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Review

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# Guard cell metabolism and $CO_2$ sensing

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## Contents



**Key words:** abscisic acid (ABA), Calvin cycle, $CO_2$ sensing, guard cell, ion channels, metabolism, signal transduction, stomatal movement.

## Summary

In this review we concentrate on guard cell metabolism and $CO_2$ sensing. Although a matter of some controversy, it is generally accepted that the Calvin cycle plays a minor role in stomatal movements. Recent data emphasise the importance of guard cell starch degradation and of carbon import from the guard cell apoplast in promoting and maintaining stomatal opening. Chloroplast maltose and glucose transporters appear to be crucial to the export of carbon from both guard and mesophyll cells. The way guard cells sense $CO_2$ remains an unresolved question. However, a better understanding of the cellular events downstream from $CO_2$ sensing is emerging. We now recognise that there are common as well as unique steps in abscisic acid (ABA) and $CO_2$ signalling pathways. For example, while ABA and $CO_2$ both trigger increases in cytoplasmic free calcium, unlike ABA, $CO_2$ does not promote a cytoplasmic pH change. Future advances in this area are likely to result from the increased use of techniques and resources, such as, reverse genetics, novel mutants, confocal imaging, and microarray analyses of the guard cell transcriptome.

## Abbreviations

$[Ca^{2+}]_{cyt}$, cytosolic free calcium concentration; ABA, abscisic acid; GCPs, guard cell protoplasts; MCPs, mesophyll cell protoplasts; PCRP, photosynthetic carbon reduction pathway; PEPC, phospho*enol*pyruvate carboxylase; RBCs, the small subunit of ribulose-1,5 bisphosphate carboxylase oxygenase; Rubisco, ribulose-1,5-*bis*phosphate carboxylase/oxygenase.

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## I. Introduction

In higher plants, water loss and $CO_2$ uptake are tightly regulated by stomata on the leaf epidermis. Rapid osmolyte accumulation (or loss) and consequent increase (or decrease) in the turgor of guard cells determine the extent of stomatal aperture. Under continuously changing environmental conditions stomata optimise gas exchange between the interior of the plant and the atmosphere. Guard cells possess complex signal transduction networks and modified metabolic pathways. These features allow rapid modulations in guard cell turgor and stomatal conductance, in response to internal and environmental signals (light intensity and quality, humidity, carbon dioxide partial pressure, water availability, hydric and developmental state). A general description of the basic features of stomatal function and regulation can be found in Willmer & Fricker (1996).

In this review, we will first focus on guard cell metabolism. Guard cells are highly specialised for solute accumulation and are well equipped to generate the energy required for the uptake of ions ($K^+$, $Cl^-$), synthesis of anions (particularly malate$^{2-}$) and accumulation of osmotically active sugars (mainly sucrose). It would appear that guard cell metabolism is modified to meet these needs rather than accomplishing the typical tasks of photosynthetic carbon fixation. The relative contribution of the three processes: photosynthetic carbon reduction pathway (PCRP), the PEPC pathway and carbohydrate import, in guard cell osmo-regulation is still a matter of debate. Guard cell metabolism is highly plastic and is dependent on the energetic state of guard cells and environmental parameters (Outlaw, 2003).

The physiological role of stomata is to prevent water loss and to facilitate $CO_2$ diffusion to mesophyll cells. Much progress has been made in our understanding of the guard cell response to stimuli such as light and water stress (Assmann, 1993, 1999; MacRobbie, 1998; Blatt, 2000; Assmann & Wang, 2001; Hetherington, 2001; Schroeder *et al.*, 2001; Hetherington & Woodward, 2003). But very few studies have discussed the way guard cells sense $CO_2$. One possible reason for this is the technical difficulty of regulating and recording the $CO_2$ partial pressure during an experiment. Despite these limitations, significant progress has been made in the understanding of the guard cell $CO_2$ signalling pathway, since the last reviews in this area (Mansfield *et al.*, 1990; Assmann, 1999).

After presenting an overview of guard cell metabolism and $CO_2$ sensing, this review focuses on short-term responses to $CO_2$ of guard cells. The long-term effects of $CO_2$ on stomatal development have been recently reviewed elsewhere (Hetherington & Woodward 2003).

## II. Guard Cell Metabolism

In early studies of guard cell function, the importance of starch–sugar interconversion in regulating stomatal aperture received considerable attention. Later, the discovery of the large changes in guard cell potassium content during stomatal opening shifted the attention to the mechanisms underlying monovalent cation influx (Willmer & Fricker, 1996). During the last decade, new evidence has again pointed to the significance of carbohydrates, in addition to potassium and anions, during the build-up of the guard cell turgor. Thus, dual processes, involving synthesis and influx of osmotica, coexist in guard cell turgor modulation; however, at present, their respective contributions to the overall control of stomatal turgor is still a matter of debate.

### 1. Guard cell bioenergetics

In the green tissues of plants, chloroplasts and mitochondria are the two potential sources of energy, providing ATP and reducing power. Aspects of guard cell bioenergetice have been reviewed previously (Assmann & Zeiger, 1987; Raghavendra & Vani, 1989; Assmann 1993; Parvathi & Raghavendra, 1997; Willmer & Fricker, 1996). The prevailing view is that guard cells possess a high respiratory rate together with limited photosynthetic capability.

**1.1. Respiration and photosynthesis** As expected for cells having a high metabolic activity, guard cells contain numerous mitochondria (Willmer & Fricker, 1996). The abundance of mitochondria, along with high respiration rates, suggests that oxidative phosphorylation is an important source of ATP to fuel the guard cell machinery (Raghavendra & Vani, 1989; Parvathi & Raghavendra, 1997). The literature suggests that the guard cell mitochondria utilise both cytochrome and alternative pathways of oxidative electron transport (Mawson, 1993; Vani & Raghavendra, 1994).

By contrast to mitochondria, guard cells contain few chloroplasts (Willmer & Fricker, 1996), about one-third of the number present in mesophyll cells (Allaway & Setterfield, 1972). Further, the guard cell chloroplasts are smaller than those found in mesophyll cells (Fig. 1a,b) with limited thylakoid structures and a few granal stacks (Sack, 1987). Their chlorophyll content represents a small fraction of that in mesophyll cell chloroplasts (1–4% on a cellular basis, Zemel & Gepstein, 1985, Shimazaki *et al.*, 1983, Reckmann *et al.*, 1990, Gautier *et al.*, 1991, Birkenhead & Willmer, 1986). On a chlorophyll basis, guard cell cyclic and noncyclic photophosphorylations were estimated to be about 80% of those found in mesophyll cells (Shimazaki & Zeiger, 1985). In the light, the reducing power produced by electron transport in chloroplasts can feed the Calvin cycle (Schwartz & Zeiger, 1984; Shimazaki & Zeiger, 1985). Biochemical studies have detected the main Calvin cycle enzymes (Shimazaki & Zeiger, 1985; Zemel & Gepstein, 1985; Shimazaki *et al.*, 1989; Willmer & Fricker, 1996), but highlighted the very low level of Rubisco present in guard cells (Outlaw *et al.*, 1979; Vaughn, 1987; Reckman *et al.*, 1990; Gautier *et al.*, 1991; Kopka *et al.*, 1997).

High-resolution chlorophyll *a* fluorescence imaging suggests that the PCRP is active, albeit at low levels in guard cells (Lawson *et al.*, 2003). Even using the highest values of Rubisco reported

 

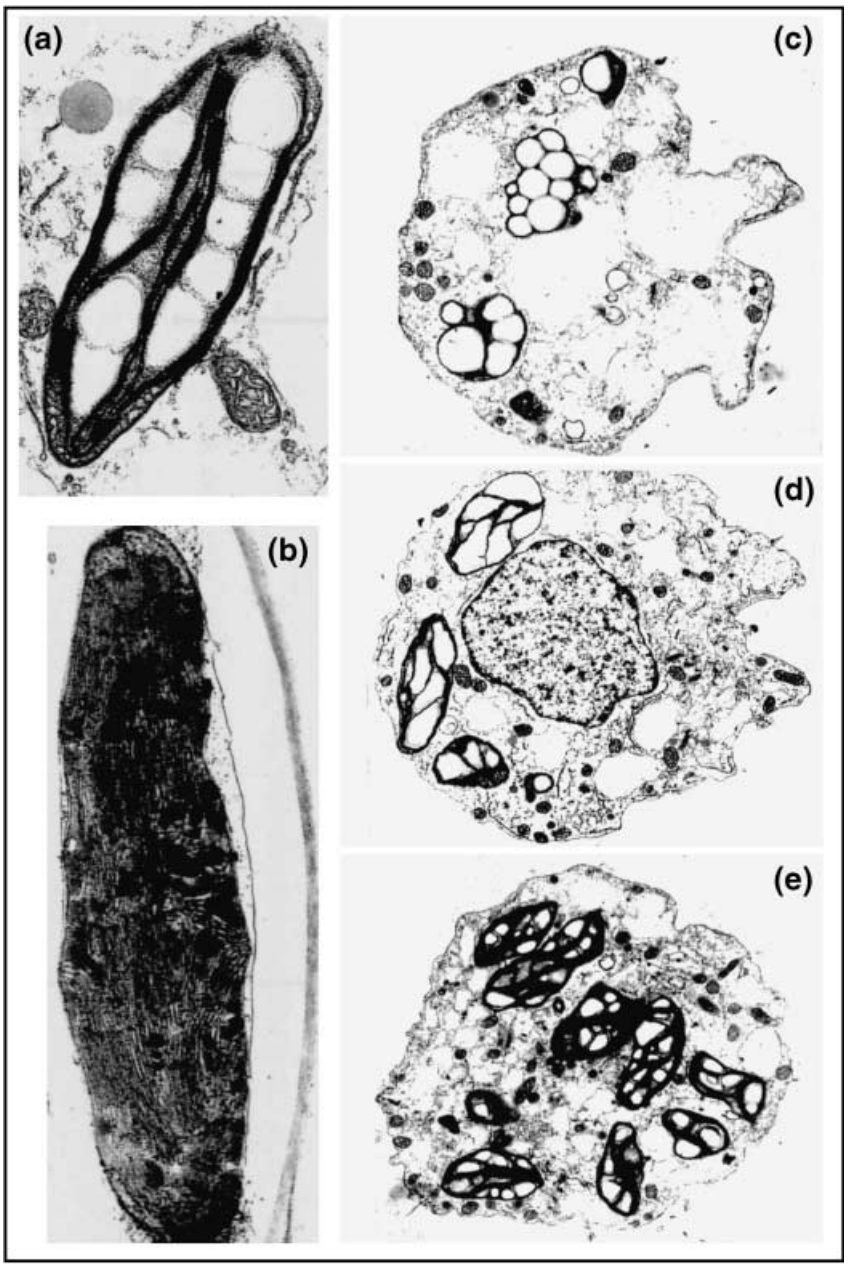


**Fig. 1** Starch content in guard cell chloroplasts of *Commelina communis*. (a, b) At the end of the night starch is abundant in guard cell chloroplasts (a) while it is absent in mesophyll cell chloroplasts (b). (c–e) Changes in starch content in chloroplasts from *C. communis* guard cell protoplasts according to the light treatment. Protoplasts were kept in darkness for 12 h (c), then illuminated with red light (30 min, 475 µmol $m^{-2}$ $s^{-1}$) (d); a blue light pulse (1 min, 70 µmol $m^{-2}$ $s^{-1}$) was applied upon the red light background and the protoplasts were fixed 7 min later (e). Note the strong decrease in starch content resulting from the blue light pulse. Bars: (a, b) 500 nm; (c–e) 1 µm.

(Shimazaki & Zeiger, 1987; Shimazaki, 1989) and taking into account the low chlorophyll content in guard cell chloroplasts, $CO_2$ fixation via the Calvin cycle should be limited to only 2%–4% of that found in mesophyll cells (Outlaw & De Vlieghere-He, 2001). Gautier *et al.* (1991) used mass spectrometry to compare the unidirectional fluxes of $O_2$ and $CO_2$ during a dark/light transition in GCPs and MCPs. In accordance with other studies, they found that GCPs display a high respiration rate (Fig. 2a,b). The major differences between the two cell types were found at the level of $CO_2$ fluxes (Fig. 2c,d). $CO_2$ exchanges from MCPs appear typical of $C_3$ plants. By contrast, in guard cells $CO_2$ fixation displays a long induction period and stays significantly lower than $O_2$ evolution. Such kinetics are consistent with a major participation of the PEPC pathway in guard cell $CO_2$ fixation, with a time-lag due to the transfer of energy between different cellular compartments. Although the importance of guard cell photosynthesis to stomatal movements is still not totally resolved, the most recent data obtained from transgenic anti-Rubisco tobacco plants (von Caemmerer *et al.*, 2004) argue for a minor role. These authors show that even a large decrease in the quantum yield of PSII in guard cells does not affect the rate of stomatal opening, steady-state stomatal conductance, or the response of stomatal conductance to ambient $CO_2$ concentration.

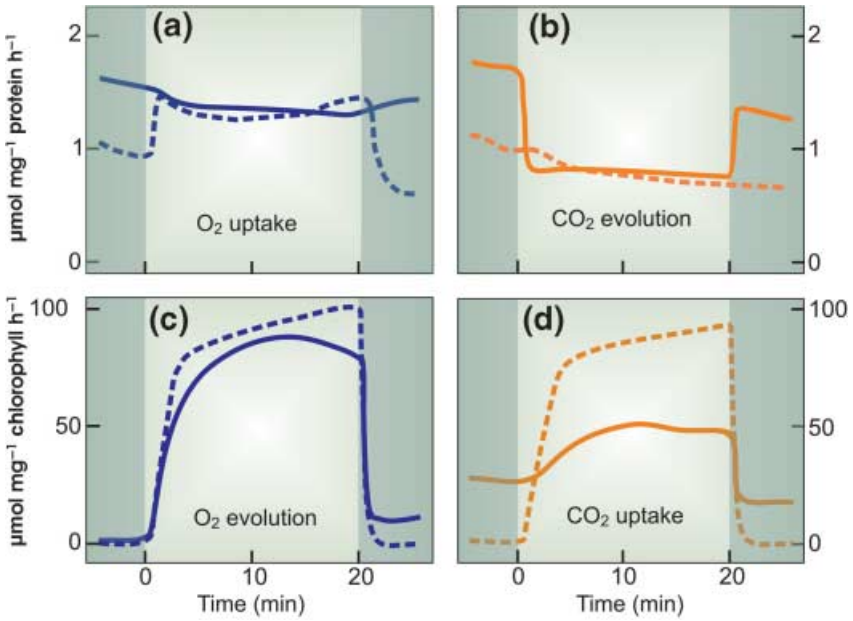


**Fig. 2** Mass spectrometric determination of unidirectional fluxes of $O_2$ and $CO_2$ from *Commelina communis* guard cell protoplasts (GCPs, solid line) or mesophyll cell protoplasts (MCPs, dashed line). After 5 min in darkness, the protoplasts were illuminated for 15 min (800 µmol $m^{-2}$ $s^{-1}$). While GCPs and MCPs exhibit a similar pattern of $O_2$ evolution under light (c), major differences can be observed in $O_2$ uptake (a), $CO_2$ evolution (b) and $CO_2$ uptake (d). Data redrawn from Gautier *et al*. (1991).

**1.2. Starch storage and mobilisation** In the light, mesophyll chloroplasts accumulate starch and lose it in the dark. By contrast, starch is present in darkness in almost all guard cell chloroplasts (Lloyd, 1908; Robinson & Preiss, 1985, fig. 2a). Despite Stadler *et al.* (2003) recent report of starch accumulation under light in Arabidopsis guard cells, which contrasts with the results of another study (Lascève *et al.*, 1997), starch content is generally inversely correlated to the degree of stomatal aperture (Outlaw & Manchester, 1979). Starch-to-sugar conversion was proposed as an osmotic motor to drive changes in guard cell turgor in the early 20th century (Lloyd, 1908; Scarth, 1927). This hypothesis was widely accepted by most physiologists until the 1960s. Later, the essential role of $K^+$ accumulation in the build up of the osmotic potential driving stomatal movements was revealed (Immamura, 1943; Yamashita, 1952; Fujino, 1967; Fischer, 1968). However, even if potassium is now currently recognised as the major osmoticum, organic anions such as $malate^{2-}$ are likely candidates to balance the positive charges due to $K^+$ accumulation while starch degradation could provide the carbon precursors needed for malate synthesis in the cytosol. Two main pathways allow carbon exchanges between mesophyll cell chloroplasts and the cytosol. Under light, the triose-phosphate/phosphate translocator of the inner envelope membrane of chloroplasts represents the major interface for the distribution of photoassimilates between the chloroplast and the cytosol. At night, maltose and, to a lesser extent, glucose are the major forms of carbon exported from mesophyll cell chloroplasts (Weise *et al.*, 2004) and a maltose transporter, MEX1, located at the chloroplast membrane, has been recently identified (Niittylä *et al.*, 2004). The phosphate translocator from guard cell chloroplasts of *Pisum sativum* has been characterised (Overlach *et al.*, 1993). Interestingly, the guard-cell phosphate translocator differs from the mesophyll cell one in that it possesses a high affinity for Glc-6-P (as high as that determined for pea-root amyloplasts (Borchert *et al.*, 1989)). This ability to exchange Glc-6-P provides a way to import reduced carbon that could be temporarily stored as starch in the guard cell chloroplast. By contrast with mesophyll cell chloroplasts, recent biochemical analysis of carbon export from illuminated guard cell chloroplasts by Ritte & Raschke (2003) indicated that starch breakdown results in substantial glucose and maltose export besides triose phosphates. This observation points to a specific adaptation/regulation of the guard cell chloroplast to allow starch breakdown under light and the release of precursors of malate and sucrose to the cytoplasm to sustain stomatal opening (Fig. 3). Interestingly, this last study reports that most of the carbon exported by guard cell chloroplasts originated predominantly from starch breakdown, reinforcing the general consensus of a low PCRP in guard cells.

Lascève *et al.* (1997) took advantage of an *Arabidopsis* mutant devoid of starch, a phosphoglucomutase mutant (*pgm*, Caspar *et al.*, 1985), to test the importance of starch in stomatal movements. Microscopic observations confirmed that the guard cell chloroplasts from the *pgm* mutant plants were starch depleted, while starch was observed at dusk in wild-type plants. In whole plant experiments, such an absence of starch in guard cells in *pgm* plants did not affect stomatal behaviour under white light and slightly reduced stomatal response to red light. By contrast, stomatal opening under blue light was severely impaired. Interestingly, a wild-type stomatal response to blue light was restored in epidermal strips of *pgm* plants at high chloride concentration. These data strongly argue that malate synthesis accompanying $K^+$-uptake under blue light is supported by

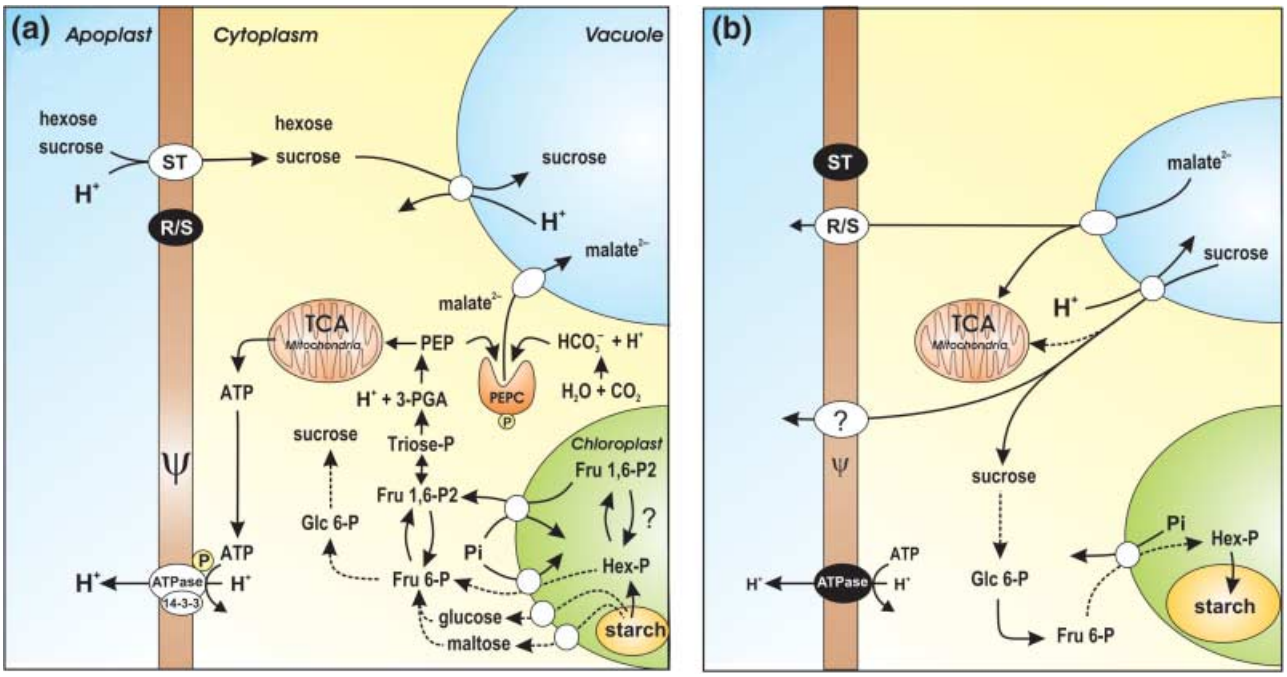


**Fig. 3** Schematic representation of guard cell metabolism during (a) light-induced stomatal opening or (b) stomatal closure; adapted with minor modifications from the model proposed by WH Outlaw (2003). Activated transporters are circled in white. Inactivated transporters are circled in black. Dashed lines represent multistep processes. (a) During opening starch degradation provides carbon skeletons for malate synthesis. In parallel, recent studies underline the importance of carbon import from the apoplast through sugar transporters and of maltose and glucose delivery from the chloroplasts to the cytoplasm during starch degradation. Carbohydrates and organic anions can be stored in the vacuole to increase guard cell turgor or be consumed by the TCA cycle in mitochondria to sustain the activity of the proton pump. (b) During stomatal closure malate is delivered from the vacuole to the cytoplasm and then to the guard cell apoplast through anion channels (R/S). Some malate can be consumed in mitochondria by the TCA cycle. An open question is the fate of the sucrose accumulated in the guard cell vacuole. Evidence is against neoglugenesis in guard cell (Outlaw, 2003) but part of the sucrose could be converted back to starch.

starch breakdown. They also demonstrate the flexibility of guard cells about the nature of the osmoticum accumulated in response to a specific stimulus.

**1.3. Guard cell PEPC pathway** Most investigations conclude that $Cl^-$ influx during stomatal opening cannot compensate for the positive charge resulting from $K^+$ accumulation (Willmer & Fricker, 1996). Thus, it has been proposed that other(s) counterion(s) such as organic anions, and mainly $malate^{2-}$ are involved. Indeed, a good correlation has been observed between stomatal opening and accumulation of malate in guard cells (Allaway, 1973; Pearson, 1973). Malate synthesis is highly dependent on phospho*enol*pyruvate carboxylase (PEPC) activity. As the $C_4$ enzyme, the guard cell PEPC is regulated by cytoplasmic pH, Glucose-6P (Glc-6P) and triose-6P (activators) and L-malate (feed-back inhibitor) (Tarczynski & Outlaw, 1990, 1993). However, a high sensitivity of guard cell PEPC to malate would be in contradiction with the large increase in malate content observed during stomatal opening. Indeed, in CAM, $C_4$ and some $C_3$ plants, PEPC is strongly regulated through phosphorylation (Nimmo *et al.*, 1995; Chollet *et al.*, 1996). The phosphorylated enzyme has an increased activity and is considerably less sensitive to retroinhibition by malate (Jiao & Chollet, 1990, 1991). Phosphorylation of the guard cell enzyme results in a 50% increase in the *Vmax* and in a large reduction in L-malate retroinhibition (Cotelle *et al.*, 1999).

Guard cell PEPC from open stomata was found to be less sensitive to L-malate than the enzyme from closed stomata (Zhang *et al.*, 1994) and later studies demonstrated that the phosphorylation state of the guard cell PEPC correlates with stomatal aperture. Stomatal opening triggered by fusicoccin promotes phosphorylation of the guard cell PEPC while abscisic acid (ABA) results in dephosphorylation (Du *et al.*, 1997). Meinhard & Schnabl (2001) observed that PEPC phosphorylation under light is up-regulated by $K^+$ and suppressed by inhibitors of the proton pump. In a recent work, Outlaw *et al.* (2002) showed that phosphorylation of the guard cell enzyme after fusicoccin treatment is much lower in the presence of chloride. Thus, guard cell PEPC activation through phosphorylation would not be a primary process but a response to cation influx in the cytosol. By contrast with the $C_4$ enzyme, cytoplasmic alkalinization does not cause PEPC phosphorylation (Outlaw *et al.*, 2002). Conversely, cytoplasmic acidification leads to guard cell PEPC activation, suggesting that cytosolic pH acts as a signal in guard cell PEPC regulation

(Meinhard *et al.*, 2001). All these results point to a specific regulation of the guard cell PEPC resulting in malate synthesis during stomatal opening.

In guard cells, the Calvin cycle and β-carboxylation pathways play complementary and redundant roles as shown by inhibitor studies. Stomatal opening is restricted in the presence of 3,3-dichloro-dihydroxyphosphinoyl-methyl-2-propenoate (DCDP), an inhibitor of PEPC. However, ribulose-5-phosphate or 3-PGA could relieve significantly the inhibition of stomatal opening by DCDP, indicating that the Calvin cycle may become significant when PEPC is restricted (Parvathi & Raghavendra, 1997). Such results were confirmed in a recent study by Asai *et al.* (2000). Thus both Calvin cycle and β-carboxylation pathways are beneficial for stomatal opening, particularly when either of these pathways are restricted.

**1.4. Carbohydrate transporters** By contrast with the limited guard cell PCRP, recent observations *in planta* point to a significant participation of sucrose in guard cell turgor under light (Talbott & Zeiger, 1996, 1998). Alternatively, carbohydrates could be imported from the guard cell apoplast as suggested by earlier studies. Epidermal strip experiments have suggested that the guard cell is able to import $^{14}$C-glucose and $^{14}$C-sucrose (Dittrich & Raschke, 1977; Reddy & Das, 1986). Recently, two distinct sugar import processes have been described in guard cells from *Pisum sativum* (Ritte *et al.*, 1999). The first was characterised as a saturable hexose proton symporter, the activity of which depends on the membrane potential. The second has the characteristics of a sucrose transporter that could contribute to sucrose import at high apoplastic sucrose contents (> 4 mM). Interestingly, the measurement of sucrose content in the guard cell apoplast in *Vicia faba* plants gives evidence for just such high concentrations (Lu *et al.*, 1995, 1997; Outlaw & De Vlieghere-He, 2001; Outlaw, 2003). Under high photosynthesis and transpiration, the sucrose content in the guard cell apoplast increased 7-fold, reaching values up to 100 mM. This sucrose accumulation in the apoplast is paralleled by an elevation of sucrose in the guard cell symplast. These results support the observations from Zeiger's lab that *in planta* a two-phase mechanism contributes to guard cell swelling during the day (Talbott & Zeiger, 1996). In the morning phase, opening is mostly correlated with $K^+$ uptake in guard cells, while during the afternoon phase $K^+$ content declines and sucrose becomes the dominant osmoticum. In such a scheme, sugar transport between the guard cell apoplast and symplast should play a crucial role (Fig. 3).

Work is still in progress concerning the characterisation of guard cell carbohydrate transporters. In *Arabidopsis*, AtSTP1 has been identified as a monosaccharide-$H^+$ symporter by functional analyses in yeast (Sauer *et al.*, 1990). Its substrate specificity is close to the hexose proton symporter observed in guard cells of *Pisum sativum* (Ritte *et al.* (1999). Recent work from Stadler *et al.* (2003) demonstrates that *AtSTP1* gene expression is guard cell specific and displays a strong nycthemeral regulation. *AtSTP1* expression is reduced during the light period and quickly up-regulated at the onset of dusk. The authors suggest that AtSTP1 could participate in guard cell import of apoplastic glucose delivered by starch breakdown in mesophyll cells at night. Additionally, it could also participate to some extent in carbohydrate import during the day. An *Atstp1* T-DNA null-mutant was analysed but did not present any obvious guard cell phenotype (Stadler *et al.*, 2003). Taking account of the multiplicity of sugar transporters in plants, other carbohydrate carriers supporting redundant or complementary functions with AtSTP1 could account for this absence of phenotype. RT-PCR performed on guard cell RNA preparations (Stadler *et al.*, 2003), and studies of promoter-dependent GFP fluorescence (Meyer *et al.*, 2004) revealed the expression of *AtSUC2* and *AtSUC3* in guard cells, guard cell specific expression of *AtSUC3* being limited to very young leaves. Further analyses of carbohydrate carriers using reverse genetics should bring more information on the role of these transporters in guard cell osmoregulation.

The contribution by $K^+$ or sucrose to guard cell osmoticum depends not only on the time of the day but also on the external stimuli (described in the following section). Thus, it is now necessary to characterise in detail the features of carbohydrate influx/efflux and the distribution between the apoplast and symplast of guard cells. While the import of glucose/sucrose into the guard cells during stomatal opening is well demonstrated, what happens to these carbohydrates during stomatal closure is not clear. Sucrose or glucose can either be exported from or metabolised in the guard cells. Further experiments are needed to examine the fate of glucose/sucrose during stomatal closure.

## 2. Responses to external stimuli

Guard cell carbon metabolism exhibits specific responses to different stimuli. For example, blue light triggers starch mobilisation, malate synthesis, activates the plasma membrane proton pump and $K^+$ accumulation. Whereas, in red light, it is carbohydrate import and to a limited extent sugars, synthesised by the limited PCRP, which support guard cell turgor. However, under $CO_2$ free-air, the response to red light becomes close to the one observed under blue light ($K^+$ uptake, malate synthesis), highlighting the flexibility of the osmoticum accumulated according to the stimulus.

**2.1. Blue/red light** Stomatal responses to light are strictly wavelength dependent with blue light more efficient (2–20 fold, Willmer & Fricker, 1996) than red light in most species. These observations suggest that there must be at least two photoreceptors. As the red light response was found to be DCMU sensitive, it was inferred that it depends on chlorophyll and electron transport in guard cell chloroplasts (Tominaga *et al.*, 2001). By contrast, the blue-light response was found to

be DCMU stimulated and rotenone sensitive, suggesting an essential role for oxidative phosphorylation (Agbariah & Roth-Bejerano, 1990) in this pathway. Excitation of the blue light photoreceptor triggers an activation of the electrogenic proton pump at the plasma membrane of guard cells (Assmann *et al.*, 1985). Recently the blue light receptor was determined (Kinoshita *et al.*, 2001). In *Arabidopsis* PHOT1 and PHOT2 (phototropins) are blue light receptors exhibiting serine/threonine kinases activity (Huala *et al.*, 1997). PHOT1 and PHOT2, which are apparently functionally redundant, mediate blue light response in guard cells. These photoreceptors undergo autophosphorylation under blue light irradiation leading to proton pump phosphorylation and interaction with 14-3-3 protein(s) (Kinoshita & Shimazaki, 2002, 2003; Kinoshita & Shimazaki, 2002) which stabilise and activate the proton pump (Maudoux *et al.*, 2000; Emi *et al.*, 2001). It would be interesting to know whether 14-3-3 proteins control other guard cell signalling pathways since 14-3-3 proteins regulate multiple metabolic key enzymes (Cotelle *et al.*, 2000). However, very recent data suggest that there may be a second blue-light signalling pathway that is independent of PHOT1 and PHOT2 (Talbott *et al.*, 2003a).

Histochemical and biochemical analyses in epidermal peels of *Vicia faba* showed that red light-dependent stomatal opening at ambient $CO_2$ concentrations was largely independent of starch breakdown and $K^+$ uptake (Tallman & Zeiger, 1988; Talbott & Zeiger, 1993; Olsen *et al.*, 2002). This would suggest that opening under red light depends on sucrose synthesis and/or import (Poffenroth *et al.*, 1992; Talbott & Zeiger, 1993). When epidermal peels were submitted to red light illumination under low $CO_2$ conditions, stomatal opening was accompanied by a net increase in $K^+$ content in guard cell and by starch breakdown (Olsen *et al.*, 2002). These features, starch breakdown, malate synthesis and $K^+$ uptake are reminiscent of the type of osmoticum accumulated during stomatal opening under blue light (Hsiao *et al.*, 1973; Ogawa *et al.*, 1978; Talbott & Zeiger, 1993). Blue light-induced starch degradation can be observed in GCPs (Fig. 1c–e. H. Gautier & A. Vavasseur, unpublished data). As discussed above, blue light activates the proton pump, hyperpolarizing the membrane potential, which drives $K^+$ uptake through inward $K^+$ channels. Additionally, the apoplastic acidification resulting from the proton pump activation could power sugar carriers (mainly sugar/$H^+$ symporters) at the plasma membrane.

Interestingly, Talbott & Zeiger (1996) observed a change in the nature of the guard cell osmoticum along the course of the day. During the 'morning' phase, stomatal opening correlates with $K^+$ accumulation, while in the 'afternoon phase' $K^+$ content declines and sucrose becomes the dominant osmoticum. Such a shift in the nature of the osmoticum is suggestive of a transition from blue light associated osmoticum ($K^+$) during the morning phase to a red light one (sucrose) in the afternoon. Such a change in the osmoticum may explain some discrepancies between observations of stomatal behaviour *in planta* and in epidermal strips as exemplified in the case of the *gork*-1 mutant. GORK is an outward rectifying $K^+$ channel from the Shaker family expressed in guard cells (Ache *et al.*, 2000). It locates to the plasma membrane and its activation through membrane depolarisation is proposed to allow $K^+$ efflux from the guard cell cytoplasm during stomatal closure. Since $K^+$ is a major osmoticum, GORK disruption was predicted to greatly affect the ability of stomata to close. Recently, the stomatal phenotype of the *gork*-1 knockout mutant has been characterised (Hosy *et al.*, 2003). It displays a higher transpiration rate and a lower rate of stomatal closure than the wild-type plant, in accordance with a defect in $K^+$ efflux. However, the *gork*-1 phenotype is much more pronounced in epidermal strips experiments than in whole plant experiments. The observation that in photosynthesising plants, guard cell $K^+$ is with time replaced by sucrose delivered by mesophyll cells could explain such a discrepancy. Osmoregulation in epidermal peels is likely to be essentially based on $K^+$ exchange with the bathing medium, which in the majority of experiments only contains KCl, explaining the strong phenotype of the mutant in these conditions. By contrast, in whole plant experiments, the accumulation of sucrose in guard cells would lead to a less pronounced phenotype. Indeed, in *gork*-1 plants, $K^+$ efflux during stomatal closure would not be the main limiting factor. This again raises the question of the fate of sucrose during stomatal closure. It could be consumed at the mitochondrial level, reconverted to starch albeit at a low rate (Outlaw, 2003) or exported to the apoplast (Figs 3b and 5b). It is clear that much information is needed before a clear picture of the role of sucrose can emerge.

**2.2. Water stress** Under water stress, guard cells display a short-term response based on osmoregulation and a long-term response involving modification of major metabolic enzymes due to alterations in guard cell gene expression. The short-term response is primarily controlled by ABA, which reduces ion uptake and promotes ion efflux. This involves changes in cytoplasmic $Ca^{2+}$ and pH (Assmann & Shimazaki, 1999; Hetherington, 2001; Schroeder *et al.*, 2001). Only a few studies have investigated the long-term effects of drought stress or ABA on guard cell metabolism. Early studies, using epidermal strips, did not find any metabolic regulation in guard cells under drought stress (Grantz & Schwartz, 1988). However, subsequent studies revealed dramatic changes in guard cell expression profile of key metabolic enzymes during a short drought stress. In *Solanum tuberosum*, Kopka *et al.* (1997) observed an up-regulation of the mRNA levels of sucrose synthase and sucrose-phosphate synthase. By contrast, the expressions of *KST1* (guard cell inward $K^+$ channel), and of *PHA2* (plasma membrane H ± ATPase) were reduced together with vacuolar invertase, UDP-glucose pyrophosphorylase, ADP-glucose pyrophosphorylase (large subunit), cytosolic glyceraldehyde-3-phosphate dehydrogenase, a sucrose/$H^+$ cotransporter and an isoform of PEPC. Interestingly, PEPC,

vacuolar invertase, and cytosolic glyceraldehyde-3-phosphate dehydrogenase were regulated specifically in guard cells. These changes in transcript levels were complete before any observation of a decrease in leaf water potential, which suggests the involvement of ABA (Gowing *et al.*, 1993).

Using microarrays covering one-third of the *Arabidopsis* genome, Leonhardt *et al.* (2004) compared guard cell expression profiles with those of mesophyll cells. They observed an ABA-modulation of many known guard cell ABA signalling components at the transcript level. Apart from modulating the expression of signalling elements, key enzymes involved in guard cell carbon metabolism were also ABA-repressed. The expression level of RBCs was severely repressed by ABA in guard cells and, to a lesser extent, in mesophyll cells. Four isoforms of PEPC are encoded within the *Arabidopsis* genome (*AtPPC1-4*, Sánchez & Cejudo, 2003). Data from microarrays indicate that at least two isoforms of PEPC are expressed in guard cells and mesophyll cells of *Arabidopsis*, *AtPPC2* and *AtPPC3*. *AtPPC2* is the most expressed in both cell types but its level of expression is far more elevated in guard cells. When plants were sprayed with ABA (Leonhardt *et al.*, 2004), a strong decrease in *AtPPC2* expression level was observed in both cell types after 4 h of treatment. These observations of a down-regulation of PEPC transcripts by ABA are in good agreement with those of Kopka *et al.* (1997), with the exception that in *S. tuberosum* the strong inhibition of PEPC expression under drought stress was guard cell specific. These data highlight the fact that more research is needed into the contribution that metabolic regulation makes to adaptation to reduced water availability stress.

## III. Guard Cell $CO_2$ Sensing

In this review, only the short-term effects of $CO_2$ on stomatal behaviour will be considered. Long-term responses to elevated carbon dioxide have been reviewed elsewhere (Woodward, 1987; Morison, 1998; Assmann, 1999; Gray *et al.*, 2000; Woodward *et al.*, 2001; Hetherington & Woodward, 2003). To optimise the water use efficiency, guard cells must monitor the plant water status and the carbon dioxide demand from the mesophyll. To perform such regulation, $CO_2$ sensing in guard cells is required. Freudenberger (1940) and Heath (1948) were the first to describe the stomatal response to elevated $CO_2$ (reduction in aperture), which was then found to be ubiquitous in higher plants (Morison, 1985; Mansfield *et al.*, 1990).

### 1. Location of $CO_2$ sensing

Studies conducted with epidermal strips or GCPs revealed that $CO_2$ sensing is an intrinsic property of guard cells (Fitzsimons & Weyers, 1986). Later, Mott (1988) observed that *in planta* guard cells respond to the intercellular $CO_2$ concentration ($C_i$), which is determined by atmospheric $CO_2$ ($C_a$) and by the mesophyll assimilation rate. Such sensitivity to $C_i$ allows a tight coupling between stomatal conductance and photosynthesis. In well-watered plants, the stomatal response to $CO_2$ is generally limited, a doubling of $C_a$ (350–700 ppm) resulting in reductions of approximately 40% of stomatal conductance (Morison, 1987).

The response to $CO_2$ is generally curvilinear and more important below 300 ppm than at higher $CO_2$ concentrations (Morison, 1987). In some studies maximal stomatal opening was higher at 100 ppm than in $CO_2$-free air, suggesting that a low $CO_2$ concentration may have a positive effect on stomatal opening (Raschke, 1976; Dubbe *et al.*, 1978). However, whether $CO_2$ may have a positive effect on stomatal opening remains an open question. Moreover, the amplitude of the stomatal response to changes in $CO_2$ partial pressure is tightly dependent on many parameters such as lighting conditions and the water status of the plant.

### 2. $CO_2$ sensing and ABA

Is there an interaction between $CO_2$ and ABA?. Some of the early studies indicated independence (Mansfield, 1976; Mansfield & Wilson, 1981; Wilson, 1981), whereas others point to a strong interaction. Raschke (1975) was the first to report such strong interaction; working with *Xanthium strumarium* plants he observed that stomata did not close in response to elevated, $CO_2$ concentrations unless the leaves had been treated with ABA. Some studies suggested that auxin could be involved in such interaction (Davies & Mansfield, 1987). Raschke (1975) also observed that stomatal responses to ABA were weak in $CO_2$-free air (Raschke, 1975) and similar results have also more recently been reported in *Arabidopsis* (Leymarie *et al.*, 1998a). As illustrated in Fig. 4,

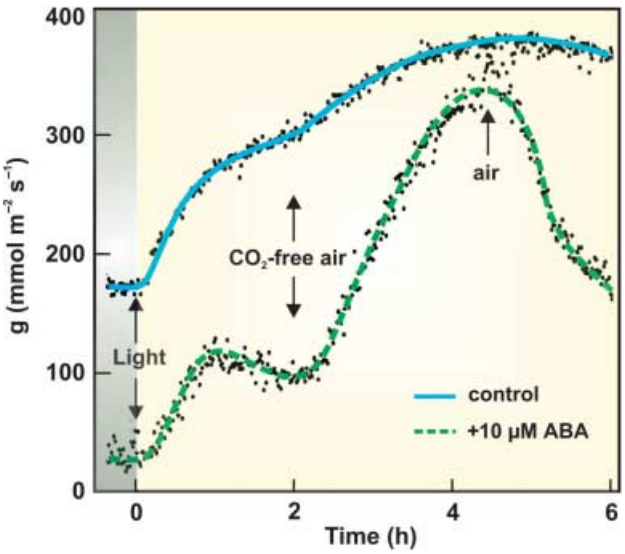


**Fig. 4** Responsiveness of *Arabidopsis* shoot conductance to light and $CO_2$-free air in control conditions (blue line) or after a 24 h treatment with 10 μM ABA in the nutrient solution (green line). Note that in the absence of $CO_2$ the effect of ABA is strongly reduced. Redrawn from Leymarie *et al*. (1998a).

$CO_2$ removal is able to fully abolish the inhibition of transpiration induced by 10 μM ABA in the nutrient solution. Additionally, stomatal sensitivity to $CO_2$ was enhanced when an osmotic stress was applied to the roots (Leymarie *et al.*, 1999). These observations point to a very strong interaction between ABA and $CO_2$ signalling pathways. To test this relationship further, studies were performed using ABA insensitive *Arabidopsis* mutants (Koornneef *et al.*, 1984). Two *Arabidopsis* mutants, *abi1*–1 and *abi2*–1 exhibit a wilty phenotype (Leung *et al.*, 1994, 1997) indicating a loss of ABA control of the transpiration rate resulting from ABA-insensitive guard cells (Roelfsema & Prins, 1995). These ABA-insensitive mutants provide a good model to test the interactions between ABA and $CO_2$ signalling. Webb & Hetherington (1997) observed that *abi1*–1 and *abi2*–1 mutants fail to respond to $CO_2$ and extracellular calcium. From these results they inferred that the signal transduction pathways for ABA, $CO_2$ and $Ca^{2+}$ converge on, or close to, the *ABI1* and *ABI2* gene products. Another study using epidermal bioassays by Leymarie *et al.* (1998b) showed that, in the *abi1*–1 and *abi2*–1 contexts, a partial stomatal response to $CO_2$ was observed when the $K^+$ concentration in the bathing medium was decreased. These authors proposed that, according to the osmoticum, ABA and $CO_2$ do not share the same signalling pathways but interact in a synergistic manner and that the *ABI1* and *ABI2* gene products are involved in this interaction. However, the results of these studies must be treated with caution since the *abi1*–1 and *abi2*–1 mutations are dominant. Further characterisation of recessive alleles and molecular studies have shown that *ABI1* and *ABI2* genes encode two protein phosphatases 2C sharing redundant functions and acting in a negative feedback regulatory loop of the abscisic acid signalling pathway (Gosti *et al.*, 1999; Merlot *et al.*, 2001). Accordingly, double *abi1-abi2* mutant plants are ABA hypersensitive at the level of stomatal response to ABA (Merlot *et al.*, 2001). A detailed study of $CO_2$ sensing in these mutants is still awaited before a definitive conclusion can be drawn about the role of these redundant PP2C in integrating ABA and $CO_2$ signalling.

The conditions under which a plant was grown have a major effect on the extent of the stomatal response to $CO_2$. Recently, Frechilla *et al.* (2002) observed that stomata of growth chamber-grown *Vicia faba* leaves have an enhanced $CO_2$ response compared with stomata of glasshouse-grown leaves. Complementary studies on the parameters driving this response led Talbott *et al.* (2003b) to propose air relative humidity as a key factor in modulating stomatal sensitivity to $CO_2$ with elevation of relative humidity resulting in an enhanced $CO_2$ response. The authors also suggested that humidity could function as a signal for leaves inside dense foliage canopies that promotes stomatal opening under low light and low $CO_2$ conditions. Detailed studies of stomatal responses to air relative humidity have resulted in the conclusion that guard cells do not directly sense RH but instead respond to transpiration rate (Mott & Parkhurst, 1991). Accumulation of sucrose in the guard cell apoplast under high transpiration level has been suggested to mediate stomatal response to RH (Outlaw & De Vlieghere-He, 2001). In any case, an up-regulation of guard cell $CO_2$ sensing by RH is difficult to reconcile with the synergistic effect of ABA and $CO_2$ in promoting stomatal closure (Raschke, 1975; Leymarie *et al.*, 1998a,b). Indeed, water stress and associated ABA synthesis are more likely to occur at low RH. However, a stomatal response to humidity is still observed in ABA-deficient and ABA-insensitive *Arabidopsis* mutants, which would suggest that ABA is not the prime mediator of the guard cell response to RH (Assmann *et al.*, 2000). It is clear that more research is needed to clarify the relationship between $CO_2$ sensing and plant water status.

## 3. $CO_2$ sensing and light

In epidermal peels and *in planta*, stomatal response to $CO_2$-free air is generally reduced under darkness and greatly enhanced in the presence of low blue light or strong red light illumination (Assmann, 1988; Vavasseur *et al.*, 1990a, 1990b; Willmer & Fricker, 1996). Accordingly, the increase in stomatal opening triggered by light under $CO_2$-free air is accompanied by a large increase in $K^+$ and $Cl^-$ accumulation in guard cells (Lascève *et al.*, 1987).

Recent studies have shown that cytosolic ATP is essential for maintaining the activity of $K^+$-uptake channels in guard cells (Goh *et al.*, 1999, 2002). ATP depletion results in an inhibition of inward $K^+$ currents and photosynthetic electron transport, while addition of ADP together with orthophosphate prevents the inhibitory effect of these treatment. These results suggest that cytoplasmic ATP provides a coupling mechanism between guard cell chloroplasts, mitochondria, and ion transport. As discussed above, low blue light illumination promotes a rapid decrease in starch content (Fig. 1c–e), which could supply carbon skeletons for malate$^{-2}$ synthesis (Ogawa *et al.*, 1978) providing negative charges to balance the $K^+$ influx and additional substrates for oxidative phosphorylation (Agbariah & Roth-Bejerano, 1990).

Under red light illumination, a significant part of the ATP produced by photophosphorylation is used for $H^+$ pumping (Tominaga *et al.*, 2001). This is consistent with the observation that red light triggers an electrogenic current sensitive to DCMU in GCPs (Serrano *et al.*, 1988). In the absence of $CO_2$, the ATP sink represented by the Calvin cycle should be limited, which should allow an increased transfer of ATP to the cytosol for proton pumping. However, subsequent studies did not confirm the activation of the proton pump under red light (Taylor & Assmann, 2001; Roelfsema *et al.*, 2002). Recent *in planta* studies combining voltage-clamp and recording of $CO_2$ partial pressure in substomatal cavity led to similar results (Goh *et al.*, 2001, 2002). These authors failed to observe any hyperpolarization of the plasma membrane upon red light illumination when the light beam was limited to the

guard cell area. Conversely, when the red light beam was extended to mesophyll cells, a lowering of substomatal $CO_2$ partial pressure was observed accompanied by an hyperpolarization of the guard cell plasma membrane. From these data it can be concluded that the guard cell response is not primarily linked to red light illumination, but more likely to a $CO_2$ lowering driven by photosynthesis in neighbouring mesophyll cells.

## 4. Second messengers in $CO_2$ signalling pathway

The involvement of calcium ions in ABA signalling has recently been comprehensively reviewed (Assmann & Shimazaki, 1999; Hetherington, 2001; and Schroeder *et al.*, 2001). ABA binding to still unidentified receptors activates a transfer of $Ca^{2+}$ from the guard cell apoplast and the vacuole to the cytosol. The increased $Ca^{2+}$ in the cytosol ($[Ca^{2+}]_{cyt}$) inhibits the $H^+$ pump depolarising the membrane, activates outward anion channels in the plasma membrane and blocks $K^+$ uptake through inward $K^+$ channels. Depolarisation and cytoplasmic alkalinization activate outward $K^+$ channels. These events conduct the loss of solutes and stomatal closure. By contrast, much less is known about the intracellular second messengers involved in stomatal response to $CO_2$.

**4.1. Cytoplasmic free calcium** By contrast to the situation with ABA, there have been relatively few investigations of the role of $Ca^{2+}$ in guard cell $CO_2$ signalling. Schwartz *et al.* (1988) demonstrated that, in epidermal strips, external application of calcium chelator (EGTA) results in diminished stomatal response to $CO_2$. Later, Webb *et al.* (1996) used fluorescence ratio-photometry to measure $[Ca^{2+}]_{cyt}$ in response to changes in $CO_2$. Elevated $CO_2$ (700 ppm) induced increases in guard cell $[Ca^{2+}]_{cyt}$ which were similar to those observed in response to ABA (McAinsh *et al.*, 1990, 1992). These increases in $[Ca^{2+}]_{cyt}$ were reversible upon removal of $CO_2$ and repeated application of $CO_2$ resulted in an additional increase in $[Ca^{2+}]_{cyt}$. Importantly, removal of extracellular calcium both prevented the $CO_2$-induced increase in $[Ca^{2+}]_{cyt}$ and inhibited the associated reduction in stomatal aperture (Webb *et al.*, 1996). The results of a pharmacological study by Cousson (2000) indicates that the $CO_2$ signal is transduced through depolarisation-mediated activation of plasma membrane voltage-gated L-type $Ca^{2+}$ channels, which would activate slow anion channels (Schroeder *et al.*, 2001). However, until now L-type $Ca^{2+}$ channels have not been identified in plants. In any case, the bulk of results underlines the potential importance of $[Ca^{2+}]_{cyt}$ in $CO_2$ signalling, as is the case for most of the effectors of stomatal responses. The transgenic lines expressing the calcium indicator yellow cameleon 2.1 (Allen *et al.*, 1999), developed to monitor $Ca^{2+}$ signalling in plant cell represent a promising approach for further studies. Despite recent debate (Köhler *et al.*, 2003), work from the Schroeder lab suggests convincingly that generating ABA-evoked $[Ca^{2+}]_{cyt}$ increases involves a reactive oxygen species regulated, voltage-dependent inward $Ca^{2+}$ channels at the plasma membrane (Kwak *et al.*, 2003). Whether such mechanism also participates in $CO_2$ signalling remains to be determined.

**4.2. Apoplastic and cytoplasmic pH** Cytoplasmic and apoplastic pH are important factors, which impact on the regulation of key guard cell enzymes, for example PEPC (Cotelle *et al.*, 1999), and ionic channels at the plasma membrane (Schroeder *et al.*, 2001). It is now well recognised that alkalinization of guard cell cytoplasmic pH is an integral component of ABA signalling, with one of the major effects being to activate $Ca^{2+}$-insensitive pH-dependent outward $K^+$ channels (Schroeder *et al.*, 2001). As $CO_2$ will form carbonic acid in water it might be predicted that $CO_2$ would induce acidification. However, recent studies fail to support this suggestion. Felle & Hanstein (2002) tracked the apoplastic pH of the substomatal cavity using pH sensitive microelectrodes. They found that application of fusicoccin, which activates the proton pump led, as expected, to a strong acidification (0.5 pH unit). An 800–0 ppm $CO_2$ transition or illumination both resulted in an acidification of the apoplastic pH by 0.2–0.3 unit. This pH change was fully reversible when the initial conditions were restored. Brearley *et al.* (1997) used BCECF and ratio fluorescence microphotometry to measure cytoplasmic pH and did not record any significant pH change during a transition from 0 to 1000 ppm $CO_2$. These observations suggest that, unlike ABA signalling, cytosolic pH changes are not an essential component in $CO_2$ signalling. and highlight the involvement of different downstream elements in both signalling pathways.

**4.3. Ionic channels and membrane potential** ABA and $CO_2$ signalling share many similarities in the way they alter the membrane potential and the main conductances at the guard cell plasma membrane. Both trigger membrane depolarisation, an inhibition of inward $K^+$-channels, and an activation of outward anion and $K^+$ currents (Brearley *et al.*, 1997; Schroeder *et al.*, 2001). A subset of these responses could be attributed to $Ca^{2+}$-signalling. As described above, ABA and $CO_2$ promote cytoplasmic calcium increases (McAinsh *et al.*, 1992; Webb *et al.*, 1996) that could drive inhibition of the proton pump (Kinoshita *et al.*, 1995), deactivate inward $K^+$ channels (Schroeder & Hagiwara, 1989; Lemtiri-Chlieh & MacRobbie, 1994) and activate anion channels (Schroeder & Hagiwara, 1989). While ABA and methyl jasmonate (Suhita *et al.*, 2004) promote an alkalinization of the guard cell cytoplasm believed to drive $K^+$ efflux through outward-rectifying $K^+$ channels (Blatt, 1992), such alkalinization seems absent in $CO_2$ signalling. How elevated $CO_2$ results in a rapid increase in the magnitude of current carried by outward-rectifying $K^+$ channels (Brearley *et al.*, 1997) is currently unresolved.

Anion channel activation plays a crucial role in driving membrane potential towards $K^+$ efflux. Hanstein & Felle (2002) studied transients in apoplastic $Cl^-$ in intact leaves during changes in substomatal $CO_2$. They noticed that after a fast rise in substomatal $CO_2$ from 150 to 800 ppm, it took

several minutes before they recorded a significant increase in apoplastic $Cl^-$. This delay is considerably longer than the one reported by Brearley *et al.* (1997) on epidermal strips using a higher $CO_2$ partial pressure. Interestingly, the extent of $CO_2$–induced $Cl^-$ efflux was the same in darkness and under light. By contrast, light-on, light-off transitions induced rapid variations in apoplastic $Cl^-$ when substomatal $CO_2$ was clamped (Hanstein & Felle, 2002). These observations point to specific control of anion channels by light and $CO_2$. Additionally, exposure to $CO_2$-free air induced a 'desensitisation' of $CO_2$-triggered $Cl^-$ efflux. These results strongly argue for an indirect effect of $CO_2$ in the regulation of anion channels that mediate the $Cl^-$ efflux and suggest that an intermediate effector has to accumulate in response to $CO_2$ to induce the full response. Malate has been proposed as such intermediary link between $CO_2$ and anion channel regulation (Hedrich & Marten, 1993; Hedrich *et al.*, 1994). Two anion conductances coexist at the guard cell plasma membrane. Slow-activating (S-type/SLAC) and fast-activating (R-type/QUACK) anion channels have been distinguished. A major role has been ascribed to S-type anion channels in ABA signalling on the basis of anion channels blockers (Schwartz *et al.*, 1995). However, recent studies in intact plants do not exclude a participation of R-type anion channels in this response (Roelfsema *et al.*, 2004). In response to extracellular malate, R-type anion channels display a shift in their activation potential to more negative values (Hedrich & Marten, 1993), increasing their opening probability in open guard cells. Such sensing of apoplastic malate delivered by photosynthesising tissues would provide guard cells with a feedback sensor of $CO_2$ availability. However, this hypothesis was challenged by further observations. First, the extent of $Cl^-$ efflux in relation with $CO_2$ has been found to be the same in darkness and under light (Hanstein & Felle, 2002), which suggests that guard cell $CO_2$ sensing is independent of photosynthesis. Second, studies by Esser *et al.* (1997) and Cousson (2000) exclude $CO_2$ sensing as primarily resulting from feedback stimulation of anion efflux *via* malate-sensitive anion channel since nonphysiological concentrations of malate need to be applied to observe an inhibition of stomatal opening. Additionally, work from Hedrich *et al.* (2001), suggests that $malate^{2-}$ and $CO_2$ could act in concert as suggested by their additive effects in stomatal closing. Raschke (2003) and Raschke *et al.* (2003) propose that malate could participate in a conversion of R-type anion currents into S-type. They observed that $CO_2$ variations between 0 and 700 ppm caused rapid and reversible increases in the activity of S-type, while R-type anion currents responded to $CO_2$ in an unpredictable manner. $CO_2$-sensitive instantaneous background currents, likely driven by anion channels (Pei *et al.*, 1998), are also candidates in transducing the $CO_2$ signal (Roelfsema *et al.*, 2002). While the molecular identity of the main $K^+$ conductances at the guard cell plasma membrane had been identified for years, the nature of proteins driving anions efflux and influx, is still unknown. Progresses in this area would be a considerable aid in understanding the way ABA and $CO_2$ drive anion exchanges at the guard cell plasma membrane.

**4.4. Redox regulation** The presence of a redox system located at the guard cell plasma membrane and regulating the activity of the proton pump has been proposed based on the observation that NAD(P)H was able to drive proton efflux from guard cells (Vani & Raghavendra, 1989; Raghavendra, 1990; Gautier *et al.*, 1992; Vavasseur *et al.*, 1995). Such redox regulation of the proton pump could link the membrane potential to $CO_2$ metabolism through a modulation of the pool of reducing power. However, Taylor & Assmann (2001) and Roelfsema *et al.* (2002) failed to confirm the presence of such a redox system in their recent patch-clamp studies.

Zeiger and collaborators (Zeiger & Zhu, 1998; Zhu *et al.*, 1998) proposed that zeaxanthin formation in guard cell chloroplasts could be a mediator of light–$CO_2$ interactions. They observed that stomatal aperture and zeaxanthin content in guard cell chloroplasts were linearly related over a wide range of $C_a$. That such a relation was absent in darkness pointed to a relation that was light dependent. Dithiothreitol, an inhibitor of zeaxanthin formation, inhibited the $CO_2$ response in the light but not in the dark. These observations have led the authors to propose separate $CO_2$-sensing mechanisms in guard cells in darkness and under light. However, this interesting proposal still needs to be confirmed. Dithiothreitol, besides inhibiting zeaxanthin formation, has a wide range of cellular effects. As already proposed by Assmann (1999), a better understanding of the situation could be gained by characterising stomatal $CO_2$-sensing in the *npq1* mutant (Niyogi *et al.*, 1998), which is affected in the xanthophyll cycle and cannot de-epoxidise violaxanthin to zeaxanthin.

**4.5. Protein (de)phosphorylation** Regulation of proteins through (de)phosphorylation plays a major role in plant development and adaptation (Xiong *et al.*, 2002; Luan, 2003). Pharmacological studies have shown the importance of such regulation in stomatal movements (Cousson *et al.*, 1995; Cotelle *et al.*, 1996; Esser *et al.*, 1997; Suhita *et al.*, 2003). The potential involvement of ABI1 and ABI2, two type 2C protein phosphatases, in integrating ABA and $CO_2$ responses has been discussed above. Other essential protein kinases and protein phosphatases have been recently identified in the ABA signalling pathway leading to stomatal closure. In *Arabidopsis*, OST1, a calcium-independent protein kinase (Mustilli *et al.*, 2002), which is an orthologue of AAPK, the guard cell-specific ABA-activated serine-threonine protein kinase in *Vicia* (Li & Assmann, 1996; Li *et al.*, 2000), is an essential element in guard cell ABA signalling but does not participate in $CO_2$ sensing (Mustilli *et al.*, 2002). Again in *Arabidopsis*, disruption of *RCN1*, encoding a protein phosphatase 2A, results in guard cell ABA insensitivity (Kwak *et al.*, 2002). Very recently, a type 2C protein phosphatase

(AtP2C-HA, Leonhardt *et al.,* 2004) has been identified as acting in an ABA regulatory feed-back loop. It is striking that the type 2C protein phosphatases presently identified are all involved in feedback regulatory loop(s) in the ABA response. To the best of our knowledge, with the exception of OST1 (Mustilli *et al.*, 2002), none of the mutants for these different protein kinases and protein phosphatases have been used in investigations of guard cell $CO_2$ signalling.

## IV. Prospects in Guard Cell Metabolism and $CO_2$ Sensing

Among the new information gained in recent years, the potential role of of carbohydrates in maintaining guard cell turgor during the course of the day is particularly exciting. It could explain some discrepancies in the results according to the level of investigation (whole plant, epidermal strips, protoplasts), since guard cells *in planta* also depend on the surrounding cells. However there are important questions to address. First, is this process general? Until now it has just been described in *Vicia* and needs to be validated in a number of species. Second, what happens to sucrose and the other carbohydrates accumulated during opening when stomata close? A precise metabolic profile of the guard cell content during the course of the day would help to understand the interplay between organic and inorganic osmoticum involved in stomatal regulation. Figure 5 proposes a schematic representation of the interactions between guard cell metabolic pathways and membrane transport during light-induced stomatal opening or ABA- and $CO_2$-induced stomatal closure.

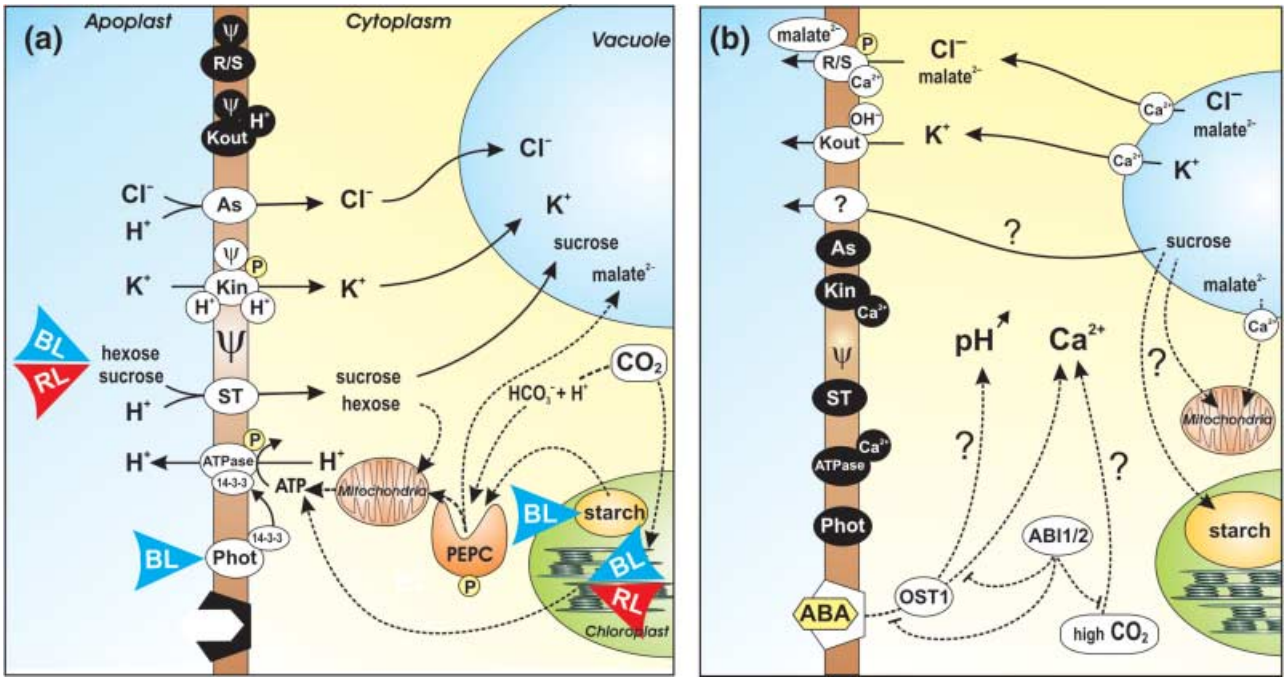


**Fig. 5** Schematic representation of the coupling between metabolic events and membrane transport during (a) light-induced stomatal opening or (b) stomatal closure induced by ABA or elevated $CO_2$. Activated proteins and their effectors are circled in white. Inactivated proteins and their inhibitors are circled in black. Dashed lines represent multistep processes.
Panel (a): red light and blue light trigger different processes during stomatal opening. Blue light promotes rapid starch degradation and PEPC phosphorylation. This allows the synthesis of malate$^{2-}$ to counterbalance $K^+$ transfer from the apoplast powered by the phosphorylation activated proton pump. Chloride is imported from the apoplast by unidentified transporters. Additionally, blue and red light allow ATP delivery to the cytoplasm through photophosphorylation and may in the long-term provide carbohydrates through limited PCRP. Carbohydrates synthesised in photosynthesising mesophyll cells are imported from the guard cell apoplast by sugar transporters. In time $K^+$, malate$^{2-}$ and $Cl^-$ accumulated in the guard cell vacuole during the first phase would be replaced by sucrose during the course of the day.
Panel (b): ABA interacts with internal and/or external ABA receptors activating PLC and PLD and triggering cytoplasmic $Ca^{2+}$ and pH rises. The rise in $Ca^{2+}$ inhibits inward rectifying $K^+$ channels and the proton pump, decreasing the driving force for $K^+$ uptake. Calcium activates anion channels leading to $Cl^-$ and malate$^{2-}$ efflux and membrane depolarisation, which, together with cytoplasmic alkalisation, triggers the activation of outward rectifying $K^+$ channels allowing $K^+$ efflux. High $CO_2$, through an undetermined signalling pathway, also results in a calcium rise but without pH change. Thus, most of the calcium dependent downstream elements of the response could be shared with ABA signalling. The main difference is that high $CO_2$ does not modify the cytoplasmic pH but triggers the activation of outward rectifying $K^+$ channels by an unknown mechanism. The question marks indicate a lack of knowledge and this is particularly evident concerning the fate of carbohydrates during stomatal closure. RL, red light; BL, blue light; As, anion sym(anti)porters; ATPase, proton pumps; Kin, inward rectifying $K^+$ channels; Kout, outward rectifying $K^+$ channels; Phot, phototropins; R/S, rapid and slow anion channels; ST, sugar transporters; PLC/PLD, phospholipases C/D.

Besides the classical biochemical and fluorescence approaches to study the role of the guard cell chloroplast other approaches can now be used. The effects of a lack of guard cell chloroplasts have been addressed in *Paphiopedilum* species. However, these orchids could have evolved compensatory mechanisms as the result of a long evolution and might not represent an actual 'disruption' of the chloroplastic pathway. The role of the guard cell chloroplast could be addressed by manipulating the number of chloroplasts using either pharmacological tools (Izumi *et al.*, 2003) or *Arabidopsis* mutants affected in chloroplast division (Robertson *et al.*, 1995; Larkin *et al.*, 1997). Modulation of the expression level of key guard cell metabolic enzymes is another way to decipher the respective roles of different metabolic pathways. This is illustrated by the work of von Caemmerer *et al.* (2004) working with anti-Rubisco plants and in Gehlen *et al.* (1996) who observed that stomatal opening was delayed in PEPC antisense *S. tuberosum* plants and accelerated in plants overexpressing PEPC from *Corynebacterium glutamicum*. Such approaches can now be undertaken specifically at the guard cell level aim to the increasing knowledge about guard cell specific promoters (Taylor *et al.*, 1995; Plesch *et al.*, 2000; Plesch *et al.*, 2001).

Following the pioneering work of Leonhardt *et al.* (2004), using genechips holding about one-third of the full *Arabidopsis* genome, the recent availability of full genome microarrays opens the way for exhaustive expression profiling in guard cells. This powerful tool will help in understanding guard cell global gene expression changes modulated by environmental signals. Screening and characterisation of null mutants has been and continues to be a powerful tool in the identification of components involved in ABA signalling. Infrared thermography (Merlot *et al.*, 2002) is an ideal approach for the identification of mutants with altered stomatal response to $CO_2$.

In the course of this review we underlined the strong interaction between ABA and $CO_2$ sensing. It is striking that, while a legion of messenger systems are now clearly identified upstream of $[Ca^{2+}]_{cyt}$ in ABA signalling, for example cyclic ADP-ribose, inositol 1,4,5 triphosphate, active oxygen species, nitric oxide, phospholipase C, phospholipase D (see Schroeder *et al.*, 2001; Garcia-Mata *et al.*, 2003; Hunt *et al.*, 2003), their participation in $CO_2$ sensing has not been investigated. The authors would like to stress that all the molecular tools developed in the course of these studies are potentially applicable to investigations of $CO_2$. For example, few of the numerous mutants affected in their stomatal response to ABA have been studied at the level of their $CO_2$ response. Such studies could determine common and independent elements in the respective signalling pathways. Additionally, they would allow us to ask whether some components are specifically involved in $CO_2$-induced stomatal closure or $CO_2$-inhibition of stomatal opening as already demonstrated for ABA (Li *et al.*, 2000). The recent identification of the *ost1* ABA-insensitive mutant is of particular interest. While this mutant is fully impaired in the guard cell ABA response it displays a wild-type response to $CO_2$. Thus, *ost1* is an ideal tool to study calcium signalling in relation with $CO_2$ without side-effects from ABA signalling. Comparison of $[Ca^{2+}]_{cyt}$ transient triggered by elevated $CO_2$ in *ost1* and wild type plants could reveal key elements in the interaction between $CO_2$ and ABA.

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