The cellular basis of guard cell sensing of rising \( \text{CO}_2 \)

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ABSTRACT

Numerous studies conducted on both whole plants and isolated epidermes have documented stomatal sensitivity to \( \text{CO}_2 \). In general, \( \text{CO}_2 \) concentrations below ambient stimulate stomatal opening, or an inhibition of stomatal closure, while \( \text{CO}_2 \) concentrations above ambient have the opposite effect. The rise in atmospheric \( \text{CO}_2 \) concentrations which has occurred since the industrial revolution, and which is predicted to continue, will therefore alter rates of transpirational water loss and \( \text{CO}_2 \) uptake in terrestrial plants. An understanding of the cellular basis for guard cell \( \text{CO}_2 \) sensing could allow us to better predict, and perhaps ultimately to manipulate, such vegetation responses to climate change. However, the mechanisms by which guard cells sense and respond to the \( \text{CO}_2 \) signal remain unknown. It has been hypothesized that cytosolic pH and malate levels, cytosolic \( \text{Ca}^{2+} \) levels, chloroplastic zeaxanthin levels, or plasma-membrane anion channel regulation by apoplastic malate are involved in guard cell perception and response to \( \text{CO}_2 \). In this review, these hypotheses are discussed, and the evidence for guard cell acclimation to prevailing \( \text{CO}_2 \) concentrations is also considered.

Key-words: \( \text{Ca}^{2+} \), \( \text{CO}_2 \), guard cell, malate, photosynthesis, stomata, transpiration, zeaxanthin.

INTRODUCTION

It is well known that when ambient \( \text{CO}_2 \) concentrations are experimentally elevated, stomatal closure results. A doubling of ambient \( \text{CO}_2 \) levels, as is expected to occur within the next century, has been predicted to reduce stomatal conductance by as much as 40% (Morison 1983). Given the potentially large impact of this conductance reduction on plant water status and atmospheric conditions, it would be of interest to elucidate the physiological basis of the \( \text{CO}_2 \) sensing and response mechanism of guard cells. Further, it could be important to ascertain whether an elevated \( \text{CO}_2 \) environment that prevails over the lifetime of a plant has the capacity to modulate the \( \text{CO}_2 \) response, namely, whether guard cells acclimate to the prevailing \( \text{CO}_2 \) concentration. This short review will summarize current knowledge regarding these two topics. Since several authors have comprehensively reviewed the literature on the stomatal response to \( \text{CO}_2 \) with emphasis on its manifestation in the whole plant (Morison 1983, 1985, 1998; Mott 1990; Eamus 1991; Drake, González-Meler & Long 1997), I focus here on the cell biology of the guard cell response (see also Mansfield, Hetherington & Atkinson 1990).

HOW DO GUARD CELLS SENSE \( \text{CO}_2 \)?

Where is the \( \text{CO}_2 \) sensor located?

It was conclusively demonstrated by Mott (1988) that stomata sense intercellular \( \text{CO}_2 \) concentrations rather than \( \text{CO}_2 \) concentrations at the leaf surface or in the stomatal pore. He used a gas exchange system with two independently controlled sides to produce changes in the intercellular \( \text{CO}_2 \) concentration (\( c_i \)) in the amphistomato-cous leaves of \( \text{Xanthium strumarium} \) and \( \text{Helianthus annuus} \) while keeping \( \text{CO}_2 \) concentration at one surface of the leaf constant, and vice-versa. Stomatal conductance consistently tracked \( c_i \) regardless of surface \( \text{CO}_2 \) concentrations or concentrations within the stomatal pore. This study conclusively demonstrates that \( c_i \) is the relevant signal but it does not address whether this signal is sensed directly by the guard cells or whether it is sensed by another cell population (mesophyll or epidermal cells) which then relays a signal to the guard cells. Actually it was demonstrated early on in studies of stomatal responses in epidermal strips removed from the mesophyll environment, that a sensor for the stomatal response to \( \text{CO}_2 \) is contained within the epidermis. Under these conditions, guard cells are still able to respond to a decrease in \( \text{CO}_2 \) with an increase in stomatal apertures (e.g. Travis & Mansfield 1979a,b; Schwartz, Ilan & Grantz 1988). With the advent of techniques by which guard cell protoplasts could be produced, it became possible to test for a \( \text{CO}_2 \) response in the guard cell itself. In the first study employing this approach, quite high (1000 mm\(^3\) dm\(^{-3}\)) \( \text{CO}_2 \) concentrations were reported to cause shrinkage of \( \text{Vicia faba} \) protoplasts (Gotow, Kondo & Syono 1982). Fitzsimmons & Weyers (1986), using guard cell protoplasts of the other species favoured by stomatal physiologists, \( \text{Cnemidophyllum communis} \), found that protoplast swelling in response to light was significantly enhanced by aeration of the incubation medium with \( \text{CO}_2 \) free air. Their results clearly demonstrate that there is a \( \text{CO}_2 \) perception and response mechanism that is intrinsic to the guard cell itself. However, the cellular basis of this mechanism remains in dispute over a decade later. Some early hypotheses regarding the mechanism of the \( \text{CO}_2 \)
response have not been disproven, and in addition there are at least three recent new proposals. These topics are discussed below.

Laying the groundwork

Some of the most informative early studies on the guard cell CO₂ response were concerned with quantifying this response in various species and determining whether CO₂-induced changes in stomatal aperture could be correlated with changes in guard cell K⁺, Cl⁻ and malate content (alterations in osmotic potential drive the guard cell volume changes that in turn regulate stomatal apertures). Travis & Mansfield (1979a) used enzymatic techniques to measure malate content of C. communis epidermal peels following incubation of the tissue in medium bubbled with either 350 mm dm⁻³ CO₂ or CO₂-free air. They found that the larger apertures produced by the CO₂-free treatment were accompanied by an increase in malate content, and that an increase in stomatal aperture upon exposure to CO₂-free air was not observed when KCl was omitted from the incubation medium. The maximal CO₂ effect was observed at an external KCl concentration of 50 mm.

The above results implicate malate, K⁺, and Cl⁻ in the regulation of stomatal aperture in response to CO₂, but do not address whether these ions are the sole osmotica involved. MacRobbie’s work indicates that solutes in addition to K⁺, Cl⁻, and malate also contribute to osmotic build-up during some conditions or phases of stomatal opening (summarized in MacRobbie 1983). Following on these studies, Tallman, Zeiger and colleagues have used high-performance liquid chromatography techniques to document a varying contribution of inorganic ions (especially K⁺) versus organic ions (especially sucrose) to guard cell osmotic content under different illumination conditions (Tallman & Zeiger 1988; Poffenroth, Green & Tallman 1992; Talbott & Zeiger 1993, 1996). It would be informative to now apply these techniques to address the contribution that non-ionic organic solutes play to osmotic changes induced by CO₂. This would help to appropriately focus attention on the downstream targets of the CO₂ response.

While the signal transduction pathways by which organic solute production is modulated in response to environmental stimuli remain largely unknown, electrophysiological studies have contributed substantial information regarding the specific ion transporters that mediate transmembrane ion fluxes during stomatal responses (Assmann 1993; Kearns & Assmann 1993; Ward, Pei & Schroeder 1995). During stomatal opening, K⁺ uptake occurs through inwardly rectifying K⁺ channels. These channels are activated by hyperpolarized (‘more negative’) membrane potentials, and hyperpolarization also provides an electrical driving force for K⁺ uptake. H⁺ extrusion by an H⁺ ATPase, as is known to occur in response to stomatal opening signals such as light and fusicoccin, contributes to this hyperpolarization. Varying degrees of malate production and Cl⁻ uptake accompany K⁺ uptake depending on the plant species and the environmental conditions. Cl⁻ uptake is against the electrochemical gradient for this ion, and is speculated to occur by H⁺/Cl⁻ cotransport or OH⁻/Cl⁻ antiport.

In order to effect stomatal closure, the membrane potential must be depolarized (‘made less negative’). Depolarization both provides the driving force for K⁺ efflux and activates outwardly rectifying K⁺ channels that serve as the conduits for this efflux. Depolarization can be achieved by inhibition of the H⁺ ATPase, by anion efflux, and to some extent by Ca²⁺ influx. Both Cl⁻ and malate (van Kirk & Raschke 1978) are lost from guard cells upon stomatal closure (malate can also be metabolized or back-converted to starch), and two types of anion channels, which are permeable to both Cl⁻ and malate (Hedrich & Marten 1993; Schmidt & Schroeder 1994) appear to be involved in this process. One is the R-type or ‘rapid’ anion channels which activate and deactivate quickly following voltage-activation. The second is the S-type or ‘slow’ anion channels which deactivate very slowly following activation. The S-type channels are not highly voltage regulated, but are strongly activated by elevation of cytosolic Ca²⁺ levels. The R-type channels show strong voltage regulation, and are also Ca²⁺-stimulated.

A recent electrophysiological study has shown that CO₂ indeed regulates ion channels in the predicted manner, suppressing inward K⁺ currents, enhancing outward K⁺ currents, and stimulating anion currents (Brealey, Venis & Blatt 1997). Now the key issue is to identify the underlying signal transduction cascades.

A role for pH?

Although the detailed picture of ion transport mechanisms that has been provided by electro-physiological studies was not available even a decade ago, already at that time researchers recognized that ion transporters might be key targets for modulation by CO₂ (Mansfield, Travis & Jarvis 1981). Edwards & Bowling (1985) performed an electrophysiological study which showed that elevated CO₂ levels depolarized the membrane potential of guard cells of Tradescantia virginiana, and suggested that the depolarization resulted from inhibition of the H⁺ ATPase, which would correlate well with CO₂-induced inhibition of stomatal opening. However, in these experiments the solution bathing the epidermal peels was exposed to pure CO₂ and it is unclear whether the response observed would have relevance to the documented ability of guard cells to sense changes in CO₂ concentration of the order of 100 mm⁻³. Blatt (1987) observed a significant depolarization of the membrane potential upon medium acidification from pH 7.5 to 4.5 and he proposed instead that CO₂-based changes in apoplastic pH might report the CO₂ concentration. However, Raschke (1972) pointed out that such a pH signal could scarcely result from CO₂ itself, since a change in dissolved CO₂ concentration from, for example, 300 to 100 mm⁻³ will alter the pH of water by only 0.2 pH units (Raschke 1972).
Raschke (1975; 1979) proposed that as CO\(_2\) concentrations increase, excess malate produced in the guard cell cytosol via carboxylation of PEP would result in a drop in the cytosolic pH. He hypothesized that both the decrease in pH and the accumulation of cytosolic malate would feed back to inhibit PEP carboxylase, and that cytosolic malate accumulation could also regulate ion transport mechanisms at the plasma membrane and tonoplast (Raschke 1979). There are some data (Talbott & Zeigler 1996) suggesting that stomatal opening in response to lowered CO\(_2\) exhibits flexibility in the use of organic versus inorganic solutes depending on other environmental conditions; an observation which might mediate against a CO\(_2\)-control mechanism tightly linked to levels of one particular metabolite, such as malate. On the other hand, the plant cell has available other mechanisms of cytosolic pH regulation as well, for example, via modulation of H\(^+\) ATPase activity. One might question whether cytosolic pH would be so tightly regulated by the cell that it could not serve as a signal transducer, but this appears not to be the case: for example, the abscisic acid (ABA) signal appears to be transduced in part by changes in cytosolic pH (Blatt & Armstrong 1993). Electrophysiological studies have shown that a decrease in intracellular pH, as predicted by the malate model of CO\(_2\) sensing, inhibits the channels that mediate K\(^+\) efflux during stomatal closure (Blatt & Armstrong 1993; Miedema & Assmann 1996) and enhances the currents associated with K\(^+\) influx (Blatt 1992; Grabov & Blatt 1997). These effects would tend to oppose, rather than promote, stomatal closure. Nevertheless, if a drop in cytosolic pH were to strongly activate the anion channels this might drive stomatal closure despite effects on K\(^+\) channels. Research on the pH dependence of R-type and S-type anion channels is required to address this possibility. In addition, now that fluorescent indicator dyes are available to monitor cytosolic pH in living cells, it is feasible to directly test whether or not increasing CO\(_2\) levels results in acidification of the guard cell cytosol. One photometric study (Brearley et al. 1994) showed no effect of 1,000 mm\(^3\) dm\(^{-3}\) CO\(_2\) on overall cytosolic pH but the possibility remains that localized pH changes could still be involved in ion channel regulation in response to CO\(_2\).

**R-type anion channels: sensors/effectors of the CO\(_2\) response?**

Hedrich and colleagues have proposed that CO\(_2\)-stimulated photosynthetically driven changes in apoplastic malate content activate guard cell anion channels, resulting in anion loss (Hedrich & Marten 1993; Hedrich et al. 1994). As mentioned previously, anion loss from guard cells both decreases cellular osmotic content, and mediates membrane depolarization which will drive K\(^+\) efflux, thus promoting water loss and effecting stomatal closure. This hypothesized mechanism of CO\(_2\) sensing is based on two observations. First, Hedrich and coworkers reported a rapid doubling of apoplastic malate levels in *Vicia faba* leaves following an increase in ambient CO\(_2\) concentrations from 362 mm\(^3\) dm\(^{-3}\) to 10,000 mm\(^3\) dm\(^{-3}\) (Hedrich et al. 1994). While such a drastic elevation in CO\(_2\) levels is unlikely to have physiological or environmental relevance, the authors also mention that an increase in CO\(_2\) concentration from 362 to 672 mm\(^3\) dm\(^{-3}\) resulted in a comparable (although quite variable and possibly non-significant) increase in malate concentration, from 1.00 ± 0.60 mm to 3.10 ± 2.30 mm. Second, their patch clamp experiments revealed that comparable experimentally imposed increases in extracellular malate levels shifted the activation of R-type anion channels to more negative membrane potentials, that is, shifted the voltage-gating of this channel such that the channels would tend to be open and thus mediate anion loss at prevailing membrane potentials (Hedrich & Marten 1993; Hedrich et al. 1994). Single channel recordings demonstrated that this shift in voltage-gating could be attributed to an increase in the mean open time of the channels at the more negative voltages, rather than to a change in the conductance of individual channels. R-type anion channels are permeable to both Cl\(^-\) and malate (as well as to other anionic species) and anion efflux could be expected to contribute to local increases in the apoplastic concentrations of these two ions. An increase in apoplastic malate would have a positive feedback effect on the voltage response of the channel. Experimental increase of extracellular Cl\(^-\) concentrations was observed by these researchers to increase the single channel conductance, and this would also positively feed back on anion loss.

The malate hypothesis of Hedrich and colleagues is appealing in its simplicity: one molecule, the R-type anion channel, is both the sensor and the effector. However, several questions remain to be addressed concerning this hypothesis. First, do the bulk leaf extracellular malate levels reported by these researchers accurately reflect the local apoplastic malate concentrations surrounding guard cells? Second, we know that the R-type anion channels mediate anion efflux over a limited voltage range, e.g. −120 to +15 mV in the absence of applied malate and −180 to −15 mV in the presence of 82 mm malate, as reported in one set of experiments (Hedrich & Marten 1993). As the membrane potential passes through this range of voltages and then perhaps becomes sufficiently depolarized for the R-type channels to again become quiescent, will sufficient anion loss have occurred to account for the extent of stomatal closure observed under elevated CO\(_2\) conditions? A direct way to address this issue would be to measure stomatal apertures in epidermal peels exposed not to different concentrations of CO\(_2\) but instead to different concentrations of malate. However, when such experiments were performed by two different groups widely different results were obtained. Hedrich et al. (1994) reported that application of 3 mm K\(_2\) malate to epidermal peels of *V. faba* was sufficient to effect stomatal closure, and calculated a K\(_m\) of 0.3 mm malate for this response, but Schroeder and colleagues found comparable effects only at malate concentrations of 40 mm and above (Esser Liao & Schroeder 1997). Esser et al. also found that malate did not begin to
inhibit stomatal opening until concentrations exceeded 10 mM. Thus, the physiological relevance of the malate effect on R-type anion channels, and its role in the guard cell response to CO₂ currently remain in question.

The guard cell chloroplast as a CO₂ sensor?

Zeiger and colleagues have stressed a possible role of the guard cell chloroplast in CO₂ sensing (Melis & Zeiger 1982; Assmann & Zeiger 1985). Most recently, they have proposed a model involving the carotenoid zeaxanthin. In this model, changes in CO₂ level lead, via CO₂ fixation, to changes in the ATP and NADPH status of the guard cell chloroplast, accompanied by an increase in the pH of the chloroplast lumen. Because of the pH sensitivity of the enzymes involved in zeaxanthin formation, this would translate into a decrease in zeaxanthin levels. The Zeiger group has shown that zeaxanthin content in the guard cell chloroplasts is altered by ambient CO₂ levels, with zeaxanthin content decreasing when either intact leaves or epidermal peels are exposed to elevated CO₂ concentrations (Zhu, Talbott & Zeiger 1998). These researchers propose that in the dark, where guard cell CO₂ sensing also operates, another, as yet unidentified, sensor is involved. Zeiger and colleagues have promoted zeaxanthin as the blue light photoreceptor that mediates blue-light-specific stomatal opening (Srivastava & Zeiger 1995a,b), and they argue that having the same molecule function as a CO₂ sensor could integrate light and CO₂ sensing.

Currently unanswered questions regarding the possible role of zeaxanthin in the CO₂ response are whether the levels of this pigment are rate-limiting for the response, as is inherent in the model of Zeiger and colleagues, and whether the rate at which zeaxanthin levels change is rapid enough to account for the rate of stomatal aperture change in response to CO₂. The latter question could be readily addressed by extant techniques. One prediction of the model would be that plants with high levels of zeaxanthin would have larger stomatal apertures. This in fact is seen in the aba mutants (Webb & Hetherington 1997), which are blocked in their ability to convert zeaxanthin to antheraxanthin, although the large apertures have been attributed to decreased levels of endogenous ABA. Since these mutants are blocked in zeaxanthin conversion, one might predict that they would be less sensitive to a decrease in CO₂ levels. To my knowledge, this prediction has yet to be tested. It would also be of interest to assess the CO₂ responsiveness of guard cells from the recently identified npq1 (non-photochemical quenching) mutant of Arabidopsis, which cannot deepoxidize violaxanthin to zeaxanthin (Niyogi, Grossman & Björkman 1998).

If zeaxanthin were to transduce the CO₂ signal, the information contained in zeaxanthin content would somehow have to be conveyed from the guard cell chloroplast to the rest of the cell, in order to effect changes in cytosolic and vacuolar osmotic content. Secondary messengers that have been proposed for the light responses of guard cells, which under this model might be also co-opted for the CO₂ response, are diacylglycerol, an unknown photosynthetic metabolite, and Ca⁡²⁺ (Zhu et al. 1998; Zeiger & Zhu 1998).

Ca²⁺ as a CO₂ signalling component?

A possible role of Ca²⁺ in the CO₂ response has been promulgated since 1988, when Schwartz and coworkers showed that perfusion of epidermal peels of C. communis with solution containing the Ca⁡²⁺-chelator EGTA resulted in a reduced CO₂ response. More recently, the group associated with Hetherington (Webb et al. 1996a) performed photometric measurements of cytosolic free Ca²⁺ levels ([Ca²⁺]cyt) in guard cells of C. communis epidermal peels perfused with solutions aerated with either CO₂-free air or 700 mm³ dm⁻³ CO₂. Seventy-three per cent of the cells (n = 33 total) exhibited an immediate increase in [Ca²⁺]cyt upon exposure to the high CO₂ concentration. In all except three of the cells, the increase in [Ca²⁺]cyt was sustained until return to the CO₂-free medium. Since we know that guard cells can sense small changes in CO₂ concentration, it will be important to repeat these experiments using steps in CO₂ concentration on the order of 100 mm³ dm⁻³, to determine whether or not these smaller steps still generate a change in [Ca²⁺]cyt of sufficient magnitude to regulate cellular processes.

A Ca²⁺-based signal transduction chain for the CO₂ response is an appealing hypothesis in that experimental manipulation of [Ca²⁺]cyt has already been shown to affect most of the major ion transport pathways of guard cells in a manner consistent with the observed effects of CO₂ on ionic currents (Brearley et al. 1997; Ward et al. 1995; Webb et al. 1996b). Thus, Ca²⁺ inhibits the inwardly rectifying K⁺ channels that mediate K⁺ uptake during stomatal opening, activates both R-type and S-type anion channels at the plasma membrane which mediate anion loss, and inhibits the plasma membrane H⁺ ATPase which functions during stomatal opening. Channels mediating K⁺ and anion fluxes across the tonoplast are also Ca²⁺-regulated (Ward et al. 1995; Pei et al. 1996).

Ca²⁺ has been implicated in all of the guard cell responses where a role for it has been tested to date, including responses to ABA, auxin, CO₂, and oxidative stress (Webb et al. 1996b). Given the participation of Ca²⁺ in many signal transduction pathways, the question arises as to how the specificity of the response is achieved, particularly since Webb et al. report that the CO₂-induced increases in [Ca²⁺]cyt are similar to those evoked by ABA. In fact, Webb & Hetherington (1997) suggest that such specificity may not actually exist beyond the level of CO₂ and ABA sensing, namely, beyond the early events upstream of [Ca²⁺]cyt; they conclude that the same effector mechanism may function in both ABA and CO₂-induced stomatal closure. This conclusion is based on stomatal aperture measurements showing that the ABA-insensitive mutant abi1 has a reduced stomatal response to 700 mm³ dm⁻³ CO₂, and the abi2 mutant shows no difference at all in stomatal aper-
ture in zero versus 700 mm$^3$ dm$^{-3}$ aerated solutions. The mutants were also impaired in their ability to respond to Ca$^{2+}$ in the incubation medium, which induces stomatal closure in epidermal peels taken from wild-type plants. These authors thereby conclude that there is a convergence of the ABA and CO$_2$ signalling pathways on Ca$^{2+}$, ABI1, and ABI2. Since both ABI1 and ABI2 encode PP2C-type protein phosphatases (reviewed in Giraudat 1995), they invoke de/phosphorylation as a component of the signal transduction pathway.

The interpretation of these data is, however, challenged by more recent results. Leymarie, Vavasseur & Lascève (1998) found that, both in measurements of stomatal conductance in whole plants and in epidermal peel assays, abi1 and abi2 plants responded to CO$_2$, although the responses were reduced in magnitude. These data therefore suggest interaction between, but not convergence of, the ABA and CO$_2$ signalling pathways. About two decades ago Raschke and coworkers demonstrated that ABA enhances the CO$_2$ response (Raschke, Pierce & Popiela 1976; Dubbe, Farquhar & Raschke 1978). If the presence of this hormone, and, necessarily, the ability to sense it, enhances the guard cell response to CO$_2$, then it is possible that the CO$_2$ and ABA responses are mediated by separate but interacting pathways, such that it simply becomes difficult to detect the CO$_2$ response in the absence of an ABA response. Leymarie et al. (1998) also provide an interesting specific hypothesis as to why Webb and Hetherington did not see the CO$_2$ response under their experimental conditions: those conditions employed relatively high (50 mM) KCl concentrations in the incubation medium, and early work with epidermal peels of V. faba showed that high KCl concentrations suppress the CO$_2$ response, with the sensitivity restored if an impermeant anion, iminodiacetic acid, is substituted for Cl$^-$ (Wardle & Short 1981). Mechanistically, these results can be understood if a high external Cl$^-$ concentration reduces the electrochemical gradient for Cl$^-$ loss through the anion channels, thereby also suppressing the membrane depolarization required for stomatal closure.

To summarize, four mediators of CO$_2$ action have been proposed: a malate-induced change in cytosolic pH; a change in the activity of R-type anion channels caused by apoplastic malate; a change in chloroplastic zeaxanthin levels; and a change in cytosolic free Ca$^{2+}$ concentrations. Since none of these responses appears mutually exclusive, it remains a possibility that they all contribute to the CO$_2$ response, or at least to the CO$_2$ response that occurs under illumination. Under darkness, there cannot be a photosynthetically driven change in apoplastic malate levels, and if zeaxanthin must be excited to serve as a signal-transducing molecule, then it also could not function as a CO$_2$ sensor in the absence of illumination. It might therefore be particularly informative to study whether changes in [Ca$^{2+}$]$_{cyt}$ are still induced by CO$_2$ when the guard cell is left in darkness (other than for the brief illumination necessary to measure [Ca$^{2+}$]$_{cyt}$). The [Ca$^{2+}$]$_{cyt}$ measurements of Webb et al. (1996a) were performed under high intensity illumination (1·0 mmol m$^{-2}$ s$^{-1}$ PAR), so that study may have no bearing on the stomatal response to CO$_2$ under darkness.

**How many CO$_2$ responses?**

Perusal of the literature leads to the conclusion that even the number of CO$_2$-sensing mechanisms employed by guard cells has not been defined. First, I have already mentioned the possibility that there may be two fundamentally different CO$_2$-sensing mechanisms that operate under light versus darkness.

Second, Jarvis & Davies (1998) have determined that stomatal responses to CO$_2$ recorded in published data sets of whole plant responses could be explained by a model in which stomatal conductance correlates with the pool size of a carbon compound that varies inversely with mesophyll photosynthetic rate. This hypothesis is based on modelling and needs to be addressed with biochemical analyses. If verified, the inescapable conclusion will be that guard cells have two independent mechanisms to sense CO$_2$, because the Jarvis and Davies model is certainly not sufficient to account for the documented sensitivity of guard cells to CO$_2$ in isolated epidermal strips or as protoplasts.

Third, even in isolated guard cells under constant illumination there may be two different CO$_2$ responses that operate. In the early literature, several researchers reported guard cell swelling or increase in stomatal aperture when protoplasts or peels were taken from CO$_2$-free to low (below ambient) CO$_2$ concentrations, with closure or shrinkage observed when still higher CO$_2$ concentrations were imposed. Thus, Gotow et al. (1982) actually saw swelling of guard cell protoplasts when the incubation medium was aerated with CO$_2$ concentrations between zero and 300 mm$^3$ dm$^{-3}$, although this response was variable. RagHAVendra and colleagues (Mrinalini et al. 1982) reported that low amounts of bicarbonate (up to 10 μM in the absence of fusicoccin and 25 μM in the presence of fusicoccin) stimulated stomatal opening in epidermal peels of Commelina benghalensis, while higher concentrations inhibited light-induced stomatal opening. Travis & Mansfield (1979b) also found that the expected CO$_2$ response of C. communis stomata was reversed in the presence of fusicoccin such that a change from zero to 350 mm$^3$ dm$^{-3}$ CO$_2$ elicited stomatal opening. We know that guard cells use CO$_2$ as a substrate for malate synthesis via PEP carboxylase, and it appears that guard cells also have a limited capacity for photosynthetic CO$_2$ fixation (reviewed in Assmann 1993). Do the results described above then simply reflect the fact that CO$_2$ is incorporated into organic solutes that drive the water potential of the guard cell to more negative values, favouring stomatal opening, or is a more complicated signal transduction pathway being activated under these circumstances? Whatever the mechanism of this ‘wrong-way’ CO$_2$ response, it is apparently the case that at still higher CO$_2$ concentrations a second CO$_2$ response is activated, resulting in ion loss and water efflux that overwhelms the wrong-way effect (if indeed the wrong-way response remains active at the
higher CO2 concentrations), leading to the classic stomatal closure event.

**IS THERE STOMATAL ACCLIMATION TO CO2?**

One of the best ways to gain information on a system is to perturb it, and to monitor the consequences. This, of course, is routinely done when short-term changes in CO2 concentration are imposed and stomatal responses are assayed. However, one may also ask whether longer-term changes in CO2 concentration result in longer-term changes in guard cell response; that is, do guard cells acclimate to the prevailing CO2 environment? While there have been numerous studies concerning the acclimation of the photosynthetic mesophyll to elevated CO2, with consequent effects on c1 and stomatal conductance, relatively few reports have addressed the question of whether the guard cell itself acclimates to CO2. This section will summarize data on this subject, focusing on acclimation at the level of the single cell. For information regarding CO2-induced acclimation in stomatal frequency over the life-span of the individual plant or over a paleobotanical time scale, the reader is referred to other reports (Drake et al. 1997; Poole et al. 1996; Woodward & Kelly 1995).

Perhaps the first experimenters to address the issue of guard cell acclimation to different CO2 environments were Jones & Mansfield (1970). These researchers grew lettuce (Lactuca sativa) and Xanthium pennsylvanicum under ambient and enriched CO2 conditions, and then used porometer measurements to assess stomatal responses over a range of CO2 concentrations. No differences in stomatal response were noted between the two growth treatments.

Both in studies where the intent is to study stomatal response, and in studies where the focus is on photosynthetic acclimation, it is customary to measure assimilation (A) as a function of c1, producing so-called ‘A–c1’ curves. The c1 is altered by manipulating the ambient CO2 concentration (c2). Taking advantage of the ready availability of such information, Sage (1994) evaluated stomatal acclimation in plants grown under different CO2 conditions by assessing c1/c2 ratios from 11 studies on 10 different C3 species grown under ambient (330 mm3 dm–3) or elevated (1000 mm3 dm–3) CO2 conditions. Overall, c1/c2 values of plants from the two growth treatments were similar, regardless of whether measurements were made at elevated or ambient CO2 conditions; indicating a lack of acclimation. However, exceptions to this generalization have also been found in studies on miscellaneous species. For example, the same author, in collaboration with Santrucek (1996) found that Chenopodium album plants grown at 750 mm3 dm–3 CO2 had a higher c1/c2 ratio at CO2 levels between 300 and 1200 mm3 dm–3 than plants grown at 350 mm3 dm–3, suggesting that growth under elevated CO2 produced a decrease in stomatal sensitivity to CO2.

Hollinger (1987) assessed the photosynthetic and stomatal responses of three tree species: Pinus radiata, Nothofagus fusca, and Pseudotsuga menziesii. Seedlings were grown under 340 or 640 mm3 dm–3 CO2 and measurements were made under both ambient and elevated CO2 conditions. A hint of acclimation was found in P. radiata: seedlings grown under ambient conditions exhibited similar stomatal conductances when measured at 310 and 620 mm3 dm–3, while seedlings grown under enriched CO2 conditions showed the more typical response of a decrease in stomatal conductance under elevated CO2. In this case, enriched CO2 growth conditions apparently had an effect that was opposite to that found in Chenopodium album. More studies of this nature are required to ascertain whether CO2 acclimation correlates with taxonomy or growth habit.

For the short-term guard cell response to CO2 it is known that the response can be enhanced by the presence of stresses such as low humidity (Bunce 1998) or stress signals such as ABA (Dubbe et al. 1978; Raschke et al. 1976). Similarly, an acclimatory response of guard cells to CO2 may only be revealed under certain environmental conditions. Rigorous control of environmental parameters will be needed to ferret out such interactions. One point that is already clear from the literature is that not all stresses will result in such enhancement. Thus, growth of Phaseolus vulgaris plants under low nutrient conditions did not differentially affect the stomatal CO2 sensitivity of 350 mm3 dm–3 versus 700 mm3 dm–3 CO2-grown plants (Radoglou, Ahalo & Jarvis 1992).

To summarize, while many studies seem to indicate a lack of guard cell acclimation to the CO2 environment prevailing during the growth of the plant, there are enough exceptions to make this a topic worthy of further study. The great majority of the studies described above were conducted in the intact plant. Elsewhere in this issue, Jarvis, Mansfield & Davies (1999) rightly emphasize that a rigorous analysis of whole plant photosynthetic acclimation is necessary in order to correctly interpret changes in stomatal behaviour observed when plants are subjected to different CO2 regimes. While such an analysis of whole plant responses obviously reflects the most physiological situation, the results of such experiments are more difficult to interpret than those of experiments conducted using a simpler system, such as the isolated epidermal peel. For example, if there is a mesophyll messenger (Wong, Cowan & Farquhar 1979; Jarvis & Davies 1998) in addition to c1 itself that regulates stomatal apertures (perhaps apoplastic malate?), then the c1/c2 relationships may not suffice to reveal direct guard cell acclimation to CO2.

A more informative approach with regard to the intrinsic guard cell response and its ability to acclimate may be to subject intact plants to different CO2 conditions during growth, but then to assay the responses of guard cells isolated either in peels or as protoplasts, and to ask whether such cells inherently differ in their CO2 responses. Such an approach has been developed by Zeiger and coworkers. They observed that guard cells from growth-chamber-grown plants of V. faba were more sensitive to manipulations in CO2 concentration than were those from greenhouse-grown plants (Talbott, Srivastava & Zeiger 1996). The differential sensitivity was observed either when epidermal peels were directly isolated from leaves of
plants subjected to short-term (2 h) CO₂ exposures, or, importantly, when epidermal peels were isolated and then subjected to the different CO₂ conditions (L. Talbott & E. Zeiger, personal communication). The latter experiment is important because it indicates that there is an altered CO₂ response in the epidermal peel (presumably in the guard cells), separate from any response that may be mediated via the mesophyll. When plants were transferred from one growth condition to the other, the guard cells gradually took on the CO₂ responsiveness characteristic of their new environment. In plants transferred to growth-chamber conditions, CO₂ sensitivity increased in 7–8 d, whereas in the plants transferred to the greenhouse, CO₂ sensitivity diminished rapidly, and was complete in 2–3 d. Zeiger and colleagues found that zeaxanthin levels also change in the predicted manner as plants acclimate from one growth condition to the other (L. Talbott and E. Zeiger, personal communication). It would be of great interest to study these plants relative to the other cellular responses described above, to ascertain whether or not there is a correlation between CO₂ sensitivity and levels of anion channel activity, and/or \([\text{Ca}^{2+}]_\text{cyt}\) and/or cytosolic H⁺ and malate concentrations. Interestingly, Talbott & Zeiger (1996) found that the guard cells from the more sensitive growth-chamber-grown plants utilized malate as an osmoticum to a greater extent that those from the less sensitive greenhouse-grown plants. Could this provide the basis of the enhanced CO₂ sensitivity of the growth-chamber-grown plants, in accordance with Raschke’s hypothesis?

The key variable differing between the greenhouse and growth-chamber environments might have been CO₂ itself which ranged over the same values in the two environments, but showed more pronounced fluctuations in the growth chambers, owing to the smaller total volume and the proportionally larger canopy (E. Zeiger, personal communication). Other variables that also differed between the two environments were the relative humidity, which was lower for the greenhouse-grown plants (40–50% versus 85% (E. Zeiger, personal communication)), and the light environment, which consisted of natural illumination in the greenhouse-grown plants, and 12 h of 0.65 mmol m⁻² s⁻¹ combined incandescent and fluorescent light under the growth-chamber conditions. Boundary layer conditions are also likely to have varied greatly between the two environments. Specific experiments must now be conducted under well-controlled conditions to identify the relevant variable. It may prove to be the case that it was the interaction between CO₂ and other environmental variables, rather than the CO₂ response in and of itself, that was influenced by the growth environment in these experiments. Such an effect would be illustrative of the true complexities of the CO₂ sensing and response system.

CONCLUSIONS

In conclusion, while the CO₂-sensing pathway(s) of guard cells remain poorly understood, there are now enough data that additional experiments, a few of which have been suggested above, could provide useful information concerning the involvement of cytosolic pH and malate, zeaxanthin, \(\text{Ca}^{2+}\), or anion channels in the short term response to CO₂. Parallel experiments on guard cells isolated from plants raised under ambient versus CO₂-enriched conditions could be performed to elucidate the roles of these parameters in stomatal acclimation to CO₂, in species where this phenomenon occurs. Differences in the CO₂ sensitivity of abaxial versus adaxial stomata have been reported (summarized in Morison 1998), and an evaluation of the responses of these two guard cell types relative to the four proposed signalling mechanisms could also prove illuminating.

It is also possible that not all plant species have the same CO₂-sensing mechanisms. Species comparisons will certainly be crucial for predicting the impact of vegetation response to rising CO₂ on crop productivity and climate. When conducting experiments on the guard cell response to CO₂, it will be important to manipulate other environmental variables, such as water availability, humidity, and illumination conditions, which impact the sensitivity of the CO₂ response, and to be cognizant that physiological parameters, including not only ABA but also other hormones such as kinetin (Wardle & Short 1981) and auxins (Davies & Mansfield 1983) also affect this response (see summary in Morison 1985). Genetic analysis of mutant phenotypes, as is being initiated with the \(\text{abi}\) mutants, could assist greatly in revealing these complexities. In fact, perhaps the guard cell response to CO₂ will be ultimately elucidated through its interactions with other signalling pathways.

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