Evidence for Involvement of Photosynthetic Processes in the Stomatal Response to CO_2[1]

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Stomatal conductance (g_s) typically declines in response to increasing intercellular CO_2 concentration (c_i). However, the mechanisms underlying this response are not fully understood. Recent work suggests that stomatal responses to c_i and red light (RL) are linked to photosynthetic electron transport. We investigated the role of photosynthetic electron transport in the stomatal response to c_i in intact leaves of cocklebur (*Xanthium strumarium*) plants by examining the responses of g_s and net CO_2 assimilation rate to c_i in light and darkness, in the presence and absence of the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and at 2% and 21% ambient oxygen. Our results indicate that (1) g_s and assimilation rate decline concurrently and with similar spatial patterns in response to DCMU; (2) the response of g_s to c_i changes slope in concert with the transition from Rubisco- to electron transport-limited photosynthesis at various irradiances and oxygen concentrations; (3) the response of g_s to c_i is similar in darkness and in DCMU-treated leaves, whereas the response in light in non-DCMU-treated leaves is much larger and has a different shape; (4) the response of g_s to c_i is insensitive to oxygen in DCMU-treated leaves or in darkness; and (5) stomata respond normally to RL when c_i is held constant, indicating the RL response does not require a reduction in c_i by mesophyll photosynthesis. Together, these results suggest that part of the stomatal response to c_i involves the balance between photosynthetic electron transport and carbon reduction either in the mesophyll or in guard cell chloroplasts.

Guard cells respond to light and to intercellular CO_2 concentration (c_i). Traditionally, the mechanisms for these two responses have been treated independently, but neither response is well understood. Recently, two hypotheses have been proposed by which c_i and light responses might be mechanistically linked through photosynthetic processes (Zeiger and Zhu, 1998; Buckley et al., 2003). Both of these hypotheses (discussed in more detail below) predict that the response of stomatal conductance (g_s) to c_i is controlled by the balance between electron transport capacity and ribulose 1,5-bisphosphate carboxylation capacity. These hypotheses are, however, controversial, and the major goal of this study is to examine the role of photosynthetic processes in the response of g_s to c_i.

The stomatal response to light has at least two components; the so-called blue light (BL) and red light (RL) responses. The BL response saturates at fluences much lower than photosynthetic saturation (around 50 μmol m^{-2} s^{-1}; Zeiger, 2000). This response has been shown to involve the activation of a plasma membrane H^+-ATPase (Kinoshita and Shimazaki, 1999), although the steps leading up to this activation are not clear. The receptor for this response has yet to be identified unequivocally, and there is evidence supporting both zeaxanthin (Zeiger and Zhu, 1998; Talbott et al., 2003) and phototropins (Kinoshita et al., 2001; Doi et al., 2004) for this role. The RL response is also poorly understood, but many data suggest the involvement of guard cell photosynthetic processes. Specifically, the RL response saturates at fluences similar to those for photosynthetic saturation. In addition, the action spectra for mesophyll photosynthesis and for the stomatal response to RL are similar to one another (Sharkey and Raschke, 1981a), and the RL response is abolished by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a PSII inhibitor (Sharkey and Raschke, 1981b; Schwartz and Zeiger, 1984; Tominaga et al., 2001; Olsen et al., 2002). For these reasons, chlorophyll is commonly assumed to be the RL receptor (Assmann and Shimazaki, 1999; Zeiger et al., 2002). Despite the accumulation of evidence showing that guard cells respond directly to RL, this conclusion has recently been challenged based on electrophysiological measurements of the guard cell plasma membrane (Roelfsema et al., 2002).

Much less is known about the stomatal response to c_i. Malate production by phospho*enol*pyruvate carboxylase was once thought to play a role in this response (Raschke, 1975). However, increased c_i should enhance malate production, leading to an increase, not

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a decrease, in guard cell osmotic pressure and thus g_s. Therefore, if malate plays a role in the c_i response, it must not involve a direct effect on osmotic pressure, but rather an indirect function on guard cell osmoregulation. Along these lines, Hedrich et al. (1994) have proposed that malate plays a role in regulating R-type anion channels and they showed how this regulation could account for stomatal response to CO_2. Another possibility is that the c_i response involves C_3 photosynthetic processes in guard cells. It now seems clear that the Calvin cycle and photosynthetic electron transport are operational in guard cell chloroplasts (see Zeiger et al., 2002; Vavasseur and Raghavendra, 2005) and that these two processes can occur at similar relative rates in guard and mesophyll cells (Cardon and Berry, 1992; Lawson et al., 2002). These results form the basis for two hypotheses that link guard cell photosynthesis with the stomatal responses to light and c_i.

The first of these hypotheses (Zeiger et al., 2002; Outlaw, 2003) is an extension of the zeaxanthin hypothesis for the BL response (Zeiger and Zhu, 1998). Zeaxanthin functions in nonphotochemical quenching. As a result, increased dissipation of photosynthetic reducing power by the Calvin cycle—promoted by increased c_i at a given level of photosynthetically active radiation (PAR)—decreases zeaxanthin concentration (Zhu et al., 1998). Conversely, at a given c_i, zeaxanthin rises with PAR because greater photon supply demands more nonphotochemical quenching. According to the hypothesis, PAR- and c_i-induced variations in the concentration of zeaxanthin regulate stomatal aperture by modulating guard cell sensitivity to BL. The second hypothesis builds on the observation that photosynthetically derived ATP is shuttled from guard cell chloroplasts to the cytosol, where it is used to drive proton pumping and hence cation uptake at the plasmalemma (Tominaga et al., 2001). The abundance of ATP, like zeaxanthin, should increase with the thylakoid pH gradient and hence PAR, and should decrease with Calvin cycle activity and hence c_i (Buckley et al., 2003). It is worth noting the existence of a third hypothesis concerning guard cell photosynthesis and g_s. This hypothesis holds that the Calvin cycle generates Suc or other osmotica within guard cells, driving osmotic swelling and stomatal opening in RL, or more generally in PAR (Zeiger et al., 2002; Outlaw, 2003). However, in contrast to observations, this mechanism predicts greater stomatal opening under elevated c_i.

A key feature of the zeaxanthin and ATP hypotheses is that the c_i and RL responses depend on the balance between photosynthetic electron transport and Calvin cycle activity in guard cells. However, several recent studies have questioned the role for guard cell photosynthetic processes in stomatal control. For example, Roelfsema et al. (2002) concluded that the RL response results from reductions in c_i caused by mesophyll photosynthesis. In addition, studies on antisense plants with suppressed expression of Rubisco (von Caemmerer et al., 2004) and Rieske FeS protein (Price et al., 1998) have found little or no difference in stomatal function between wild-type and antisense plants, suggesting neither mesophyll nor guard cell photosynthesis plays a role in the c_i response. Finally, it has also been argued for many years that the existence of a CO_2 response in darkness and of functional nonchlorophyllous guard cells in some orchids (Nelson and Mayo, 1975) disproves any necessary role for photosynthesis.

In this study, we used several techniques to explore the roles of both mesophyll and guard cell photosynthetic electron transport and Calvin cycle activity in the response of g_s to c_i. We monitored spatial and temporal changes in photosynthetic capacity and g_s concurrently after treating a leaf with DCMU, a compound known to interrupt photosynthetic electron transport. We also compared the stomatal response to c_i between a leaf in the dark and a leaf treated with DCMU in the light. Finally, we examined the responses of g_s and photosynthesis to c_i using O_2 and light to alter the balance between electron transport and Calvin cycle activity.

RESULTS

Effects of DCMU on Photosynthesis and g_s

When 100 μM DCMU was applied to a leaf via the transpiration stream, photosynthetic CO_2 uptake declined gradually over several hours. This decline in photosynthetic rate was accompanied by a decline in g_s and a slight decline in c_i (Fig. 1). Fluorescence images (Fig. 2, top row) were taken concurrently with the gas-exchange measurements to assess the spatial distribution of photosynthesis in response to DCMU. These images show distinct areas with near-zero quantum efficiency for PSII (ϕ_{PSII}) spreading out from the veins. The proportion of the leaf with near-zero ϕ_{PSII} was directly proportional to the percent reduction in photosynthesis as measured by gas exchange (Fig. 3). This supports the conclusion that the areas with near-zero ϕ_{PSII} had near-zero photosynthesis. These results show that the gradual decline in photosynthesis observed in gas-exchange data was caused by an increase in the proportion of the leaf for which photosynthesis was severely inhibited, rather than a slow uniform decline in photosynthesis for the entire leaf. This experiment was repeated three times with similar results.

Thermal images were used to assess the spatial distribution of g_s in response to DCMU treatment. Leaf temperature, and hence infrared emission, should be higher in areas of lower conductance as a result of reduced evaporative heat loss. These images (Fig. 2, bottom row) show areas of high temperature spreading out from the veins in a very similar pattern to that observed in the fluorescence images. The area of the leaf with higher than average temperatures was proportional to the percent reduction in g_s (Fig. 3), and the pixel intensity of the bright areas did not increase appreciably as the g_s of the entire leaf approached zero.

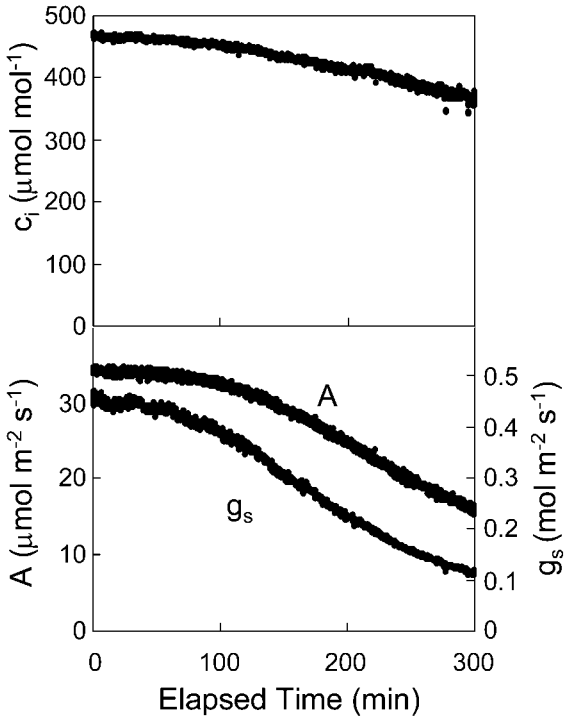

Figure 1. Responses of c_i, A, and g_s to DCMU (100 μM) fed through the transpiration stream of a detached leaf. DCMU was applied at time zero. Other conditions were as described in "Materials and Methods."

These results show that, similar to photosynthesis, the gradual decline in g_s observed with gas exchange was caused by an increase in the proportion of the leaf with near-zero conductance rather than by a slow uniform decrease in conductance. Furthermore, the spatial pattern of photosynthesis inhibition was very similar to the pattern for conductance inhibition.

The Responses of Assimilation Rate and g_s to c_i Change Slope Concurrently

The relationship between g_s and electron transport was further explored by examining the responses of g_s and photosynthesis to CO_2. These experiments were carried out at 21% and 2% O_2 using a high photon flux density (PFD; 1,000 μE m^{-2} s^{-1}) and a low PFD (300 μE m^{-2} s^{-1}), yielding four separate treatments. In all four treatments, conductance declined steeply with increasing CO_2 at high values of c_i (Fig. 4). However, in the two high-PFD treatments, the response of conductance to CO_2 was less steep at low c_i than at high c_i and, in some cases, the slope was actually positive at low c_i. This was not true for the low-PFD treatments. Furthermore, the transition from a shallow slope at low c_i to a steep slope at high c_i under high PFD occurred at approximately the same c_i values as the transition from Rubisco-limited to electron transport-limited mesophyll photosynthesis, as determined by fitting the photosynthesis model of Farquhar et al. (1980) to the assimilation rate (A) versus c_i curves (Table I). At low PFD, CO_2 assimilation was limited by electron transport at all but the lowest c_i value, and g_s declined more or less steadily with increasing c_i. Although there were differences in g_s between the upper and lower surfaces, the stomatal responses of the two surfaces to all treatments were qualitatively similar.

The change in the slope of the A versus c_i curve associated with the transition between Rubisco and electron transport limitation was larger at 2% O_2 than at 21% O_2, which makes it easier to identify unambiguously the transition point between Rubisco and electron transport limitation. It is noteworthy that the slope change in the g_s versus c_i curve was also more pronounced at 2% O_2 (Fig. 4A) and conductance actually increased slightly as the c_i values approached the transition point from below.

Photosynthetic Electron Transport Alters the Stomatal Response to c_i

Finally, we investigated the response of g_s to CO_2 in darkness and following the application of DCMU. Both treatments should reduce photosynthetic electron transport to near zero and CO_2 uptake rates were negative for both treatments at all c_i (data not shown). The response of g_s to CO_2 was essentially identical for leaves in darkness (Fig. 5A) and DCMU-treated leaves (Fig. 5B). Furthermore, there was no effect of O_2 on the response of conductance to CO_2 in darkness (Fig. 5C) or in DCMU (Fig. 5D).

To demonstrate that the difference in g_s in light and darkness was not the result of lowered c_i, as suggested by Roelfsema et al. (2002), we examined the response of photosynthesis and g_s to wavelengths greater than 500 nm while holding c_i constant by adjusting ambient CO_2 mole fraction. Both A and g_s increased approximately linearly with increasing PFD in the steady state despite a constant value of c_i (Fig. 6). Results with only wavelengths greater than 600 nm were similar to those shown in Figure 6.

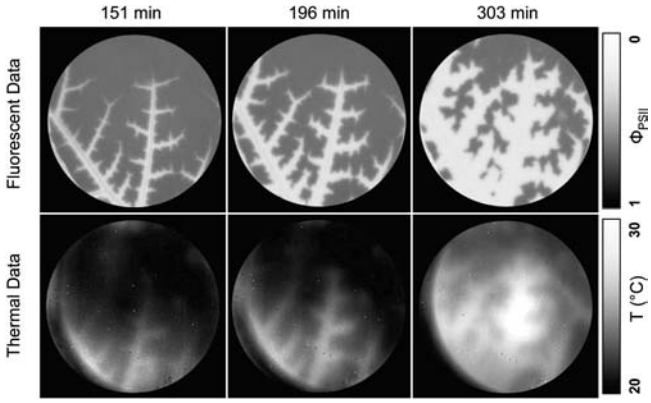

Figure 2. Fluorescence and thermal images at three times following DCMU application. Images we taken of the leaf for which data are shown in Figure 1.

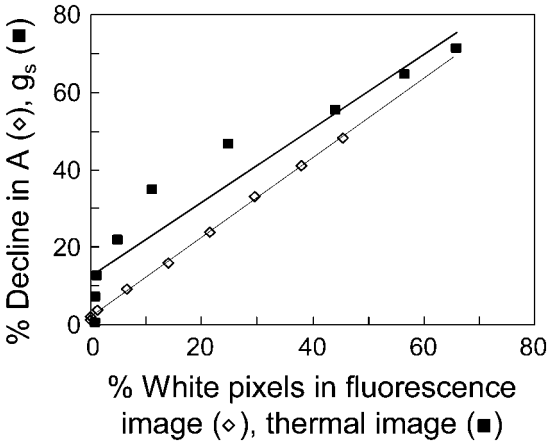

Figure 3. *A* and g_s plotted versus the percent of image with near-zero ϕ_{PSII} and percent of image with temperatures above approximately 27°C. Data were calculated at 30-min intervals as DCMU was added to the leaf via the transpiration stream.

DISCUSSION

Many previous studies have examined the role of photosynthesis in guard cell processes. However, most of these have centered on establishing the presence of photosynthetic CO_2 fixation in guard cells (Gotow et al., 1988) or the involvement of guard cell photosynthesis in providing Suc as an osmoticum (Talbott and Zeiger, 1998). In this study, we used several approaches to investigate the role of photosynthetic processes in determining the response of g_s to CO_2.

Our first approach was simply to compare stomatal responses to c_i with photosynthetic responses to c_i at two PFD values and two O_2 concentrations. These data (Fig. 4) show that the stomatal response to c_i in cocklebur (*Xanthium strumarium*) changes slope at the same c_i value as the transition from Rubisco to electron transport limitation in photosynthesis. Specifically, the stomatal response is steeper at c_i values above the transition (i.e. when mesophyll photosynthesis is limited by the supply of ATP and NADPH). The relationship holds true as the transition c_i changes with PFD, and it is clearer at 2% O_2 than at 21% O_2 because the transition between the Rubisco- and electron transport-limited portions of the curve is more distinct at 2% O_2. It is noteworthy in this regard that both the g_s versus c_i curve and the *A* versus c_i curve show a larger change in slope at 2% O_2 than at 21% O_2, making the changes in slopes less ambiguous. Collectively, these data strongly suggest that the stomatal response to c_i is somehow influenced by the balance between the light reactions and the carbon reactions in photosynthesis.

The data do not, however, indicate how or where this balance might be sensed. The photosynthesis model of Farquhar et al. (1980), as extended by Farquhar and Wong (1984), predicts that mesophyll chloroplastic [ATP] should decline as c_i increases, with a steeper slope at high c_i—as reported here for the response of g_s to c_i—so variations in ATP associated with guard cell photosynthesis are one possible sensory mechanism (Buckley et al., 2003). Zeaxanthin, one of the putative BL receptors in guard cells is another possibility. The equilibrium for interconversion of violaxanthin and zeaxanthin depends on thylakoid pH, which in turn depends on the balance between the light reactions and the carbon reactions in photosynthesis (Zeiger et al., 2002). Our data do not appear to rule out guard cell chloroplastic zeaxanthin, ATP, or any other photosynthetic intermediary, nor do they indicate clearly that the putative sensor is located in mesophyll or guard cells.

If the response of g_s to c_i is affected by the balance between electron transport and Rubisco limitations to photosynthesis, then the cessation of electron transport should markedly change the response of stomata

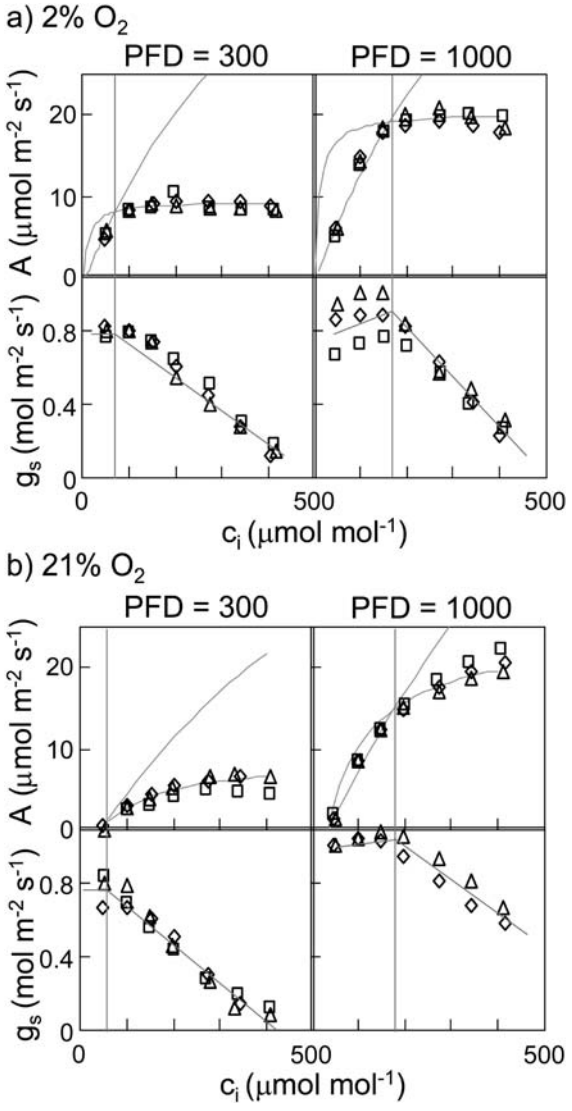

Figure 4. Responses of *A* and g_s to c_i at 2% (a) and 21% (b) O_2. Lines drawn through the *A* data are best fits to the photosynthesis model of Farquhar et al. (1980) as described in "Materials and Methods." Lines drawn through the g_s data are hand-drawn approximations. Different symbols represent different replicate experiments. Parameters for the fitted *A* versus c_i curves are given in Table I.

Table I. *Parameters of the photosynthesis model of Farquhar et al. (1980) fitted to the relationships shown in Figure 4 between net CO_2 A and c_i*

Values of the parameters Γ_* and K' (photorespiratory compensation point and effective K_m for Rubisco, respectively) were calculated from expressions given by Farquhar et al. (1980) using values of Rubisco K_m and turnover rates for carboxylation and oxygenation given by de Pury and Farquhar (1997) for 25°C.

Parameter	Symbol	Units	PFD/[O_2]			
			300/2	1,000/2	300/21	1,000/21
			$\mu E\ m^{-2}\ s^{-1}$/%			
Maximum velocity of carboxylation	$V_{c,max}$	μmol m^{-2} s^{-1}	68 ± 6	90 ± 6	105 ± 38	109 ± 6
Potential electron transport rate	J	μmol e^- m^{-2} s^{-1}	40 ± 1	84 ± 3	39 ± 7	105 ± 5
Mitochondrial respiration rate	R_d	μmol m^{-2} s^{-1}	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.4	1.1 ± 0.1
Intercellular CO_2 mole fraction at transition point	c_i	μmol mol^{-1}	79 ± 10	145 ± 20	35.9	181 ± 33
Photorespiratory compensation point	Γ_*	μmol mol^{-1}	3.4		35.9	
Effective K_m for carboxylation	K'	μmol mol^{-1}	502.3		811.9	

to c_i. We tested this idea by inhibiting electron transport with DCMU. Using high c_i to ensure that photosynthesis was electron transport limited, we found that photosynthesis and g_s declined in parallel over time as DCMU spread throughout the leaf. This confirms the earlier finding of Wong et al. (1979). It is possible that this parallel decline was caused by a slow uniform decrease in photosynthesis and g_s over the entire leaf. However, previous studies using chlorophyll fluorescence imaging have shown that DCMU inhibition of photosynthesis spreads slowly from the veins into the mesophyll, with affected areas having near-zero photosynthesis and nonaffected areas retaining high photosynthesis rates (Daley et al., 1989; Genty and Meyer, 1994).

To resolve this issue, we mapped both electron transport and g_s by imaging chlorophyll fluorescence and thermal emission simultaneously. Fluorescence images confirmed previous studies showing that areas in which photosynthetic electron transport was completely inhibited spread slowly from the veins. More importantly, thermal images clearly show that g_s had also dropped to nearly zero in areas for which electron transport was reduced to zero (see "Results"). Thus, as DCMU spread throughout the leaf, it inhibited electron transport and caused g_s to decline to zero. These two effects were simultaneous within the spatiotemporal resolution of the images. It is unlikely that the decline in conductance was caused by an increase in c_i resulting from the decline in mesophyll photosynthesis, because the average c_i for the leaf changed only slightly as both A and g_s declined by more than 50% (if mesophyll photosynthesis had declined before g_s in leaf regions affected by DCMU, the ratio of A to g_s would have declined in those regions, causing the whole-leaf estimate of c_i to increase).

DCMU was fed to leaves until the photosynthesis rate was constant and negative. At that point, we assumed that electron transport was uniformly inhibited throughout the leaf. Fluorescence images confirmed this (data not shown). Under these conditions, stomata retained a small, but measurable, response to c_i (Fig. 5B). However, several lines of reasoning suggest that this represents a separate response of stomata to CO_2 that is independent, and perhaps complementary, to the response that occurs in the presence of photosynthetic electron transport. First, the response in the presence of DCMU was not altered by the presence or absence of light and it was similar to the responses that occur in darkness and in the absence of DCMU. All responses in the absence of photosynthetic electron transport (either in darkness or after DCMU treatment) were unaffected by oxygen concentration and were qualitatively and quantitatively different from the stomatal response in the presence of photosynthetic electron transport. Thus, our data suggest that there are two mechanisms by which stomata respond to CO_2: one

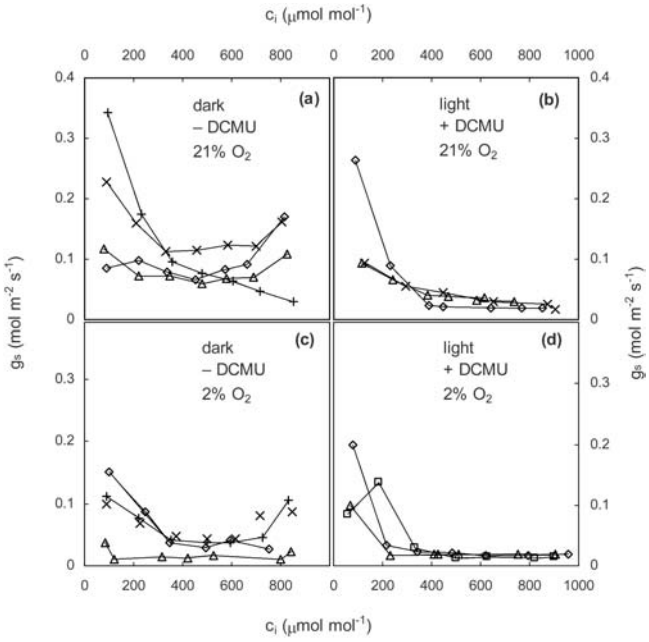

Figure 5. Responses of g_s to c_i at 21% oxygen in darkness (a) and for a DCMU-treated leaf at a PFD of 1,000 μE m^{-2} s^{-1} white light (b); and at 2% oxygen in darkness (c) and for a DCMU-treated leaf at a PFD of 1,000 μE m^{-2} s^{-1} white light (d). Different symbols represent different replicate experiments.

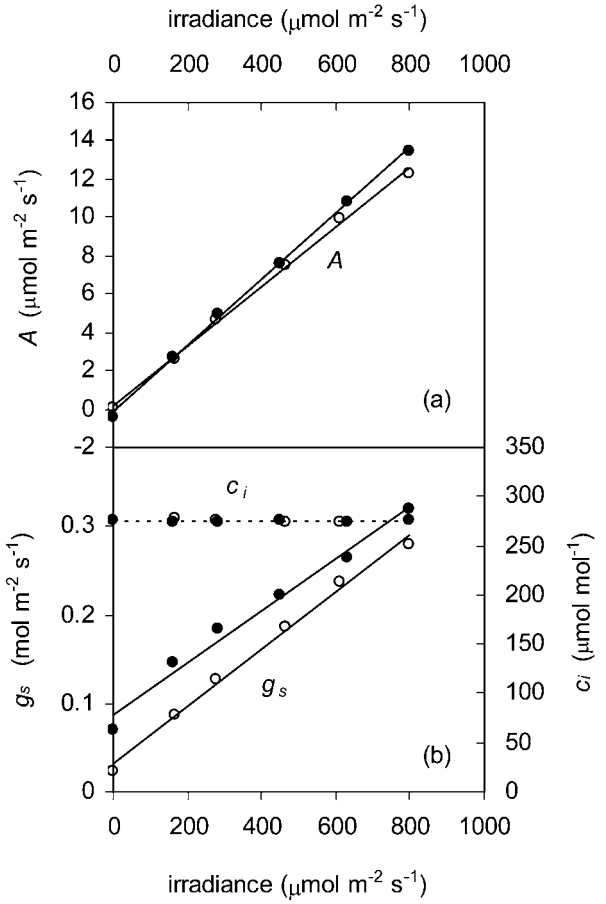

Figure 6. Responses of A (a) and g_s (b; solid lines), and c_i (dashed lines) to PFD with wavelengths above 500 nm. Ambient CO_2 was manipulated to keep c_i constant. Different symbols represent different replicates.

that depends on photosynthetic electron transport and one that does not. The idea of multiple CO_2 response mechanisms has been suggested before (Assmann, 1999) and it explains numerous previous reports that stomata can respond to c_i in the dark (Heath and Russell, 1954; Dale, 1961; Mansfield and Heath, 1961).

Although DCMU is well known to inhibit photosynthetic electron transport, its effects on g_s have not been studied in detail. It has been shown to inhibit sugar production in guard cells (Poffenroth et al., 1992) and to inhibit opening under RL in epidermal peels (Schwartz and Zeiger, 1984; Olsen et al., 2002). More recently, it has been shown to inhibit the effect of RL on H^+ pumping at the plasma membrane of guard cells of *Vicia faba* and *Commelina benghalensis* (Tominaga et al., 2001). These results suggest that DCMU should cause stomata to close in intact leaves and are therefore consistent with our data. Our results appear contradictory to those of Sharkey and Raschke (1981b), who found little effect of DCMU and cyanazine (a herbicide that blocks photosynthetic electron transport at PSII) on g_s in cocklebur. However, in *Gossypium hirsutum*, Sharkey and Raschke (1981b) found a reduction in g_s and a change in the response of conductance to c_i in response to cyanazine, and these effects were qualitatively similar to our results (compare their Fig. 1 with our Figs. 4 and 5B). Part of the discrepancy between their results and ours may be because they fed the photosynthetic inhibitor only until whole-leaf photosynthesis reached the compensation point, which would suggest that at least some areas of the leaf were still photosynthesizing. In our study, CO_2 uptake was negative and constant at all c_i values, indicating that photosynthetic electron transport was completely blocked in all areas of the leaf.

There are numerous studies showing that guard cells respond to RL directly (Heath and Russell, 1954; Sharkey and Raschke, 1981a; Zeiger, 1990). However, this finding has recently been called into question by Roelfsema et al. (2002), who concluded that the RL response of stomata in intact leaves was strictly indirect and was mediated by a reduction in c_i caused by mesophyll photosynthesis. It is critical to the central arguments of this study that guard cells respond directly to PAR independent of the BL response and independent of mesophyll-induced changes in c_i. We verified this by examining the g_s response to RL while holding c_i constant by adjusting ambient CO_2 concentration. Our results show that, in our leaves, the major RL response was not caused by mesophyll-induced reductions in c_i (Fig. 6), but instead either by a c_i-independent signal from the mesophyll (Lee and Bowling, 1995) or by processes located within guard cells.

It is unclear from our data whether guard cells are responding directly to electron transport in the guard cells or indirectly to mesophyll electron transport through an unknown signaling mechanism. If the former, our data suggest that the balance between electron transport and Rubisco capacities is similar for guard cells and mesophyll cells. This is supported by data showing that fluorescence responses to CO_2 and O_2 are similar in guard and mesophyll cells (Cardon and Berry, 1992; Lawson et al., 2002, 2003).

The suggestion that a large part of the stomatal response to c_i is closely linked to photosynthetic processes within guard cells contrasts with the findings of several studies on antisense plants with impaired photosynthetic functioning, which have generally found little difference in g_s or its response to c_i between normal and antisense plants. Price et al. (1998) found similar g_ss in wild-type plants and antisense plants with up to a 90% reduction in Rieske FeS protein (a component of the chloroplast cytochrome b_6f complex). Since these plants presumably had reduced electron transport relative to Rubisco capacity, they should have shown reduced g_s to be consistent with our results. However, although fluorescence data suggest guard and mesophyll cells possess similar photosynthetic competency on a chlorophyll basis (Lawson et al., 2002), guard cells have much less chlorophyll per cell and hence lower content and activity of photosynthetic components than mesophyll cells on a per-cell basis. As a result, it is possible that systemic reductions

in the expression of Rieske FeS protein may have little or no effect in guard cells except at very low expression levels. In any event, the C_3-type fluorescence responses of guard cells in the C_4 species *Amaranthus caudatus* (Lawson et al., 2003) suggest that photosynthetic coordination may be regulated by entirely different means in guard and mesophyll cells.

Von Caemmerer et al. (2004) likewise found no effect of reduced Rubisco content on either g_s or its response to c_i. Rubisco antisense plants possess increased electron transport capacity relative to Rubisco capacity, which should increase both g_s and the value of c_i at which the transition from Rubisco to electron transport occurs. However, our data suggest that these effects will be subtle and limited to conductances at low values of c_i. Since the experiments by von Caemmerer et al. (2004) covered a wide range of c_i values and included relatively few points at low c_i, it is unclear whether their data show a transition point effect similar to that reported in this study.

CONCLUSION

This study has shown that the response of steady-state g_s to c_i in intact leaves of cocklebur is qualitatively different when photosynthetic electron transport is eliminated, either by removal of light or by addition of DCMU, a PSII inhibitor. In the presence of photosynthetic electron transport, the response changes slope markedly at values of c_i very close to the transition of whole-leaf photosynthesis from Rubisco limitation to photosynthetic electron transport limitation: The response is shallow (small slope) at low c_i and steeper at high c_i. In contrast, the response in darkness or under DCMU is relatively small and does not show a distinct change in slope.

These data suggest there are at least two mechanisms by which stomata respond to CO_2. One of these depends on photosynthetic electron transport and is therefore sensitive to the balance between the light and dark reactions of photosynthesis; the other is independent of photosynthetic electron transport and is therefore present in darkness. Both mechanisms may contribute to normal stomatal responses to CO_2 in the light.

MATERIALS AND METHODS

Cocklebur (*Xanthium strumarium*) plants were grown in a controlled-environment greenhouse as described previously (West et al., 2005). Gas-exchange measurements were made using a standard, single-pass gas-exchange system that has also been described previously (West et al., 2005). For all experiments, leaf temperature was maintained at 25°C ± 0.2°C by changing air temperature, and the water vapor mole fraction gradient between the leaf and air was maintained at 15 mmol mol^{-1} by changing the mole fraction of water in the chamber air.

To examine the effects of DCMU on photosynthesis and g_s, a leaf was detached under water and the petiole was placed in distilled, degassed water. The leaf was brought to steady state in a clamp-on gas-exchange chamber that enclosed a circular area of leaf (diameter = 2.54 cm). PFD was maintained at 1,000 μE m^{-2} s^{-1}, O_2 concentration at 21%, and ambient CO_2 concentration at 600 μmol mol^{-1} to ensure that photosynthesis was electron transport limited. At time zero, the petiole was removed from the water and quickly placed in 100 μM DCMU (or in water for control experiments). Gas-exchange data were recorded every 10 s, and fluorescence and thermal images were captured every 3 min. The details of the methods for fluorescence and thermal images have been described previously (West et al., 2005).

The responses of g_s and A to c_i were determined by placing a leaf (attached to the plant) in a clamp-on chamber that enclosed a square area of the leaf (2.54 × 2.54 cm). The leaf was brought to steady state at an ambient CO_2 concentration of 360 μmol mol^{-1}, O_2 concentration of either 2% or 21%, and a PFD of either 1,000 or 300 μE m^{-2} s^{-1}. PFD was gradually increased to these levels over a period of several hours to avoid damaging the leaf. After gas exchange reached steady state (usually within 2–3 h), c_i was manipulated by varying ambient CO_2 concentration.

To determine the response of g_s to c_i in darkness, a detached leaf was enclosed in the square gas-exchange chamber and allowed to reach steady state at 2% or 21% O_2 at the lowest CO_2 concentration. After steady state was achieved, ambient CO_2 concentration was increased in steps, allowing the leaf to reach steady state at each concentration. DCMU experiments were done as above, except that 100 μM DCMU were fed to the leaf the day before the experiment.

A long-pass filter with a cutoff at 500 nm was used to investigate the response of stomata to RL. An attached leaf was allowed to reach steady state in the square chamber at a PFD of 800 μE m^{-2} s^{-1} of RL, 21% O_2, and 360 μmol mol^{-1} CO_2. The PFD was then lowered in steps, allowing the leaf to reach steady state at each value. As g_s and photosynthesis changed in response to the new PFD value, ambient CO_2 was adjusted to maintain c_i constant at the value observed at the highest PFD.

The A versus c_i response curves obtained from the light and oxygen experiments were analyzed to estimate the transition point between Rubisco-limited and electron transport-limited photosynthesis. The datapoints on the A versus c_i response curves were identified as electron transport limited or Rubisco limited by fitting them with the biochemical photosynthesis model of Farquhar et al. (1980). In that model, the shape of the A versus c_i response curve and, consequently, the transition point, depends sensitively on the values of two parameters—the maximum carboxylation rate of Rubisco, $V_{c,max}$, and the maximum potential electron transport rate, J_{max}. While the other model parameters can be decided a priori, the values of $V_{c,max}$ and J_{max} must be estimated from the A versus c_i response curve. For each dataset, the values of $V_{c,max}$ and J_{max} were estimated by minimizing the sum of square error of the model. For each of the four treatments, the parameter values were averaged to estimate the transition point.

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