Are diurnal patterns of stomatal movement the result of alternating metabolism of endogenous guard cell ABA and accumulation of ABA delivered to the apoplast around guard cells by transpiration?

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Abstract
Abscisic acid (ABA) prevents opening of closed stomata and causes open stomata to close. A dual-source model is proposed linking ABA to diurnal stomatal movements. Darkness would favour guard cell biosynthesis of endogenous ABA and disfavour ABA catabolism. At first light, xanthophyll cycling, isomerization of ABA precursors, and activation of a cytochrome P450 mono-oxygenase (CytP450) would deplete endogenous guard cell ABA. The NADPH-requiring CytP450 would be activated by elevated O₂ and reduced CO₂ concentrations resulting from mesophyll photosynthesis. An increased O₂-to-CO₂ ratio would limit the Calvin cycle in guard cells, diverting NADPH produced by photosynthetic electron transport to the cytosol where, along with elevated O₂, it would activate CytP450. Depletion of endogenous ABA would liberate guard cells to extrude protons and accumulate the ions and water needed to increase guard cell turgor and open stomata. By midday, stomata would be regulated by steady-state concentrations of ABA delivered to the apoplast around guard cells by transpiration. In temperate conditions, ABA would reach concentrations high enough to trigger ion efflux from guard cells, but too low to defeat the accumulation of sugars used to maintain opening. In dry conditions, ABA would reach effective concentrations by midday, high enough to trigger ion efflux and inhibit sugar uptake, reducing apertures for the rest of the day. At sunset, conditions would again favour biosynthesis and disfavour catabolism of endogenous guard cell ABA. The model can be used to reconcile proposed cellular mechanisms for guard cell signal transduction with patterns of stomatal movements in leaves.

Key words: ABA, abscisic acid, guard cells, metabolism, stomata.

Introduction
The plant hormone abscisic acid (ABA) can prevent the opening of closed stomata and cause open stomata to close. Elegant molecular studies are revealing the signal transduction mechanisms by which ABA governs guard cell turgor (Assmann and Xi-Qing, 2001; Hetherington, 2001; Schroeder et al., 2001a, b). Even so, few attempts have been made to reconcile the patterns of ABA localization, distribution, and/or metabolism with the patterns of diurnal stomatal movements in intact plants. Nor have light and CO₂ responses of guard cells in isolated epidermis been adequately reconciled with those of stomatal responses in intact leaves (for a recent review see Outlaw Jr, 2003). In this opinion paper, which is meant to integrate existing literature and provoke new thinking, a dual-source model consistent with extant data is proposed that links ABA to diurnal patterns of stomatal movement in C₃ plants such as Vicia faba L. (faba bean) growing in temperate or dry conditions. It is suggested that these patterns are the result of triphasic alternation of (i) early daylight depletion of endogenous guard cell ABA; (ii) midday delivery of ABA by transpiration to the apoplast around guard cells at steady-state concentrations that vary with transpiration rates; and (iii) accumulation of endogenous ABA by guard cells in the dark period following each daylight period.

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A paradigm based on the metabolism of endogenous guard cell ABA is also presented to reconcile stomatal responses observed in detached leaf epidermis with those observed in intact leaves.

**Dual-source ABA regulation of diurnal stomatal movements**

A dual-source model for ABA-mediated regulation of diurnal stomatal movements is illustrated in Fig. 1. At dawn, increasing light levels, increasing intercellular leaf oxygen ($O_2$) concentrations, and decreasing concentrations of leaf intercellular carbon dioxide ($C_i$), all of which signal the beginning of photosynthetic carbon assimilation, would activate the depletion of endogenous, unconjugated (Grantz et al., 1985) guard cell ABA (Fig. 1, bottom; Singh et al., 1979; Weiler et al., 1982; Grantz et al., 1985). Destruction of endogenous ABA would liberate guard cells to extrude protons ($H^+$) using the P-type plasma membrane $H^+$-translocating ATPase ($H^+$-pump; Fig. 2; Assmann et al., 1985; Shimazaki and Kondo, 1987) and accumulate the ions ($K^+$, $Cl^-$, and malate) and water needed to generate turgor and open stomata (Schwartz et al., 1994; Outlaw Jr, 2003).

An increase in the ratio of $O_2$ to $CO_2$ in guard cells would favour the activation of a cytosolic cytochrome P450 mono-oxygenase (CytP450) that would catalyse the first step in endogenous guard cell ABA catabolism to 8′-hydroxy-ABA (Fig. 2; Krochko et al., 1998; see the author’s interpretation below of Trejo et al., 1993). Activation of the haem-containing, light-absorbing CytP450 requires both $O_2$ and NADPH (Krochko et al., 1998). Inside guard cells of illuminated leaves, falling $CO_2$ and increased competition by $O_2$ for what are often small amounts of guard cell Rubisco (Hamp et al., 1982; Outlaw Jr et al., 1982; Gotow et al., 1988; Outlaw Jr, 1989; Shimazaki, 1989; Tarczynski et al., 1989; Reckmann et al., 1990; Gautier et al., 1991; Cardon and Berry, 1992; Mawson, 1993; Talbott and Zeiger, 1993; Goh et al., 2002b; Lawson et al., 2002) would limit carbon fixation by the Calvin cycle (Fig. 2). Without Calvin cycle intermediates as reductants, some fraction of the accumulating stromal NADPH produced by linear photosynthetic electron transport in guard cell chloroplasts (Zeiger et al., 1980; Outlaw Jr et al., 1981; Tsionsky et al., 1997; Goh et al., 2002a, b) would be diverted to the cytosol where, along with high $O_2$ concentrations produced mainly by water-splitting in mesophyll chloroplasts, it would increase the activity of a guard cell CytP450 to accelerate the conversion of ABA to 8′-hydroxy-ABA (Fig. 2; Krochko et al., 1998).

Another fraction of the NADPH accumulating in the stroma would be used to reduce oxaloacetate (OAA) to malate using a chloroplastic, NADPH-dependent malate dehydrogenase (MDH; Fig. 2; Gotow et al., 1985). The activity of this MDH is enhanced by blue light (Gotow et al., 1985). OAA would be produced by carboxylation of phosphoenol pyruvate (PEP) in a reaction catalysed by guard cell PEP carboxylase (Outlaw Jr, 1990; Denecke et al., 1993; Tarczynski and Outlaw Jr, 1993; Wang et al., 1994; Meinhard and Schnabl, 2001; Outlaw Jr et al., 2002). By virtue of its relatively high affinity for bicarbonate ions, active PEP carboxylase would ensure that $CO_2$ concentrations in guard cells would remain low during stomatal opening, thereby minimizing the potential for Calvin cycle activity and maximizing NADPH availability for CytP450 activation and malate biosynthesis catalysed by the NADPH-dependent MDH.

At the same time, guard cell ABA biosynthesis would be restricted by the removal of the ABA precursor, violaxanthin (V; Fig. 2; Xiong and Zhu, 2003) through light-driven xanthophyll cycling which converts V to zeaxanthin (Z; Eskling et al., 1997; Gilmore, 1997). While Z is proposed to be the blue-light-specific photoreceptor of guard cells (Frechilla et al., 1999; Talbott et al., 2003b), conversion of V to Z as part of a mechanism to regulate endogenous guard
cell ABA turnover would offer an additional explanation for the high correlation between guard cell Z formation and stomatal opening induced by increased light levels and/or reduced CO$_2$ concentrations (Quinones et al., 1996; Zeiger and Zhu, 1998; Zeiger, 2000). Low fluences of blue light would also inhibit the biosynthesis of the active isomer of ABA (cis-(+)-S-ABA) by causing photo-isomerization of the ABA carotenoid precursors 9-cis-neoxanthin, 9-cis-violaxanthin, and/or cis-xanthoxin to their trans isomers (Fig. 2; Schwartz et al., 2003). Metabolism of these trans isomers yields physiologically inactive trans-ABA instead of the active cis-(+)-S-ABA (Fig 2; Schwartz et al., 2003).

In a later, midday daylight phase, ABA from foliar tissue (Cornish and Zeevart, 1985) or arriving through the xylem transpiration stream (Davies and Zhang, 1991; Mansfield and De Silva, 1994; Hartung et al., 1998; Davies, 2002) and concentrating in the apoplastic surrounding guard cells (Zhang and Outlaw Jr, 2001a, b, c) would determine the time of day at which ions would leave guard cells (Fig. 1; Talbott and Zeiger, 1998). Under conditions favourable for photosynthesis and apoplastic sucrose loading (Outlaw Jr and De Vlieghere-He, 2001; Outlaw Jr, 2003), ABA might reach steady-state concentrations in the apoplast (and possibly in guard cells) sufficient to trigger ion efflux (Talbott and Zeiger, 1996; Zhang and Outlaw Jr, 2001a, c) but below a threshold effective concentration required to defeat guard cell H$^+$-sugar symporters (Fig. 1, bottom; Kopka et al., 1997; Ritte et al., 1999; Outlaw Jr, 2003; Stadler et al., 2003). Under such conditions, ions lost from guard cells would be replaced by sucrose (Fig. 1, top; Lu et al., 1995, 1997; Talbott and Zeiger, 1998) and stomata would remain open over the rest of the day (Fig. 1, top; Talbott and Zeiger, 1998).

Under dry conditions, effective concentrations of ABA might become high enough both to trigger ion efflux and to inhibit guard cell sucrose uptake. If so, stomata would open less and/or close earlier in the day (Macdowall, 1963; Tenhunen et al., 1980). As one possibility, ABA-induced suppression of the guard cell H$^+$-translocating ATPase (below; Assmann, 1993; Kinoshita et al., 1995) combined with alkalinization of the apoplast (Wilkinson and Davies, 1997; Felle and Hanstein, 2002) could reduce the availability of H$^+$ for H$^+$-sugar symporters. While an alkalinizing apoplast would be expected to decrease ABA transport into guard cells (Wilkinson and Davies, 1997; Felle and Hanstein, 2002), ABA might still act externally (Homberg and Weiler, 1984; Curvetto and Delmastro, 1986; Anderson et al., 1994; Assmann, 1994; MacRobbie, 1995, 2000; Yamazaki et al., 2003) through an oscillatory amplification cascade (Ng et al., 2001b) to reach effective concentrations sufficient to inhibit H$^+$ extrusion by the guard cell H$^+$-pump (Kinoshita et al., 1995). A combination of apoplastic alkalinization and pump inhibition might then deprive H$^+$-sugar symporters (Stadler et al., 2003) of the H$^+$ needed to drive proton-dependent sugar accumulation. Alternatively,
at lower concentrations, ABA might facilitate sugar uptake by guard cells through its effects on membrane potential created by ABA-activated ion efflux, but thwart sugar accumulation at higher concentrations (Gibson, 2004). Under dry conditions, sucrose concentrating in the apoplast could also significantly reduce midday stomatal apertures (Lu et al., 1995, 1997). Under these conditions, high apoplastic ABA concentrations (Zhang et al., 2001) might exceed the capacity of guard cells to catabolize ABA internalized from the apoplast (Zhang and Outlaw Jr, 2001b) in daylight hours. If so, internalized ABA could increase the accumulated pool of ABA that would have to be degraded the next morning to trigger stomatal opening.

At sunset, conditions favouring endogenous guard cell ABA biosynthesis would prevail once again, maintaining stomata in the closed position until the next daylight period. In darkness, net O₂ consumption by mesophyll and guard cell respiration and lack of NADPH would disfavour guard cell ABA degradation, while the formation of V from Z would favour endogenous ABA biosynthesis by guard cells. Under dry conditions, guard cells might also contain additional ABA accumulated from the apoplast over daylight hours (above).

Under temperate conditions, the first early daylight phase would guarantee that a certain amount of the transpiration needed for nutrient transport would occur early, in the cooler hours of the day, when plant water potentials were least negative and evaporative demand was at its lowest. The later, midday daylight phase, regulated by steady-state concentrations of apoplastic ABA around guard cells, would enable plants to gauge whether leaves could withstand the maximum rates of transpirational water loss over the remainder of the day or whether reduced rates would be required to maintain plant turgor. Through this connection to hydraulic regulation of plant water potential (Tardieu et al., 1992; Tardieu and Davies, 1993), stomata would exert control over rates of photosynthetic carbon assimilation and diurnal net carbon gain (Jarvis and Davies, 1998), at least at certain times of the day. Finally, the third, dark phase would ensure that stomata would remain closed to maximize tissue rehydration and dilution, absorption, conjugation, and/or metabolism of residual apoplastic ABA before the next early daylight phase occurred. Tightly closed stomata would also ensure a rapid increase in intercellular leaf O₂ concentrations and lowered C₄ before stomata opened at the beginning of the next daylight period. Both would favour depletion of endogenous guard cell ABA and stomatal opening.

Why do guard cells have chloroplasts?

The model suggests why (i) guard cells of C₄ plants growing in temperate conditions have chloroplasts (Outlaw Jr et al., 1981; Zeiger et al., 2002) while other epidermal cells do not; and (ii) why guard cell chloroplasts often (but not always; Gotow et al., 1988; Tallman and Zeiger, 1988; Cardon and Berry, 1992; Lawson et al., 2002) possess limited Calvin cycle capacity. In daylight hours, guard cells would catabolize both endogenously produced ABA and at least some of the ABA absorbed from the apoplast, and so guard cells would require chloroplasts to synthesize internally the ABA required to regulate the basal activity of the plasma membrane H⁺-pump and various ion channels required to maintain stomata in the closed position in dark periods when no transpiration was desired. At dawn, light and falling C₄ would initiate the depletion of endogenous guard cell ABA and stomatal opening by disturbing multiple equilibria reactions for biosynthesis, isomer interconversion, and/or catabolism of ABA and its precursors (Fig. 2). The limited Rubisco activity of guard cell chloroplasts would maximize the dissipation of light energy through the conversion of V to Z, decreasing the supply of carotenoid precursors for ABA biosynthesis and maximizing NADPH accumulation from photosynthetic electron transport at relatively low light levels. Both Z formation and NADPH accumulation would favour the depletion of guard cell ABA in daylight (above; Fig. 2), even on cloudy days or in shaded environments.

In the model presented here, ABA catabolism in guard cells would be in an inverse proportion to guard cell Calvin cycle capacity. Studies made with various methods report a wide range of photosynthetic carbon fixation rates by the Calvin cycle in guard cells from an assortment of species grown under a wide array of seasonal and environmental conditions (Hampp et al., 1982; Outlaw Jr et al., 1982; Gotow et al., 1988; Outlaw Jr, 1989; Shimazaki, 1989; Tarczynski et al., 1989; Reckmann et al., 1990; Gautier et al., 1991; Cardon and Berry, 1992; Mawson, 1993; Talbott and Zeiger, 1993; Goh et al., 2002; Lawson et al., 2002). Recently, it has been suggested (Outlaw Jr, 2003) that sucrose uptake by guard cells might suppress expression of genes for the photosynthetic carbon reduction pathway. This hypothesis has not been tested, but would be consistent with this model and published reports. For example, on days that favoured high rates of photosynthesis and sucrose loading into the apoplast, sucrose accumulating in guard cells could repress the transcription of Rubisco genes as it does in mature leaves of greenhouse-grown plants of sugar beet (Kovtun and Dale, 1995) and other plants (Lalonde et al., 1999; Pego et al., 2000; Roland et al., 2002). The next day, guard cells would have little Calvin cycle activity and would readily catabolize both internally produced and any exogenously supplied ABA, favouring stomatal opening. On days when guard cells could not accumulate sucrose (e.g. a day on which transpiration rates, and therefore, apoplastic ABA concentrations, were high), guard cell Rubisco levels might rise, reducing the capacity of guard cells to catabolize ABA in the next light period and reducing stomatal opening. The hypothesis is attractive, but apparent exceptions have already been reported (Lawson et al., 2002). Such exceptions might be explained by the observation that sugars
regulate other genes with the potential to regulate stomatal movements as well.

In *Arabidopsis* sugars and ABA act synergistically to induce the transcription of ABA2/GIN1, the only gene known to encode the unique short-chain alcohol dehydrogenase/reductase, SDR1, that catalyses the conversion of cis-xanthoxin to ABA-aldehyde during ABA biosynthesis (Cheng et al., 2002; Xiong and Zhu, 2003). This gene is not expressed at high levels in guard cells of *Arabidopsis* seedlings grown on glucose-containing media (Cheng et al., 2002). *Arabidopsis* guard cells do apparently contain the aldehyde oxidase, AAO3, that catalyses the conversion of ABA-aldehyde to ABA (Koiwai et al., 2004), raising the question of why guard cells would have one of the cytoplasmic enzymes for ABA biosynthesis but not the other. One possible explanation is that expression of guard cell ABA2/GIN1 homologues is diurnally regulated; the diurnal pattern of ABA2/GIN1 expression in guard cells in plants growing in less contrived environments has not been investigated. Hypothetically, ABA in the transpiration stream and sugars entering the guard cells from midday onwards could act synergistically (Cheng et al., 2002) to increase the expression of guard cell ABA2/GIN1 homologues so that guard cells would be prepared to synthesize the ABA required to close stomata and maintain stomatal closure in the ensuing dark period. If so, sugar accumulation in the guard cells might simultaneously suppress Rubisco formation and increase guard cell capacity for ABA catabolism while acting in concert with ABA in the transpiration stream to increase the capacity for ABA biosynthesis. When superimposed upon the developmental history of the plant, the integrated balance among these processes under any particular set of conditions might regulate the sensitivity of guard cells to various environmental signals and thereby regulate stomatal dimensions.

The model suggests that endogenous guard cell ABA catabolism signalled initially by an increased ratio of O₂ to CO₂, and not activation of the guard cell plasma membrane proton pump (Outlaw Jr, 2003), may be the primary event in stomatal opening. If so, signalling would be from the ‘inside, out’ through the chloroplast to the plasma membrane, rather than from the ‘outside, in’ from the plasma membrane to the chloroplast. Both in intact leaves (Assmann, 1988; Doi et al., 2004) and in detached leaf epidermis (Schwartz and Zeiger, 1984; Tallman and Zeiger, 1988), stomatal opening induced by low fluences of blue light is enhanced by pre-illumination with background red light. In intact leaves, saturating red light alone produces stomatal conductances that are 60–70% of those that can be achieved with added blue light (Assmann, 1988; Doi et al., 2004). In *Arabidopsis*, stomatal conductances produced by saturating red light are substantially increased by superimposing blue light on the red light background. Despite this significant increase in conductance, however, only very small increases in rates of photosynthetic carbon assimilation are observed (Doi, 2004), suggesting that red light and reduced Cᵢ can produce conductances that are non-limiting for photosynthesis. In detached leaf epidermis, the guard cell H⁺-pump can be activated by red light (Serrano et al., 1988), especially in a reduced CO₂ atmosphere (Olsen et al., 2002; Roelfsema et al., 2002). Under saturating red light with reduced CO₂, stomatal opening is both ion- and chloroplast-dependent (Olsen et al., 2002). Taken together, these data suggest that the guard cell chloroplast may have to ‘license’ the guard cell plasma membrane before processes like phototropin-induced activation of the H⁺-pump can occur (Kinoshita et al., 2001; Doi et al., 2004). Depletion of endogenous guard cell ABA in response to the increased ratio of O₂ to CO₂ in the early daylight hours could license the H⁺-pump to activate initial stomatal opening. Such a mechanism would link early stomatal movements with leaf metabolism and identify the O₂ to CO₂ ratio as a potential ‘mesophyll messenger’ (Dittrich and Raschke, 1977; Wong et al., 1979).

**Other plant correlates**

A dual-source model of alternating endogenous guard cell and apoplastic ABA concentrations could account for a number of interesting adaptation responses of stomata observed in various studies with leaves of intact plants.

The CO₂ sensitivity of stomata in leaves of *V. faba* plants grown at a lower humidity in a greenhouse is less than that of plants grown at higher humidity in a growth chamber (Talbott et al., 1996). When greenhouse plants are misted or moved to the chamber, their sensitivity to reduced CO₂ levels increases within 5–7 d (Frechilla et al., 2002; Talbott et al., 2003a). Conversely, the CO₂-sensitivity of stomata of plants moved from the growth chamber to the greenhouse decreases within 2–3 d (Frechilla et al., 2002). Low humidity equates to higher vapour pressure differences (VPD) between leaves and air (Assmann and Grantz, 1990; Mott and Parkhurst, 1991) and potentially higher rates of transpiration, while high humidity equates to lower VPD and potentially lower transpiration rates.

In plants moved from the greenhouse to the growth chamber, higher humidity might be expected to decrease rates of transpiration so that lower steady-state concentrations of ABA would accumulate in the apoplast around guard cells. Over a few days, guard cells would come to contain a new, lower combined concentration of endogenously-synthesized ABA and ABA accumulated from the apoplast than that which they harboured in the greenhouse under lower humidity at higher transpiration rates. Thereafter, this new, reduced, basal concentration of endogenous guard cell ABA would allow stomata to open more fully initially when CO₂ concentrations around leaves were reduced.

At low humidity with high transpiration rates in the greenhouse, ABA might reach higher steady-state concentrations in the apoplast. Under such conditions, guard cells...
of chamber-grown plants moved to the greenhouse would come to contain a new, higher combined concentration of endogenously-synthesized ABA and ABA accumulated from the apoplast than the concentration they harboured in the growth chamber under higher humidity at lower transpiration rates. If so, stomata flanked by guard cells with higher basal concentrations of endogenous ABA would not open as fully initially in response to reduced CO₂ concentrations around leaves.

The osmotica used for stomatal opening vary with time of day (Talbott and Zeiger, 1996). Early in the daylight period, ions are used in the initial phase of stomatal opening (Talbott and Zeiger, 1996). Later in the day, ions are replaced by sucrose (Talbott and Zeiger, 1996). If elevated external CO₂ concentrations are used to delay opening until late in the light period when guard cells would normally use sucrose as the major osmoticum, guard cells accumulate ions when CO₂ concentrations are finally lowered to allow stomata to open (Talbott and Zeiger, 1996). Later in the day, however, CO₂-induced closing and reopening depends on the time of day, with ions used early in the light period and sucrose used later in the light period (Talbott and Zeiger, 1996).

Delaying the initial stomatal opening by elevating CO₂ might be expected to increase the Calvin cycle in guard cells, lowering cytosolic NADPH and shifting xanthophyll cycling to produce some V as more light energy is dissipated by guard cell carbon fixation. Even in the O₂-rich environment of the illuminated leaf, diversion of the NADPH required for cytosolic CytP450 mono-oxygenase activity into guard cell chloroplastic carbon fixation, coupled with Z to V interconversion as light energy is reallocated to photochemistry, could restore guard cell ABA to levels just high enough to block stomatal opening.

After initial stomatal opening, upon subsequent rounds of CO₂-induced closing and reopening, which osmoticum is used would be a function of ABA concentrations created by combined endogenous ABA biosynthesis by guard cells and ABA accumulation in and/or from the apoplast. In morning hours before much transpirational ABA had concentrated in the apoplast, endogenous concentrations of ABA upon closing would be mainly from internal guard cell biosynthesis. Upon reopening, apoplastic concentrations at these hours of the day might still be too low to prevent either ion accumulation or sucrose uptake by guard cells. Later in the day, however, CO₂-induced closure might result in a combined concentration of ABA produced endogenously and accumulated in and/or from the apoplast that would inhibit ion uptake but permit sucrose accumulation (Fig. 1, bottom).

Stomata on the abaxial (bottom, shaded) surfaces of V. faba leaves come to full opening at a light intensity approximately 10 times lower than that required to produce full opening on their adaxial (top) surfaces (Yera et al., 1986). When leaves are inverted in a dark period between light periods so that the abaxial surface faces up at the beginning of the next light period, this pattern of light sensitivity is reversed immediately in the very next light period (Yera et al., 1986). There are intrinsic differences in the sensitivity of abaxial and adaxial guard cell protoplasts to blue light (Goh et al., 1995) and Ca²⁺-mediated responses to external ABA (Wang et al., 1998). However, immediate adaptation of whole leaves to a new light environment created by altered leaf orientation could also be explained by the regulation of stomatal movements through the O₂/CO₂ control of endogenous guard cell ABA turnover. Stomata on both surfaces might be expected to reach maximum opening at a light level saturating for mesophyll photosynthesis that also provides light of sufficient intensity on the lower leaf surface to saturate guard cell xanthophyll cycling and maximize guard cell NADPH production at the lowest C₄ possible (Fig. 2). Diurnal patterns observed in these experiments (Yera et al., 1986) included a midday ‘depression’ in stomatal conductance which may reflect the net opening achieved as guard cell ion efflux triggered by apoplastic ABA and sucrose uptake cross over each other in time (Fig. 1, top; Talbott and Zeiger, 1998).

Some plants undergo stomatal oscillation during the daylight hours, periodically opening, closing, and reopening their stomata throughout the day (Cowan, 1972; Farquhar and Cowan, 1974; Hirose et al., 1994; Rose et al., 1994; Herppich and von Willert, 1995; Kaiser and Kappen, 2001). The model could explain these observations if the hydraulic conductivity of the plant were such that leaf tissues could accumulate ABA in the apoplast rapidly, and then, upon stomatal closure, rehydrate rapidly to dilute the apoplastic ABA to allow stomata to reopen. Similarly, the uneven distribution of transpirational ABA concentrations across leaf area might explain the patchy stomatal opening and closing observed in some plants (Mott, 1995; Buckley et al., 1997; Haefner et al., 1997; Mott et al., 1999; Santrucek et al., 2003).

Even in the late stages of leaf senescence, the chloroplasts of guard cells remain green and functional (Zeiger and Schwartz, 1982; Heaton et al., 1987). The maintenance of guard cell viability in yellowing leaves is thought to hold stomata in the closed position to ensure that senescing leaves survive long enough to allow nutrient recycling back into stems before leaf abscission occurs (Heaton et al., 1987). Stomata in epidermis detached from senescing leaves of Nicotiana glauca open in response to light and reduced concentrations of CO₂, but stomata of illuminated senescing leaves remain closed, even under high light at very low C₄ (Ozuna et al., 1985; Heaton et al., 1987; Thomas et al., 1991). While senescing leaves might not have the capacity to generate the high concentrations of O₂ needed to activate the mono-oxygenase activity of guard cell CytP450, the concentrations required in vitro are well below ambient (Krochko et al., 1998). Thus, intact senescing leaves may
have high levels of apoplastic ABA or, for lack of a transpirational source of ABA, use sphingosine-1-phosphate or other signals to maintain stomata in the closed position (Ng et al., 2001a; Coursol et al., 2003).

Under continuous light, free-running stomatal circadian rhythms (Dodd et al., 2004) could be explained by oscillatory changes in the activity of enzymes like Rubisco activase that would affect guard cell Rubisco activity; the gene for this enzyme is under circadian control in other plant tissues (Harmer et al., 2000).

Extending the model to microclimates of radiation and water vapour surrounding leaves at various positions within the canopy, seasonal variations in photoperiods and water availability, diurnal variations in light quality, and plant developmental processes regulated by hormones (e.g. flowering and leaf senescence) could explain the diversity of stomatal responses (and some discrepancies among experiments) reported in the literature (Olsen et al., 2002; Zeiger et al., 2002).

**Reconciling stomatal responses in isolated epidermis and leaves**

To reconcile the responses of guard cells to light and CO₂ in experiments with detached leaf epidermis with the stomatal responses of intact leaves, the following assumptions are made.

(i) There is a basal constitutive level of activity of the guard cell plasma membrane H⁺-pump that contributes to the resting membrane potential of guard cells in darkness.

(ii) By mechanisms yet to be fully elucidated, endogenous guard cell ABA attenuates the activity of the pump in darkness so that solute transport rates that depend on H⁺ extrusion are low enough that no stomatal opening occurs against the counterforce of neighbouring epidermal cells.

(iii) Initial stomatal opening in intact leaves derives mainly from increased activity of the H⁺-pump.

(iv) The pump is ‘activated’ by stabilizing it in its phosphorylated state.

(v) Multiple mechanisms involving kinases and phosphatases regulate the amount of time the pump is phosphorylated and, hence, active (i.e. in its stabilized, phosphorylated state).

Two physiologically-relevant conditions activate the guard cell H⁺-pump, guard cell K⁺ uptake, chloroplastic starch catabolism, and stomatal opening in isolated *V. faba* epidermis: low fluences of blue light (Schwartz and Zeiger, 1984; Tallman and Zeiger, 1988; Talbott and Zeiger, 1993) and reduced concentrations of CO₂ (Fischer, 1968; Olsen et al., 2002; Roelfsema et al., 2002). To distinguish the components of the blue light and CO₂ signal transduction pathways, the effects of both of these signals have been measured under saturating red light (above; Schwartz and Zeiger, 1984; Tallman and Zeiger, 1988; Talbott and Zeiger, 1993; Olsen et al., 2002; Talbott et al., 2003b).

The high correlation between Z levels and both blue light-induced stomatal opening in detached epidermis and CO₂-induced opening in whole leaves led to the hypothesis that both blue light and CO₂ responses of guard cells are mediated through Z as a blue light photoreceptor (Zeiger and Zhu, 1998; Zeiger, 2000). As envisioned in that model, declining CO₂ would limit the guard cell Calvin cycle, further driving up Z levels to dissipate excess light energy, and thereby increasing guard cell sensitivity to blue light (Zeiger, 2000). The green-light-reversibility of the specific blue light response of guard cells also led to the hypothesis that interconversion of Z between cis and trans isomers might allow Z to act as a signalling switch for stomatal opening (Zeiger and Zhu, 1998).

While there is evidence for Z as a specific blue light photoreceptor in guard cells (Frechilla et al., 1999; Talbott et al., 2003), blue light is not required to activate the plasma membrane H⁺-pump (Serrano et al., 1988; Olsen et al., 2002; Roelfsema et al., 2002) or to initiate stomatal opening, either in intact leaves (Assmann, 1988; Doi et al., 2004) or in detached leaf epidermis (Olsen et al., 2002). Under levels of red light saturating for photosynthesis, both in intact plants (Assmann, 1988; Doi et al., 2004) and in detached leaf epidermis (Olsen et al., 2002), reduced concentrations of CO₂ effectively induce stomatal opening. Furthermore, in intact leaves of *Arabidopsis*, stomatal opening produced by saturating levels of red light appears to be non-limiting for photosynthetic carbon assimilation (Doi et al., 2004).

The hypothesis linking Z to guard cell CO₂ responses would also be explained by the modified model advanced here in which processes affecting equilibria of various reactions governing turnover of endogenous guard cell ABA would control stomatal opening. In this model (Fig. 2), the mechanism by which the proton pump would be licensed for activation would depend on whether blue light or reduced CO₂ was used to alter ABA activity in guard cells.

Three processes would regulate the turnover of endogenous guard cell ABA: (i) removal of V by light-driven xanthophyll cycling (Gilmore, 1997) and consequent reduction of the pool of 9-cis-epoxycarotenoid and cis-xanthoxin precursors available for conversion to ABA (Fig. 2; Schwartz et al., 2003); (ii) blue-light-induced conversion of 9-cis-epoxycarotenoid precursors or cis-xanthoxin to their trans isomers (Fig. 2; Schwartz et al., 2003); and (iii) activation of a CytP450 mono-oxygenase in guard cells (Fig. 2; Schwartz et al., 2003).

To trigger stomatal opening, combinations of these processes would have to deplete guard cell ABA concentrations sufficiently to allow activation of the guard cell H⁺-pump. Under saturating red light at ambient CO₂ concentrations, guard cells would convert V to Z by xanthophyll...
cycling (Fig. 2; Zeiger and Zhu, 1998; Zeiger et al., 2002). Superimposing low fluences of blue light on a saturating background of red light would isomerize at least a fraction of the remaining small pool of V-derived cis precursors of ABA (9-cis-epoxycarotenoid precursors and cis-xanthoxin) to trans isomers, resulting in the production of inactive trans-ABA (Fig. 2; Schwartz et al., 2003). Combined with endogenous CytP450 activity, ABA concentrations would fall below the threshold concentration required to maintain the guard cell H+-pump in its constitutive state of low activity, and the pump would be activated through a phototropin-mediated mechanism (Fig. 2; Kinoshita et al., 2001; Doi et al., 2004). Reducing CO2 concentrations under saturating red light would limit guard cell Calvin cycle activity, causing even more V to be converted to Z to dissipate light energy no longer being dissipated by photosynthesis and increasing the pool of NADPH available from linear photosynthetic electron transport to activate CytP450 (Fig. 2). Along with O2 from water splitting by guard cell chloroplasts, NADPH would stimulate CytP450 mono-oxygenase activity and thereby accelerate ABA catabolism (Fig. 2; Krochko et al., 1998). By a combination of these processes, reduction of ABA below the threshold for pump activation would result in stomatal opening. Opening under red light at low CO2 is not inhibited by dithiothreitol (Olsen et al., 2002), which inhibits conversion of V to Z (Srivastava and Zeiger, 1995). Thus, CytP450-mediated catabolism of ABA would be the primary mechanism used to reduce endogenous ABA concentrations. Maximum ion-dependent stomatal opening in detached epidermis would be realized with blue light applied over a background of saturating red light in a reduced CO2 environment (Fig. 2; Schwartz and Zeiger, 1984).

Under saturating red light, regardless of whether low-fluence blue light or reduced CO2 was used to activate ion-dependent stomatal opening, convergent signal transduction pathways (Olsen et al., 2002) for blue light and CO2 would activate the H+-pump by stabilizing its C-terminal in a phosphorylated state (Kinoshita and Shimazaki, 1999; Emi et al., 2001) through binding of a 14-3-3 protein (Emi et al., 2001; Kinoshita and Shimazaki, 2002).

In ambient CO2, saturating red light would cause stomatal opening through photosynthetic sugar production (Gotow et al., 1988; Tallman and Zeiger, 1988). The Calvin cycle would consume NADPH, preventing activation of CytP450 and depletion of endogenous ABA, explaining why guard cells do not accumulate K+ and other ions under these conditions (Tallman and Zeiger, 1988). Very low fluences of blue light can trigger modest stomatal opening in ambient CO2 (Tallman and Zeiger, 1988; Talbott and Zeiger, 1996). Under this condition, starch is depleted from guard cell chloroplasts (Tallman and Zeiger, 1988), sugars appear early in opening (Talbott and Zeiger, 1996), and K+ appears later in guard cells (Tallman and Zeiger, 1988). Under these low fluences of blue light, a rapid release of glucose from starch could provide substrate for glucosylating ABA to its inactive conjugate (Grantz et al., 1985; Fig. 2).

Both Z and phototropin have been implicated as blue light photoreceptors of guard cells (Frechilla et al., 1999; Kinoshita et al., 2001). Arabidopsis mutants have been used (Frechilla et al., 1999; Eckert and Kaldenhoff, 2000; Kinoshita et al., 2001; Talbott et al., 2003b) in an attempt to distinguish the role(s) of these two putative photoreceptors in stomatal opening and any model advanced should be consistent with results of those studies.

The npq1 mutant of Arabidopsis, which does not convert V to Z (Frechilla et al., 1999), possesses a far-red-light reversible (but not green-light-reversible) blue light response under background levels of red light that are sub-saturating for guard cell photosynthesis, but this mutant has no blue light response under saturating levels of red light (Talbott et al., 2003b). Because npq1 guard cells can not convert V to Z they might be expected to have higher levels of endogenous ABA in daylight than guard cells of wild-type plants. If so, studies (Talbott et al., 2003b) would suggest that in this particular mutant at appropriate ratios of red to far-red light, phytochrome-mediated signal transduction pathways trigger stomatal opening by interfering with ABA signalling downstream of its biosynthesis or through processes that are ABA-insensitive (e.g. interconversion of guard cell starch to sugars). Phytochrome has also been implicated in stomatal opening in orchids, which have a chlorophyllous, but carotenoid-containing, plastids (Talbott et al., 2002).

The phot1phot2 double-phototropin mutant of Arabidopsis has no stomatal response to blue light under levels of red light that are sub-saturating for guard cell photosynthesis (Kinoshita et al., 2001), but does have a green-light-reversible (but not far-red-reversible) blue light response at saturating levels of red light (Talbott et al., 2003b). These data can be interpreted to mean that phototropin is required to activate the H+-pump, and recent experiments in which a phot1 transgene restored blue light-induced stomatal opening in the phot1phot2 double mutant of Arabidopsis are persuasive (Doi et al., 2004). They do not preclude the possibility, however, that depletion of endogenous guard cell ABA is required to license phototropin-mediated activation of the guard cell H+-pump.

Guard cells of phot1phot2 subjected to sub-saturating levels of red light that would yield less-than-complete conversion of V to Z (Kinoshita et al., 2001) might fail to isomerize enough of the pool of cis carotenoid precursors of ABA (e.g. 9-cis-epoxycarotenoids and cis-xanthoxin) to their trans forms under low fluences of blue light to prevent formation of enough cis-(+)-S-ABA to block stomatal opening. A similar argument could be used to explain why dithiothreitol blocks blue light-induced stomatal opening (Srivastava and Zeiger, 1995). Under saturating red light, the size of the cis carotenoid precursor pool would be further reduced by a more complete conversion of V to Z so
that very low fluences of blue light would be sufficient to accomplish photo-isomerization of the entire precursor pool to trans isomers which would be converted to the inactive trans isomer of ABA. Under a saturating red light background, a combination of xanthophyll cycling and blue light-induced photo-isomerization of epoxycarotenoid ABA precursors might reduce ABA levels sufficiently to allow activation of the guard cell H⁺-pump and stomatal opening in phot1phot2 (Talbott et al., 2003b). As envisioned, trans isomers of ABA carotenoid precursors created by blue light irradiation could be converted back to their cis isomers by irradiation with green light (Frechilla et al., 2000). Maximum green light reversibility in Arabidopsis appears to require a 2:1 fluence ratio of green to blue light (Frechilla et al., 2000; Talbott et al., 2003b). It should be noted that Cytp450 is, itself, a light-absorbing enzyme, and inhibition of Cytp450 by CO in vitro is substantially reversed both by blue light and by red light (Krochko et al., 1998). Still, all of the experiments with Arabidopsis epidermis reported above were performed in ambient CO₂ which would be expected to minimize the activity of this enzyme in guard cells (above).

In epidermis from Mesembryanthemum crystallinum plants induced with NaCl to develop CAM, failure of stomata to open under blue light is correlated with failure of guard cell chloroplasts to convert V to Z (Tallman et al., 1997). These data can be interpreted to mean that failure to create Z is a failure to create the specific blue light photoreceptor required to activate the H⁺-pump (Tallman et al., 1997). However, those data could be reinterpreted to mean that blue light can not obviate the effects of endogenous ABA in guard cells unless ABA turnover is first altered by at least removing a major precursor to its biosynthesis. Through destruction of endogenous guard cell ABA, both light and CO₂ would activate or inactivate the appropriate cation and anion channels to modulate ion uptake and prevent ion efflux during stomatal opening (Outlaw Jr., 2003). Proton extrusion must precede activation of voltage-gated channels like the inward-rectifying K⁺ channels of guard cells (Assmann, 1993; Schroeder et al., 2001a). Destruction of endogenous ABA would reduce dephosphorylation of the plasma membrane H⁺-pump to stabilize it in the active state through binding of a guard cell-specific 14-3-3 protein to its phosphorylated C-terminus (Emi et al., 2001; Kinoshita and Shimazaki, 2002; Kinoshita et al., 2003). Guard cell protoplasts contain protein phosphatases 1 and 2A that regulate K⁺ channel activity in whole-cell patch clamp experiments (Li et al., 1994), and mutation of a guard cell protein phosphatase 2A (PP2A) regulatory subunit gene, RCNI, confers ABA-insensitivity on Arabidopsis guard cells (Kwak et al., 2002). In guard cell protoplasts in the whole-cell patch clamp configuration, the mutation inhibits Ca²⁺-mediated activation of outward-rectifying anion channels required for stomatal closure (Kwak et al., 2002). The effects of this enzyme could extend to, or even be exercised in part, through regulation of the guard cell plasma membrane H⁺-pump. At least one plant PP2A is known to inactivate the plant plasma membrane H⁺-pump in vitro by catalysing pump dephosphorylation, thereby preventing binding of stabilizing 14-3-3 proteins to its C-terminus (Camoni et al., 2000).

At ambient CO₂ concentrations, saturating red light may activate stomatal opening by other mechanisms. For example, significant increases in the sucrose content of isolated guard cells have been measured that were dependent on guard cell photosynthetic electron transport and unaccompanied by detectable K⁺ uptake (Poffenroth et al., 1992; Talbott and Zeiger, 1993). These studies and others (Gotow et al., 1988; Cardon and Berry, 1992; Lawson et al., 2002) suggest that guard cells of plants grown under some conditions either have significant capacity for carbon fixation through the Calvin cycle and/or the capacity to convert starch to sugars under red light (Tallman and Zeiger, 1988; Outlaw Jr., 2003). Under saturating light levels, the chloroplast can provide the ATP required for the guard cell H⁺-pump (Mawson, 1993; Tominaga et al., 2001). It has been suggested that photosynthetic electron transport in guard cells may be required to supply enough ATP to activate the H⁺-pump fully, but full stomatal opening can be achieved in darkness with fuscoceocin (Turner, 1972; Squire and Mansfield, 1974). Furthermore, addition of DCMU at the cytoplasmic side of the membrane abolished red-light-induced H⁺ pumping in experiments in which patch-clamped guard cell protoplasts were simultaneously supplied with ATP through the pipette (Serrano et al., 1988), suggesting that chloroplast photosystem activity, but not chloroplast-derived ATP, is required to activate the guard cell H⁺-pump.

Two studies indicate that auxin-induced stomatal opening is mediated by auxin-induced ethylene production in guard cells (Levitt et al., 1987; Merritt et al., 2001). It is possible that auxin-induced ethylene induces stomatal opening by acting as an antagonist of endogenous guard cell ABA (Cheng et al., 2002). The model presented here is consistent with literature suggesting that in isolated epidermis, conditions that more closely mimic those of intact leaves produce the greatest degree of stomatal opening. In intact leaves, guard cells are enveloped in an O₂-rich, reduced CO₂ environment lustrous with both red and blue light. Thus, the synergism between blue and red light in maximizing stomatal opening in detached leaf epidermis (Ogawa et al., 1978; Schwartz and Zeiger, 1984; Tallman and Zeiger, 1988) is probably more than coincidental.

**Future opportunities**

Many opportunities for interesting experiments remain: whether there are conditions under which guard cells synthesize ABA is not known conclusively. One report...
suggests that guard cell protoplasts can ‘form’ ABA (Weiler et al., 1982) under osmotic stress; another indicates that guard cell protoplasts maintain ABA levels under osmotic stress (Lahr and Raschke, 1988). In another experiment, application of xanthoxin to epidermal strips from Commelina communis leaves did not cause stomatal closure but application of ABA-aldehyde did cause closure (Raschke et al., 1975). These results are consistent with the observations that Arabidopsis guard cells do not express high levels of ABA2/GIN1 (Cheng et al., 2002), but that they do contain AA03. Nevertheless, expression patterns for ABA2/GIN1 and the capacity of guard cells for ABA biosynthesis throughout an entire diurnal cycle have not been measured.

Guard cells in isolated epidermis can accumulate (Cornish and Zeevaart, 1986), catabolize (Singh et al., 1979; Grantz et al., 1985), or conjugate exogenously-supplied ABA (Grantz et al., 1985), but, to date, none of the experimental protocols employed is sufficient to determine whether conditions that cause stomata to open initially include, or require, depletion of endogenous or endogenously-produced guard cell ABA.

Only one paper describes the effect of CytP450 mono-oxygenase inhibitors on stomatal function (Trejo et al., 1993). When ABA was fed through the midrib to leaves with open stomata, its effectiveness in inducing stomatal closure was not as great as when it was applied directly to detached epidermis or leaf pieces in which stomata were open initially, suggesting that it was absorbed and/or catabolized by leaf tissue en route to guard cells. Adding tetcyclasis, a CytP450 mono-oxygenase inhibitor, along with ABA through the midrib increased stomatal closure, but increased it more than would have been predicted from experiments with ABA applied directly to epidermis. A possible interpretation is that tetcyclasis acted both to preserve exogenous ABA levels in the transpiration stream and to prevent catabolism of endogenously-produced guard cell ABA during closing.

The model suggests that in C3 plants growing in temperate climates, photosynthetic carbon assimilation is subservient to plant hydraulics (water potentials and hydraulic conductivity), at least in the afternoon hours. The opposite might be predicted for plants with CAM. In CAM plants, decarboxylation of malate in the daylight hours combined with stimulation of respiration by the high temperatures in which many CAM plants grow could provide large amounts of CO2 for guard cell photosynthesis, especially since these plants do not open stomata through the day to liberate gases to the surrounding air. These conditions would greatly favour Calvin cycle activity in the guard cells. Consumption of NADPH from guard cell photosynthetic electron transport by guard cell carbon fixation would prevent destruction of endogenous guard cell ABA and would ensure that stomata remain closed throughout the day. Near the end of the day, mesophyll malate would be depleted and cooling would slow cellular respiration. The decrease in intercellular CO2 near the end of the daylight period might favour the formation of a large enough pool of guard cell NADPH to trigger a guard cell CytP450 just before sunset. The enzyme would catalyse catabolism of endogenous guard cell ABA and allow stomata to open under the control of an activated guard cell plasma membrane H+-pump. Transpiration is usually minimal in the cooler hours of darkness. Thus, in contrast to plants with C3 metabolism, diurnal stomatal movements in plants with CAM might be regulated mostly through the control of endogenous guard cell ABA levels in response to plant net carbon metabolism more than by accumulation of ABA in the transpiration stream. If so, then in CAM plants, plant hydraulics would be subservient to photosynthetic carbon assimilation.

Caveats and summary

Many other scenarios can, and have been, constructed to explain the experimental results described in this paper, and future experimentation will undoubtedly negate many of the hypotheses presented herein. In a recent review, Outlaw Jr (2003) pointed to the paradoxes surrounding current knowledge of how Ca2+ signalling acts to regulate stomatal aperture. This model contains unexplained paradoxes as well. For example, ABA presented at the outside of a guard cell works through a different pathway than ABA presented at the inside of the cell (Anderson et al., 1994; Assmann, 1994; MacRobbie, 1995, 2000), and in various experimental systems, ABA can either promote sugar uptake by plant cells or inhibit it (Gibson, 2004). It is certainly possible that regulatory molecules other than NADPH connected with the activity of guard cell photosystems could release guard cells to open stomata or that endogenous ABA is not catabolized, but conjugated (Grantz et al., 1985) to render it ineffective as an inhibitor of stomatal opening. Furthermore, in some of the systems described, sphingosine-1-phosphate (Ng et al., 2001a; Coursol et al., 2003) or other molecules or signals that exert effects on guard cells similar to those of ABA could be responsible for the phenomena observed.

Nevertheless, a dual-source paradigm in which a block to initial stomatal opening is removed early in the day and guard cells are subsequently surrendered for the remainder of the day to external control by leaf water status would explain many patterns of stomatal movements observed in C3 plants like V. faba. A model in which the effects of ABA delivered through the transpiration stream are superimposed on the effects of ABA being metabolized in guard cells would be consistent with such a paradigm and would explain why guard cells have both intracellular and external mechanisms for ‘perceiving’ ABA. The predicted effects of light and reduced CO2 concentrations on endogenous guard cell ABA turnover are also consistent with data on stomatal responses in isolated epidermis. While the effects of ABA...
on plant growth and development certainly justify its classification among the five ‘classical’ plant hormones (Kende and Zeevaart, 1997), it may eventually become useful to think of endogenous guard cell ABA as a second messenger for light and CO₂ signals that regulate the initial phase of stomatal opening and ensure that stomata of C₃ plants remain closed in darkness.

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