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〈 Review 〉

Three Possible Mechanisms for Stomatal Opening in Response to Light

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ABSTRACT: Environmental factors such as light and low CO_2 concentrations trigger events which may result in stomatal opening. Stomatal aperature is largely controlled by the solute contents of guard cells, but not exclusively, by through changes in their content of potassium salts, with K^+ balanced either by Cl^- or malate, depending on the species and conditions. However, how these signals are sensed and how they are transduced into driving the ion fluxes that control stomatal movements is not still fully understood. The basic role of stomata is regulating transpiration and photosynthesis. Photosynthesis plays a central role in the physiology of plants and an understanding of its response to light is, therefore, critical to any discussion of how plants sense and respond to light. It had been proposed that the evidences pointed three possible mechanisms for the light response. Firstly, there is a direct response of stomata to light. Secondly, there is an indirect response of stomata to light through the effect of CO_2. Lastly, there are some evidences for a third effect of light on stomata. However, attempts to investigate how these three possible mechanisms explained in detail in response to light have not been made. Therefore, this study is examined the differences among these three possible mechanisms.

Key words: CO_2, Light, Mesophyll cells, Stomata

INTRODUCTION

Stomata are pores formed by a pair of specialized guard cells, which exist in the surface of aerial parts of most higher plants. The most conspicuous role of stomata is the regulation of transpiration and photosynthesis. The transpiration stream may facilitate uptake and transport of salts necessary for the nutrition of the plant. It also increases the uptake of carbon dioxide for photosynthesis (Willmer 1983). However, if water supplies are limited the plant's priority changes from maximizing assimilation to restricting transpiration, while maintaining as much assimilation as possible (Mansfield *et al.* 1990). Stomata therefore close when water is limiting and open under conditions favoring photosynthesis, thus preventing excessive, deleterious water loss. This balance between CO_2 uptake and water loss was achieved by the evolution of gas permeable openings in the epidermis and cuticle. In their simplest design stomata are small, permanently open pores; in more advanced designs they are hydraulically operated valves whose openings are adjustable depending on specific demands (Ziegler 1987).

Stomata usually open when leaves are moved from darkness to light. However, the response of stomata to light is not straightforward. The observations of light responses in epidermal strips (Kuiper 1964, Hsiao *et al.* 1973, Ogawa *et al.* 1978) and in isolated guard cell protoplasts (Zeiger and Hepler 1977, Zeiger 1983) provide evidence that stomatal responses to light can be separated from those to changes in C_i (internal CO_2 concentration). In addition, stomata respond markedly to experimentally manipulated CO_2 concentrations in whole leaves and in epidermal strips (Morison 1987). The ability of carbon fixation by guard cells has been reported. Gotow *et al.* (1988) showed that the rates of carbon fixation in guard cells were from 5 to 8-fold higher in the light than in the dark. They also reported that CO_2 sensing by a metabolic reaction in guard cells can be expected to be the first step of the sensory transduction process regulating the quantitative modulation of stomatal apertures by CO_2 (Zeiger 1986, Zeiger *et al.* 1987). The CO_2 sensor for stomatal action is located in the epidermis, presumably in the guard cells (Mouravieff 1958, Pallaghy 1968) of the inner lateral walls which are permeable to CO_2 (Meidner and Mansfield 1965). The remarkable conservation of chloroplasts in guard cells (Zeiger 1983) suggests that the photosynthetic carbon reduction pathway could function as a sensing mechanism (Gotow *et al.* 1988). Heath and Russell (1954) reported that the surface of the guard cells facing the substomatal cavity appears to be the site of perception. However, it is known that a general correlation exists between light intensity, mesophyll assimilation, and stomatal conductance (Morison 1987). This correlation seems to indicate the possibility that mesophyll cells might have a sensing function in response to light and CO_2, even though there is no direct evidence to favour it.

The hypothesis that is now widely accepted to explain stomatal activity involves fluxes of inorganic cations and anions across the

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plasmalemma and degradation of organic anions. Edwards *et al.* (1988) reported that previously darkened leaves exposed to light showed quenching of fluorescence in the apoplast surrounding the guard cells up to 20 min. before stomatal opening. They showed that proton efflux originating from the guard cells preceded stomatal opening, confirming earlier work that suggested that proton efflux was a necessary precursor of stomatal opening (Raschke and Humble 1973). Therefore, when stomata open, protons are first pumped out from the guard cell, resulting in hyperpolarization of the plasmalemma PD (potential difference). Consequently, K^+ may then passively enter the guard cells to lower the osmotic potential. The primary osmotic species involved in stomatal activity is now recognized as the potassium ion. It has been known since the beginning of this century that K^+ is present in guard cells (Macallum 1905), but it was not until much later that Fujino (1967) put forward the theory that K^+ was transported in and out of cells as stomata opened and closed. Fischer (1968) showed that K^+ was necessary to open stomata on epidermal strips of *Vicia faba*, and that observed uptake could account for increasing aperture. Raschke and Fellows (1971) found that in *Zea mays* there was a shuttle of K^+ between guard and subsidiary cells as stomata open and close, results confirmed by Penny and Bowling (1974) who, using K^+-ion specific microelectrodes, found a decrease in vacuolar K^+ activity across the stomatal complex from guard cells to surrounding cells of *Commelina communis* when stomata open, and the reverse when closed. Using radioactive tracers and K^+-ion specific microelectrodes it has been established that K^+ accumulation could determine the aperture of wide open stomata of *Commelina communis*, but accumulation of other solutes is required in the initial stages of opening (MacRobbie 1980, MacRobbie and Lettau 1981, MacRobbie 1983).

Cl^- also enters the guard cells, but complete charge balance of the excess K^+ is accomplished by synthesis of malate (Wilmer 1983). In *Zea mays* Cl^- ions accumulated about half the concentration of K^+ ions (Raschke and Fellows 1971). In *Vicia faba*, however, Humble and Raschke (1971) found that only 5% of K^+ was balanced by Cl^-. In the same species accumulation of the organic anion malate could account for half of the K^+ uptake (Allaway 1973). The results for *Vicia* are conflicting, depending on whether leaves or epidermal strips are used, but accumulation of malate appears to depend on the availability of Cl^- and if more Cl^- is available to the cell then more is taken up and a smaller proportion of K^+ is balanced by malate (Van Kirk and Raschke 1978). Schnable and Kottmeier (1984) reported that malate accumulated in GCPs (guard cell protoplasts) of *Vicia* when they swell in K^+, but not in swelling isolated vacuoles suggesting the participation of the cytoplasm for malate synthesis.

A 'malate-switch' hypothesis attempting to explain aspects of ion transport into and out of guard cells was proposed by Bowling (1976). The hypothesis is based upon the change of ionization of malate with pH and assumes that the monovalent ($malate^{1-}$) form is able to move out of cells while the divalent ($malate^{2-}$) form is not. Bowling observed in *Commelina communis* that a gradient of vacuolar pH occurred across the epidermal, subsidiary and guard cells, being highest in guard cells (5.8) and lowest in epidermal cells (5.1) when stomata were open and, in the reverse direction when stomata were closed. Therefore, when stomata close, guard cell pH values initially fall (become more acidic) and, as the monovalent form enters these cells, it is converted to the divalent form and withheld. The reverse would occur upon stomatal opening. It corresponds with observations that malate levels in guard cells fall as stomata close and increase as stomata open (Willmer, 1983).

Blue light-dependent proton extrusion by guard cell protoplasts of *Vicia faba* was recently reported by Shimazaki *et al.* (1986). In plant cells, including guard cells, proton pumping is widely thought to depend on the activity of a plasmalemma ATPase (Poole 1978, Spanswick 1981), and chemiosmotic proton pumping has been proposed as the basis of active ion uptake during stomatal movements (Zeiger 1983). It is believed that a pigment, possibly a flavin or flavoprotein situated in or on the guard cell plasmalemma or tonoplast, may be a sensor of blue light to initiate H^+ excretion (Willmer 1983). The low saturation levels of the response, about 10 $\mu mol\ m^{-2}\ s^{-1}$ (Zeiger *et al.* 1985, Shimazaki *et al.* 1986), imply that it is light-saturated throughout most of the day (Zeiger *et al.* 1987). Hence, the blue light response could function as a "light on" signal during daylight, with its metabolic correlates, including proton pumping (Assmann *et al.* 1985, Shimazaki *et al.* 1986) and stimulation of malate biosynthesis (Ogawa *et al*, 1978), gearing stomatal responses for the functional requirements of the daylight activity of the leaf (Zeiger *et al.* 1987).

The last twenty years have been a great increase in knowledge of the physiological and biochemical processes responsible for stomatal functioning and the majority of the work has concentrated on the isolated guard cell protoplast and the processes at work within it. However, to a large extent the important area of stomatal physiology which has been relatively neglected is the role of mesophyll cells which fix carbon dioxide through photosynthesis and contribute carbon to epidermal cells over a long period. Many investigators attribute the observed correlation between photosynthesis and stomatal conductance to parallel responses of guard cells and mesophyll cells to environmental stimuli. Therefore, light response on stomata *in vivo* will be different from that of guard cell protoplasts. Futhermore, there have been a number of reports indicating a different response to environmental stimuli between the isolated epidermis

and the intact leaf. Therefore, in order to understand the influence of the mesophyll on the stomata, the three possible mechanism of stomatal opening in response to light had been compared.

STOMATAL RESPONSES TO LIGHT

Stomata usually open when leaves are transferred from darkness to light. However, the response of stomata to light is not straightforward. Sharkey and Ogawa (1987) suggested that the evidences pointed three possible mechanisms for the light response. Firstly, there is a direct response of stomata to light first demonstrated by the work of Heath and Russell (1954). Light appears to be absorbed by pigments in the guard cells because stomata in isolated epidermis and isolated guard cell protoplasts show light reponses (Zeiger *et al.* 1987). Guard cells respond to red light and blue light and there is evidence for the existence of two distinct photoreceptors. The receptor for the red light is thought be located in the guard cell chloroplasts while the blue light is postulated to be absorbed by a flavin on the plasmalemma (Sharkey and Ogawa 1987).

Secondly, there is an indirect response of stomata to light through the effect of CO_2. Scarth (1932) first suggested that light could increase photosynthesis resulting in a decreased level of CO_2 in the intercellular spaces leading to stomatal opening. Heath and Russell were able to separate an indirect CO_2 effect from the direct effect of light. It is a matter of debates as to which one of these two effects plays the important role in controlling stomata in the field.

Heath and Russell (1954) also obtained some evidence for a third effect of light on stomata. They suggested that there was an indirect effect transmitted either from the epidermal cells or through them from the mesophyll cells by some agent of a chemical or electrical nature. Since this possibility was first put forward there have been hints from the results of other investigators of a link between the mesophyll and stomatal aperture. Wong *et al.* (1979) found that the diffusive conductance of the leaf epidermis to CO_2 transfer changed proportionately with the rate of assimilation. They suggested that the stomata responded to metabolites of photosynthesis in the mesophyll. They were in effect proposing that photosynthesis was regulating stomatal aperture in contarst to the orthodox idea that stomata control photosynthesis.

DIRECT EFFECT OF LIGHT ON STOMATA

Kuiper's (1964) action spectrum for opening on epidermal strips of *Senico odoris* Defl. showed a marked peak in the blue region, substantially higher than that in the red. When guard cell protoplasts from *Vicia faba* are irradiated with blue light under background red-light illumination, the pH of the suspension medium becomes more acidic. This blue light induced acidification is blocked by inhibitors that dissipate pH gradients, uch as CCCP, and by inhibitors of the proton pumping H^+-ATPase, such as vanadate (Amodeo *et al.* 1992, Kinoshita *et al.* 2001). Futhermore, observations of light responses in epidermal strips (Kuiper 1964, Hsiao *et al.* 1973, Ogawa *et al.* 1978) and in isolated guard cell protoplasts (Zeiger and Hepler 1977, Fitzsimons and Weyers 1986) indicate the effects are on guard cell itself.

Direct stomatal response by light at the level of the epidermis can be divided into two categories, one being a red light response and the other being a blue light response. The evidence for two photoreceptors was very well summarized by Sharkey and Ogawa (1987).

Stomatal sensitivity to blue light is much greater than can be explained by chlorophyll responses alone. Some of the available information on the red light response showed that stomatal opening by red light in epidermal strips was inferior to that by blue light (Mansfield and Meidner 1966, Meidner 1968, Hsiao *et al.* 1973, Permadasa 1982).

INDIRECT EFFECTS OF LIGHT OF STOMATA

Two central questions that have emerged over the last decade are whether C_i is the important signal for light induced stomatal opening and what influence C_i exerts on stomatal regulation (Jarvis and Morison 1981). Certainly, a general correlation exists between light intensity, mesophyll assimilation, and conductance (Morison 1987).

Since C_i declines as assimilation increases, and since conductance in many cases increases with decreasing C_i, it has been supposed that assimilation controls conductance by affecting changes in C_i (Raschke 1976). Likewise, stomata have been shown to respond to changes in CO_2 concentration in over 50 species, including angiosperms and gymnosperms, dicots and monocots, and species with C_3, C_4 and CAM photosynthetic pathway. It can, therefore, be assumed that stomatal response to CO_2 is a general phenomenon (Morison 1987). It appears to be appropriate to refer to C_i as the effective CO_2 concentration controlling stomata (Mansfield *et al.* 1990). A study by Mott (1988) has recently confirmed earlier deductions by Heath (1948) that stomata respond to C_i, but not to the concentration at the surface of the leaf. With respect to the provision of CO_2 for photosynthesis, Farquhar and Sharkey (1982) deduced that the stomata of C_4 plants should not ideally respond to C_i when it is below the saturation level for photosynthesis; but they should become very sensitive to C_i above this level, because then as they close transpiration but not assimilation will be reduced. Similar reasoning can be applied to C_3 plants, but in their case there is no level of C_i at which partial somatal closure will curtail transpiration

without some obstruction of assimilation. In C4 species the typical value of C_i/C_a (ambient CO_2 concentration) is lower than that in C_3 species and as Ramos and Hall (1982, 1983) have argued, it may be that plants with the C_4 photosynthetic pathway show a large sensitivity of stomatal aperture to C_i. However, at high humidity and moderate light intensities, stomata of two C_4 and C_3 grass species showed the same quantitative response to CO_2 (Morison and Gifford 1983), indicating that there is little inherent differences in the CO_2 sensitivity of stomata between two groups of plants.

There is a general view that C_i is nearly constant in the light. For the C_3 species examined, researchers have agreed that in well-watered conditions the contribution of the CO_2 response is slight (Farquhar *et al.* 1978, Wong *et al.* 1978, Morison and Jarvis 1983, Ramos and Hall 1983). In simple qualitative terms, stomatal conductance in *Eucalyptus*, *Commelina* and *Triticum* leaves was least sensitive to C_i at low quantum flux densities (250~2,000 μmole $m^{-2}s^{-1}$), conductance was more sensitive to CO_2, but in these ranges C_i did not decrease further with increasing light in normal atmospheric CO_2 concentrations. The analyses of Farquhar *et al.* (1978), Wong *et al.* (1978), Sharkey and Raschke (1981), Ramos and Hall (1983) indicate that, Quantitatively, the response of stomata to the changes in CO_2 is indeed slight. In spite of the uncertainty over the exact role of changes in C_i in the daily behaviour of stomata, we know that high CO_2 concentrations cause reductions in stomatal aperture and conductance. As the mean atmospheric CO_2 concentration is increasing (Clark 1982), stomatal conductance will decrease, with a consequent increase in the plant's water-use efficiency. Spanswick and Miller (1977) reported that in *Nitella* CO_2 inhibits a proton pump which is stimulated by light. Edwards and Bowling (1985) also showed that enhanced levels of CO_2 affected the cells of the leaf epidermis by depolarizing the membrane PD.

Accordingly, we have to be cautious in stating that the carbon dioxide response of the stomata is not important. For example, under natural conditions low light is correlated with low temperatures and low VPD (vapour pressure difference). The CO_2 response may be more important under these conditions in stomatal opening in the early morning or closure in the evening (Morison 1987).

THE POSSIBILITY OF ANOTHER INDIRECT EFFECT BY LIGHT ON STOMATA

There is a possibility of an indirect effect of light on stomata which could be mediated through the mesophyll cells. Analyses of stomatal response to CO_2 and light led to the conclusion that, in most cases, stomata responded to changes in the intercellular CO_2 concentration only to a small extent and most of the response to light was "direct" by not mediated by CO_2 (Wong *et al.* 1979). They suggested a possible mechanism that the stomata responded to another metabolite of photosynthesis in the leaf mesophyll tissue suggesting that ATP or NADPH could be the substance involved.

There have been several reports to suggest that stomata in isolated epidermis behave both quantitatively and qualitatively differently from those in the intact leaf. Some of the reports also demonstrated that the responses of guard cells to environmental stimuli were absent in isolated epidermis. Willmer *et al.* (1990) reported that the apparent Km for PEP (phosphoenolpyruvate) and V_{max} of PEPC (phosphoenolpyruvate carboxylase) from GCP of *Comellina communis* and the sensitivity of the enzyme to malate were not changed on exposure of the protoplasts to light or dark. Raschke *et al.* (1988) also did not detect differences of V_{max} for light or dark-treated GCPs of *Pisum sativum*. However, V_{max} for PEPC from some C_4 species is increased by light (Karabourniotis *et al.* 1983). Futhermore, the activity of electron transport in guard cells was observed (Lawson *et al.* 2002). The results obtained by Willmer *et al.* (1990) are unexpected in that the kinetics of PEPC activity from C_4 and CAM leaves changes in response to light or darkness because guard cells are expected to fix CO_2 while they are exposed to light. However, the apparent absence of the sensitivity of the enzyme in guard cells in response to light can be explained in two ways. Firstly, CO_2 fixation in guard cells seems to be negligible and the carbon source for the epidermis has to be imported from the mesophyll. Secondly, the photosensor for stomatal control is not situated in the guard cells themselves, indicating the importance of the mesophyll

Fischer (1970) carried out experiments on water stress in leaves. He showed that a minor part of the post-stress damage was located in the mesophyll in beans while the major part (approximately two-thirds) being located in the guard cells themselves. He thought that something could be transmitted from the mesophyll to guard cells. Grantz and Schwartz (1988) demonstrated that isolated epidermis may show rather different stomatal responses from those found in the intact leaf. They found that guard cells of *Commelina communis* did not respond metabolically to osmotic stress in isolated epidermis. However, in intact disks, stomata exhibited clear, hydroactive stomatal responses. They concluded that their results were consistent with the view that signal metabolites from the mesophyll mediate stomatal responses.

Willmer and Mansfield (1969) reported that in *Vicia faba*, the light effect was very apparent on attached epidermis, but on detached epidermis, the effect was largely obscured by stomatal opening that occurred in darkness. They also found that effects of carbon dioxide concentration were detectable on epidermal strips, particularly in darkness, but were of smaller magnitude than those on attached epidermis. It is known that stomatal sensitivity to CO_2

can increase as water deficit develops in intact plants (Heath and Mansfield 1962) and it may be the case that in fully hydrated epidermis, the closing response to CO_2 is minimal. Travis and Mansfield (1979) found that stomatal responses to light and CO_2 in isolated epidermis from *Commelina communis* were dependent on the KCl concentration in the incubation medium. They could eliminate the light and CO_2 effects altogether by manipulation of the medium. Fricker *et al.* (1991) measured stomatal aperture in the isolated epidermis of *Commelina communis* using a liquid flow porometer and observed that there was no response of the stomata to light. They suggested that the lack of light stimulated opening is not unique to *Comellina communis* or to their system as similar results were found with *Vicia faba.*

All the above reports seem to imply that mesophyll cells might be much more important than we originally thought. Normal stomatal responses to environmental stimuli might be less sensitive in isolated epidermis than in intact leaf. This suggests that another indirect contribution from the mesophyll to stomatal opening in response to light is quite possible.

Futhermore, there have been a number of electrophysiological studies, suggesting a possible signal transduction from the mesophyll. Gunar *et al.* (1975) found that a fairly rapid polarization of the PD by 10~15mV was observed upon the switching-on of the light in intact leaves of *Tradescantia albiflora.* They suggested that since epidermal and subsidiary cells do not have chlorophyll, changes in their PD induced by light could be associated with electrical excitation propagated from the mesophyll cells. Cheesman *et al.* (1982) also, found that membrane potential in isolated strips was considerably lower than those in intact sections and was insensitive to light. In addition, there are a number of reports suggesting that photoinduced ion fluxes in green plant tissue are probably associated with pumps regulated by photosynthesis (Jeschke 1970, Higinbotham 1973), photo-synthetic electron transport (Hartmann 1975) and cyclic-photophosphorylation (Spanswick 1973). The above results evoke the question that membrane hyperpolarization has been reported following localized wounding by heating or burning, in many species, including *Lupinus* (Paszewski and Zawadzki 1976), *Gossypiumn*, *Cucurbita*, *Zanthium* (Van Sambeek and Pickard 1976), *Vicia*, Mimosa (Roblin 1985, Roblin and Bonnemain 1985), and *Lycopersicon* (Van Sambeek and Pickard 1976, Roblin 1985, Wildon *et al*, 1989). Roblin (1985) stated that "the slow wave appears general in herbaceous plants". Similar slow waves of apoplastic depolarization may follow other type of wounding, including pricking of the midrib in leaves or petioles of *Bidens pilosus* (Frachisse *et al.* 1985) and crushing or squeezing of leaf or petiole in various species (Wildom *et al.* 1989, Van Sambeek and Pickard 1976). Researches over many years (Pickard 1973, Van Sambeek and Pickard 1976, Roblin and Bonnemain 1985, Frachisse *et al.* 1991) have established that a signal is transmitted from the wounded region with an apparent velocity of about 1~6mm. s^{-1} and that the signal, when received by metabolically active tissue, causes rapid depolarization of the extracellular potential as measured with surface contact electrodes (Van Sambeek and Pickard 1976) or with noble-metal wires inserted into apoplast (Roblin 1985). The transmitted signal is termed "Ricca' s factor" (Van Sambeek and Pickard 1976) after its proposer (Ricca 1916). Pickard (1973) suggested that Ricca' s factor may represent a hormone or a group of hormones of widespread distribution in higher plants which coordinate aspects of the plant response to breakage, abrasion, and perhaps water deficit. Malone and Stankovic (1991) suggested that arrival of the wave alters leaf water potential and thereby induces stomatal activity. Likewise, it could be imagined that there is an intial stomatal opening response caused by an electrical signal of a hormone propagated form the mesophyll in response to light.

Above all, the most important evidence suggesting the importance of the mesophyll came from *Paphiopedium.* In the case of *Paphiopedium* (Nelson and Mayo 1975, Rutter and Willmer 1979), the Calvin cycle is not present in guard cells since chloroplasts are absent although the stomata are functional. This species was referred to as an evidence that guard cell chloroplasts may not be an important source of ATP and reducing power for ion transport and other processes essential to the functioning of stomata. Nelson and Mayo (1975) observed that the stomata of *Paphiopedium*, which have guard cells devoid of chloroplasts, opened normally in light. The stomata were sensitive to both red and blue light, and this raises the possibility that the red photoreceptor may have been located in the chloroplasts of the mesophyll. They suggested that guard cell chloroplasts are not necessary to absorb the energy for the CO_2-independent opening reaction. Zeiger and Hepler (1977) reported that red light was ineffective in the swelling of GCPs and therefore, concluded that blue light was the only wavelength able to generate significant changes in the water potentials of the protoplasts. They also showed that intact onion stomata in paradermal slices, which include several layers of mesophyll cells responded to both blue and red light, but stomata in epidermal peels responded only to blue light. Hedrich and Neher (1987) reported that blue light activated that blue light activated pumps in the plasmalemma but red light had no effect on the change of PD of guard cell protoplasts. It is generally accepted that the changes of membrane PD is the result of proton efflux (Edwards and Bowling 1984, Moody and Zeiger 1978). This indicates that red lights does not stimulate proton efflux by the guard cell membrane which is the phenomenon prior to stomatal opening. This third view implicates another indirect involvement of mesophyll cells, not mediated by internal CO_2

concentration changes in response to light.

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