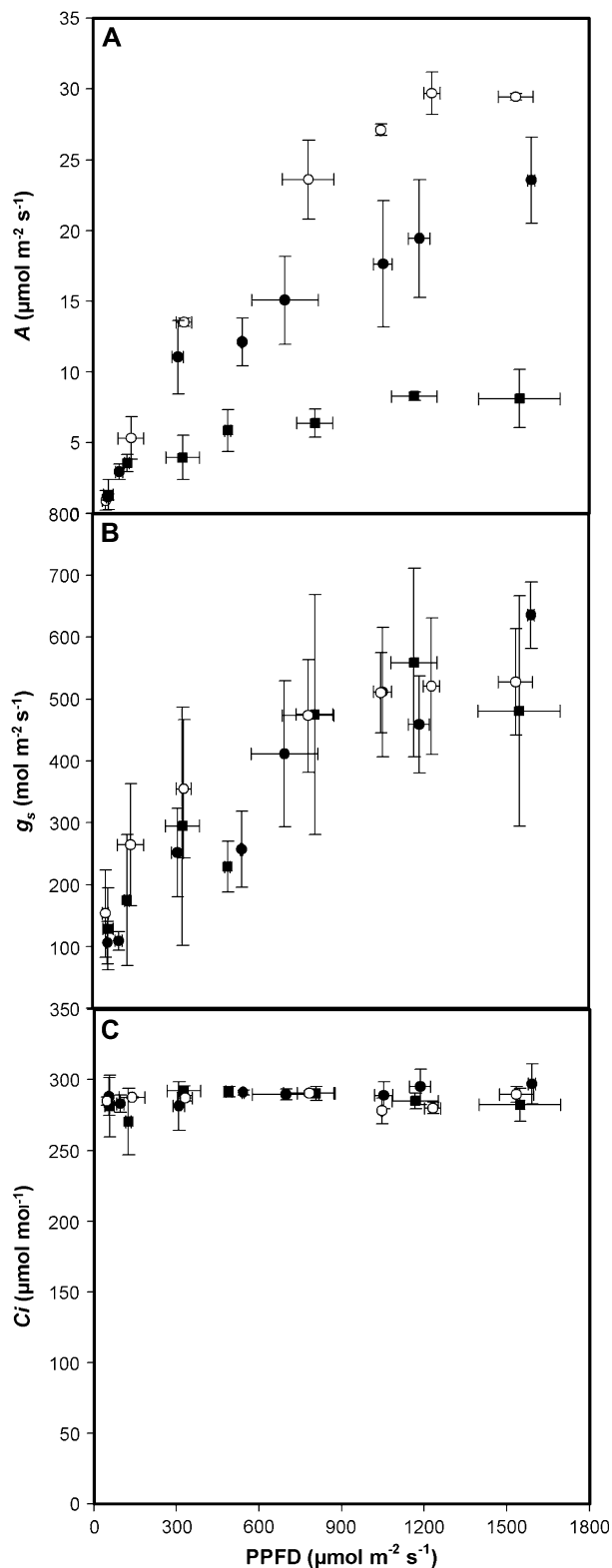
Despite reductions in both guard and mesophyll cell photosynthetic electron transport and the consequent higher  $C_i$ , the stomatal opening and closing responses to either mixed blue/red or red light in the antisense SBPase plants followed a similar pattern to those in WT plants (Fig. 4). These data provide further support for the suggestion from previous antisense studies that photosynthetic  $\text{CO}_2$  fixation in guard cells is not essential for light-induced stomatal opening in tobacco (von Caemmerer



**Fig. 7.** (A) Net  $\text{CO}_2$  assimilation rate and (B) stomatal conductance as a function of internal  $\text{CO}_2$  concentration ( $C_i$ ). Values were allowed to stabilize for at least 30 min at each  $\text{CO}_2$  concentration. (C) The relationship between ambient  $\text{CO}_2$  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.

*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<sub>2</sub> assimilation rate and (B) stomatal conductance as a function of an increase in red photon flux density whilst maintaining (C) internal CO<sub>2</sub> concentration ( $C_i$ ) at  $c. 300 \mu\text{mol mol}^{-1}$ . Values were allowed to stabilize for at least 30 min at each light level, whilst external CO<sub>2</sub> 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

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 dual 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 (Talbot 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  $230\text{--}250 \mu\text{mol mol}^{-1}$  (Mott, 1988). However, the internal CO<sub>2</sub> 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  $>250 \mu\text{mol mol}^{-1}$  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<sub>2</sub> 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).

squares (45% WT activity,  $n=4$ ). Error bars are the standard error of the mean.

An alternative explanation of these results is that guard cells respond to CO<sub>2</sub> concentrations nearer C<sub>a</sub> rather than the estimated bulk mesophyll C<sub>i</sub> (von Caemmerer *et al.*, 2004). In support of this suggestion, many of the small differences between the stomatal CO<sub>2</sub> response curves in WT and antisense plants in Fig. 7B are removed if the abscissa is C<sub>a</sub> not C<sub>i</sub> (data not shown).

Recently it has been proposed that stomatal responses to light and C<sub>i</sub> 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<sub>i</sub> was reduced compared with WT plants. Reduction of Calvin cycle activity, whether through reduced substrate availability (C<sub>i</sub>) 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<sub>i</sub>, 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<sup>+</sup> 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 photo-

synthetic 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<sub>2</sub> 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<sub>2</sub>.

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