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Do Stomata Respond to CO_2 Concentrations Other than Intercellular?[1]

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ABSTRACT

Most studies on stomatal responses to CO_2 assume that guard cells respond only to intercellular CO_2 concentration and are insensitive to the CO_2 concentrations in the pore and outside the leaf. If stomata are sensitive to the CO_2 concentration at the surface of the leaf or in the stomatal pore, the stomatal response to intercellular CO_2 concentration will be incorrect for a 'normally' operating leaf (where ambient CO_2 concentration is a constant). In this study asymmetric CO_2 concentrations for the two surfaces of amphistomatous leaves were used to vary intercellular and leaf surface CO_2 concentrations independently in *Xanthium strumarium* L. and *Helianthus annuus* L. The response of stomata to intercellular CO_2 concentration when the concentration at the leaf surface was held constant was found to be the same as the response when the surface concentration was varied. In addition, stomata did not respond to changes in leaf surface CO_2 concentration when the intercellular concentration for that surface was held constant. It is concluded that stomata respond to intercellular CO_2 concentration and are insensitive to the CO_2 concentration at the surface of the leaf and in the stomatal pore.

Although stomata have been known to respond to CO_2 for almost 90 years (3), the mechanism of this response and its role in determining stomatal conductance are still being debated today. During most of this controversy it has been assumed that guard cells sense the concentration of CO_2 inside the leaf, *i.e.* in the intercellular spaces, rather than outside the leaf. More specifically, most studies assume that guard cells perceive and respond only to the concentration of CO_2 calculated as 'intercellular.' Although it is reasonable to assume that cutinization of the outer surfaces of guard cells prevents rapid diffusion of CO_2 from the ambient air, there is no obvious rationale for assuming that guard cells perceive only intercellular CO_2 concentration and are insensitive to the CO_2 concentration in the pore. Indeed, the validity of this important assumption has never been tested.

Sensing of C_i[2] by guard cells is an attractive hypothesis because as the mesophyll demand for CO_2 increases, C_i will decrease, promoting stomatal opening and increasing C_i. At one time this feedback loop was thought to account for the relative constancy of C_i as photosynthetic rate varied with light intensity (9). However, the gain of this loop, as calculated using control theory principles, is too small to account for the response of stomata to changes in light intensity (5, 10, 14). Investigators have suggested that most of the stomatal response to light is due to a direct effect on guard cells (10) or to metabolite transfer from mesophyll cells to guard cells (15).

Recently, stomata have been shown to maintain a constant C_i/C_a ratio as ambient CO_2 concentration is varied. This was first discussed by Wong (13) as a personal communication from J. Berry, and subsequently by Bell (2) who postulated that stomatal behavior could be predicted based on a constant C_i/C_s. Ball and Berry (1) confirmed that C_i/C_s was conserved as light was varied, and have since extended their analysis to an empirical model for stomatal responses to light, CO_2, and humidity (personal communication). The tendency of stomata to maintain a constant C_i/C_s ratio has led to speculation that guard cells respond to both C_i and C_s, and possess a feedback loop to maintain a constant ratio of the two parameters (1, 2).

Although most investigators assume that C_i is the sensed parameter, the notion that stomata perceive some combination of C_i and C_s is not without precedent in the literature. Raschke (8) suggested that guard cells might exchange CO_2 along the entire wall of the stomatal pore and therefore would sense approximately the average of the C_i and C_a. The only data in the literature concerning this question are those of Heath (6) who found that in darkness, tightly closed stomata would not open in response to CO_2-free air. He hypothesized that guard cells responded to the CO_2 concentration inside the leaf, which could not be affected by C_a if stomata were tightly shut. This observation, however, does not indicate that guard cells respond only to C_i and not to C_p.

If the stomatal response to CO_2 is dependent on the concentration outside the leaf or in the stomatal pore (except as these concentrations influence C_i), it would have important implications. Stomatal responses to C_i are typically determined by varying C_a in order to change C_i. Therefore as C_i changes in one direction, C_s changes in the same direction, thus creating relatively large fluctuations in C_p. This is quite different from the situation in a 'normally' functioning leaf where ambient CO_2 concentration is a constant, and changes in C_i are induced by changes in mesophyll demand for CO_2. Here, C_s would fluctuate only slightly, and C_p would change much less in response to a change in C_i. Sensitivity to a CO_2 concentration other than that in the intercellular spaces would mean that the stomatal response to C_i, determined as described above, could be incorrect. Conclusions about the importance of the C_i response in stomatal adjustments to light intensity (5, 10) that were based on this response would then be invalid.

In this study C_i and C_s were varied independently using asymmetric CO_2 concentrations on the two sides of amphistomatous leaves. For example, by changing C_a on the upper surface it was possible to produce changes in C_i for the lower surface without significantly altering C_s for the lower surface. Using similar techniques, the C_s for one surface could be varied without altering

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[2] Abbreviations: C_i, intercellular CO_2 concentration; C_a, ambient CO_2 concentration; C_s, concentration of CO_2 at the surface of the leaf; C_p, CO_2 concentration in the stomatal pore.

its C_i. This allowed a rigorous test of the hypothesis that guard cells respond only to C_i and are independent of the CO_2 concentration outside the leaf or in the stomatal pore.

MATERIALS AND METHODS

Plant Material. Sunflower (*Helianthus annuus*) and cocklebur (*Xanthium strumarium*) in one gallon nursery pots containing a 1:1 mixture of perlite and sterile potting soil were grown in controlled environment growth chambers. Pots were watered as necessary with one-eighth strength modified Hoagland solution. The light intensity at the top of the plants was approximately 350 μE $(m^2s)^{-1}$, with photoperiods of 12 and 16 h for sunflower and cocklebur, respectively. Leaves for gas exchange experiments were selected for uniformity of age and appearance.

Gas Exchange. A clamp-on type chamber that produced separate circular chambers above and below the leaf was used for gas exchange measurements. Each chamber was 2.5 cm in diameter and 1.0 cm deep. The small volume and relatively high flow rates used (435 μmol s^{-1}, 0.75 L/min) created a boundary layer conductance of 0.65 mol $(m^2s)^{-1}$ for each chamber, or a total boundary layer conductance for the leaf of 1.3 mol $(m^2s)^{-1}$. Light was provided to the leaf by a fiber optic illuminator (Schott, model KL1500) utilizing a 150 W xenon lamp, and was attenuated with neutral density filters.

Two identical gas exchange systems were used to independently control and measure the environment of the chambers above and below the leaf. In each system N_2 and O_2 were mixed to normal atmospheric concentrations (21% O_2, 79% N_2) using mixing valves. O_2 concentration was measured using an O_2 electrode (Rank Brothers). The N_2:O_2 mixture was split between two 5 L/min mass flow controllers (Datametrics, model 825) calibrated at ambient atmospheric pressure. The gas stream from one of the mass flow controllers was humidified by bubbling it through warm CO_2-free distilled water, condensed at 22°C, and remixed with the unhumidified gas stream. A 0.5 L/min mass flow controller was used to add 1% CO_2 in air to the mixed gas stream. A portion of the final mixed gas was passed through a metering valve and a mass flow meter (Datametrics, model 831) before going to the leaf chamber. The rest of the gas was used for the reference cell of the CO_2 analyzer or exhausted.

This mixing arrangement made it possible to vary CO_2 concentration between 0 and 1000 μl/L, and water vapor pressure between 0.5 and 26.5 mbar, independently and at any flow rate. Because CO_2 was added after humidification, there was no equilibration of CO_2 with the humidification water, and changes in CO_2 concentration were achieved with the response time of the CO_2 mass flow controller. The CO_2 concentration and water vapor pressure of the mixed gas were calculated based on the readings of the mass flow controllers and the vapor pressure of the humidified air. Changes in CO_2 and humidity were substantially independent of each other in this system, but there was a small change in total flow through the system (not to the chamber) associated with alterations in CO_2 or humidity. The resulting small changes in mixture were calculated and corrected.

Gas from the leaf chamber was picked up at positive pressure and pumped through the analysis circuit, which consisted of a chilled mirror dewpoint hygrometer (General Eastern, model DEW-10) and a differential infra-red CO_2 analyzer (Analytical Development Co., model Mark III). Leaf temperature was measured using 36 gauge thermocouples.

The mixing and analysis portions of both systems were monitored by a portable datalogger (Campbell Scientific, model 21X), and values for C_a, C_i, photosynthesis, etc. were updated to the screen of a microcomputer every 5 s. Values were computed for both surfaces independently and for the total leaf using the equations given by von Caemmerer and Farquhar (12). C_s was calculated from C_a and photosynthetic rate.

RESULTS

All of the experiments described below were performed on both sunflower and cocklebur. Data from the two species were very similar, and to prevent redundancy only those from cocklebur are shown.

Whole leaf responses to C_i were produced by varying C_a in parallel for the two surfaces of a leaf. To control for the effects of humidity and temperature on stomatal conductance, leaf temperature was held at 25.0 ± 0.1°C and ambient humidity at 15.0 ± 0.1 mbar, creating a Δw of 17.2 ± 0.4 for each surface. At air level C_a (340 μl/L) photosynthetic rate saturated at, or slightly lower than, 1150 μE $(m^2s)^{-1}$ (data not shown), and this intensity was used for subsequent experiments in which CO_2 concentration was varied.

Figure 1 shows the response of total leaf photosynthesis, stomatal conductance, and C_i/C_s to C_i for cocklebur. As explained above, these data were produced by parallel changes in C_a for the two surfaces; therefore, they are comparable to data produced using a chamber that encloses the whole leaf. The data shown are for eight plants; data for a single leaf had much less scatter. Photosynthetic rate and stomatal conductance of the plants used in this study responded such that C_i/C_s remained fairly constant as C_s and C_i changed, but some increase in C_i/C_s was observed at low CO_2 concentrations.

Total leaf stomatal conductance was calculated as the sum of the stomatal conductances of the upper and lower surfaces; the magnitudes of the two conductances were similar, but that of the upper surface was usually slightly greater than that of the lower surface at a particular C_i value. The response of total leaf stomatal conductance to CO_2 concentration (Fig. 1) was produced by parallel changes in the upper and lower stomatal conductances, and these are shown by the open symbols in Figure 2, a and c, respectively. The open symbols of Figure 2, b and d are identical to those in Figure 2, a and c, and are repeated for comparison with the response at constant C_s (solid points), discussed below. The percentage of the total photosynthetic rate that occurred across each surface was proportional to the conductance of that surface (data not shown). Therefore, little or no gradient in CO_2

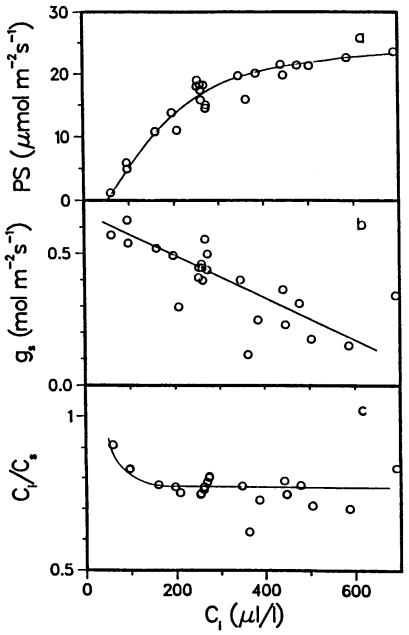

FIG. 1. Whole leaf photosynthesis (a), stomatal conductance (b), and C_i/C_s (c) for cocklebur plants. The data shown are from eight different plants and were generated using parallel changes in C_a for the upper and lower surfaces of the leaf.

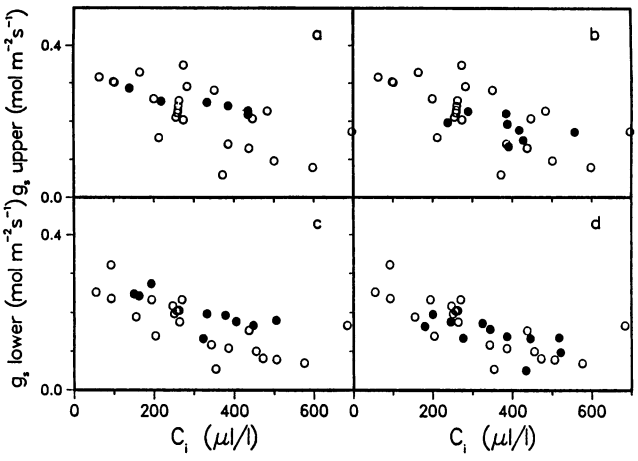

FIG. 2. Responses of upper and lower surface stomatal conductances to the C_i value calculated for that surface. Upper surface, (a) and (b); lower surface (c) and (d). The open symbols are the response generated by varying C_a in parallel for the two surfaces. The solid symbols are the response generated when C_s was held constant at 340 μl/L (a and c) or 600 μl/L (b and d) as C_i was varied.

concentration existed through the leaf, and C_i values for the two surfaces were usually within 5 μl/L of each other.

Before C_i was varied independently of C_s for a surface, a leaf was first brought to steady state at air level C_a for both surfaces to be sure that conductances and photosynthesis values were similar to those shown in Figures 1 and 2. C_a for the lower surface was then altered, producing changes in C_i for both surfaces. Although C_s for the upper surface was substantially unaffected by this process, the altered CO_2 flux across that surface did cause a small change in C_s. The CO_2 concentration of the air entering the upper chamber was then adjusted to maintain a constant C_s for that surface. Thus, C_i values for both surfaces were changed, but C_s for the upper surface remained constant. The closed symbols of Figure 2a show the response of the upper stomata to changes in the C_i value for that surface when C_s for that surface was held at 340 μl/L. They can be compared to the open symbols in that figure, which show the response of the upper stomata to C_i when C_s is varied. Similarly, the solid symbols of Figure 2b show the response of the upper stomata to upper surface C_i when C_s (for the upper surface) is held constant at 600 μl/L. The entire procedure was reversed for the lower surface, and those data are shown in Figure 2, c and d.

The asymmetric CO_2 concentrations used in these experiments produced a significant gradient in CO_2 through the leaf, resulting in different C_i values for the two surfaces. In every case the higher C_i value was associated with the surface having the higher C_a value, and the magnitude of the gradient in C_i was consistent with an intercellular conductance across the leaf of approximately 1.0 mol $(m^{-2}s)^{-1}$ (see Ref. 7 for a discussion). For example, with C_s for the lower surface held at 340 μl/L, C_s for the upper was raised to 673 μl/L. Under these conditions, C_i for the lower and upper surfaces were 323 and 391 μl/L, respectively, creating a 68 μl/L gradient across the mesophyll. This set of conditions is represented by a solid symbol on Figure 2c at a C_i value of 323 μl/L. In many cases the asymmetry in C_a was such that a reverse gradient in CO_2 existed for one surface, resulting in a net diffusional flux out of the leaf through that surface.

To further investigate the influence of CO_2 concentrations other than C_i on stomatal conductance, the effect of C_s at a constant C_i was determined using a single leaf. In these experiments, the C_a, and consequently the C_s, was changed for the lower surface, but the C_a for the upper surface was adjusted such that C_i for the lower surface remained constant. Therefore, guard cells on the lower surface experienced changes in C_s but C_i remained constant. The solid symbols of Figure 3 show the response of lower surface stomatal conductance to lower surface C_s when C_i for that surface was held constant. The open symbols of Figure 3 represent the response of the upper surface stomata to changes in C_s when C_i for the upper surface was constant. Because asymmetric C_a values resulted in a CO_2 concentration gradient through the leaf, C_i could then be maintained constant for only one surface at a time. Therefore, each line in Figure 3 was generated from one surface of a single leaf. Stomata on the surface for which C_i was not being held constant did respond to the changes in C_i that occurred for that surface. After each experiment the sensitivity of the stomata on both surfaces to C_i was checked and found to be consistent with the previously determined response.

DISCUSSION

Whole leaf gas exchange parameters were determined to verify that photosynthesis and conductance responses to CO_2 were similar to those previously reported. Also, it was necessary to determine conductance responses of the individual surfaces to C_i when C_s was varied for comparison with the responses when C_s was constant. Whole leaf photosynthesis and stomatal conductance varied with C_i in a manner consistent with data reported previously (14). C_i/C_s was conserved as C_a was varied, except at very low CO_2 concentrations when the ratio increased slightly. The increase in C_i/C_s has been observed at low CO_2 concentrations and low light intensities and occurs because C_s must equal C_i at either compensation point (T. Ball, J. Berry, personal communication).

Although nonparallel responses of stomata on the upper and lower surfaces have been reported for both light (11) and CO_2 (4), responses to C_i in this study, and to light in a previous study (7), were parallel for the two surfaces. In both of these studies, stomatal conductance of the upper surface was approximately equal to that of the lower surface, and it is possible that very different stomatal conductances on the two surfaces might have resulted in nonparallel responses. The nearly identical C_i values for the two surfaces observed in this study are also consistent with results of previous studies (7). Significant CO_2 gradients through the mesophyll, resulting in different C_i values for the two surfaces, were created when there were large differences in C_a across the leaf. This is consistent with the findings of Mott and O'Leary (7) who concluded that the conductance for gas diffusion across the leaf was large enough to preclude significant

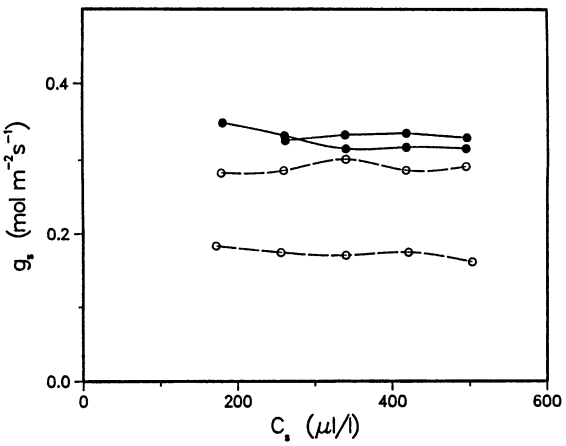

FIG. 3. Stomatal responses to C_s for a given surface when C_i for that surface was held constant. Each line represents data from a single leaf. The solid symbols are lower surface conductances; the open symbols are upper surface conductances.

differences in C_i in a normally operating amphistomatous leaf.

In previous studies on stomatal responses to C_i, it has been impossible to separate the effects of C_s or C_p on stomatal conductance from those of C_i because a change in C_a was used to alter C_i. The stomatal response observed could therefore have been to C_i, C_s, C_p, or some combination of the three parameters. To separate the response of stomata to C_i from that to C_s or C_p, C_s was maintained constant at 340 or 600 μl/L for one surface as C_i for that surface was varied. If guard cells perceive and respond to any CO_2 concentration in addition to C_i, then the stomatal response to C_i at a constant C_s of 340 μl/L (solid symbols, Fig. 2, a and c) should differ from that observed at a C_s of 600 μl/L (solid symbols, Fig. 2, b and c), and both should differ from the response when C_s is varied (open symbols, Fig. 2, all panels). The data in Figure 2 show that the stomatal response to C_i with constant C_s is the same as that observed with varying C_s, indicating that stomata do not respond to C_s. Because these experiments resulted not only in different C_s values for the same C_i, but also in different C_p values for the same C_i, the data also show that guard cells do not respond to C_p.

The nature of the experiment described above limited the amount of data that could be gathered from one leaf, therefore many plants were necessary to generate the data in Figure 2. Interplant variability was rather low, but it was still large enough to obscure small effects of C_s or C_p on conductance. This was not true for the experiment shown in Figure 3, where each line was generated from a single leaf. In this experiment large changes in C_s for a surface had no effect on stomatal conductance when C_i for that surface was held constant. As in the previous experiment, large changes in both C_s and C_p were created for a given C_i, therefore the data indicate that guard cells did not respond to C_s or C_p. Furthermore, the data also indicate that stomata of a given surface respond to the C_i value calculated for that surface and not to the average C_i for the leaf. Conductance for a given surface remained constant as long as the C_i for that surface was constant, despite changes in the C_i for the other surface and the average C_i for the leaf.

The complete insensitivity of guard cells to C_s or C_p was somewhat surprising and led to some concern that the stomata of these particular plants might not be sensitive to CO_2, as was found by Raschke (1978). However, when C_i was allowed to vary for a surface, the stomata on that surface always responded. Presumably, stomata sense the CO_2 concentration inside the guard cells, which is influenced by the CO_2 concentration of the guard cells' environment. In view of the uncertainty about both the conductance of the inner walls of guard cells to CO_2 and the exact location of the CO_2 concentration calculated as intercellular, it is remarkable that no effect of other CO_2 concentrations was found. It is possible that cutinization of the guard cells is such that C_i is the only CO_2 concentration which significantly influences the CO_2 concentration of the guard cells. An alternative explanation is that at least part of the C_i response depends on a signal from adjacent epidermal or mesophyll cells that are exposed only to the CO_2 concentration inside the leaf. This has been suggested by Wong *et al.* (15), but there is no direct evidence for such a message. The data in this study suggest that if a mesophyll signal is involved, it must be only from mesophyll cells exposed to the calculated C_i value and not from the mesophyll as a whole.

In summary, the data presented in this study confirm the observation of Heath (6) that guard cells are insensitive to the CO_2 concentration outside the leaf. In addition, they indicate that of all the different CO_2 concentrations that will exist at various points along the diffusional pathway for CO_2, guard cells apparently perceive and respond only to that calculated as C_i. Regardless of the mechanism by which C_i is exclusively sensed, this result confirms that it is a useful and valid parameter for quantifying stomatal responses to CO_2.

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