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The Parallel Measurements of Stomatal Apertures and Apoplastic pH on Guard Cells from Epidermal Strips and Intact Leaves of *Commelina communis*

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Abstract - The effects of light and darkness on stomatal aperture and guard cell apoplastic pH in the intact leaf and in the isolated epidermal strips of *Commelina communis* have been investigated. Stomata in the intact leaf opened wide in the light. In contrast, stomata in the isolated epidermal strips did not respond clearly to light. To eucidate the relationship between the stomatal aperture and the guard cell apoplastic pH, apoplastic pH was measured. In the light the guard cell wall of intact leaf was acidified by pH 1.9 units, falling from pH 7.3 to pH 5.4 in the first 10 minutes. On the contrary, apoplastic pH of isolated epidermal strips changed slowly from pH 7.3 to pH 6.9 at 20 min. Stomata in the intact leaf closed rapidly in the dark. On the other hand, stomata in the isolated epidermal strips failed to close in dark. There was a slow increase in apoplastic pH on transfer to the dark after incubation for 1.5 h in the light and the level observed before the experiment was regained after around 40 min. When the isolated epidermal strips were transferred to the dark, apoplastic pH maintained a uniform level of around pH 7.2~7.4. These results indicate that the mechanism of stomatal opening and closing from isolated epidermal strips and intact leaves could be different.

Key words : apoplastic pH, *Commelina communis*, stomata.

Introduction

Environmental factors such as light and low CO_2 concentrations trigger events which may result in stomatal opening. How these signals are sensed and how they are transduced into driving the ion fluxes which control stomatal movements is not fully understood. It is now widely believed that stomatal activity involves fluxes of inorganic cations and anions across the plasmalemma and tonoplast of guard cells associated with the synthesis 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 at the guard cells preceded stomatal opening, confirming earlier work which 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 potential difference. This gradient stimulates opening of inward K^+ channels which may allow K^+ influx to guard cells resulting in an increase in water potential. This is the theory which is now widely accepted to explain stomatal opening. However, in last twenty years the majority of works for stomatal study has

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concentrated on the isolated epidermals strips, isolated guard cell protoplast and the processes at work within it. To a large extent the important area of stomatal physiology which has been relatively reglected is the *in vivo* stomatal response.

Many studies have shown that stomata in isolated leaf epidermis behave differently, both quantitatively and qualitatively, from those in the intact leaf (Cheeseman *et al.* 1982; Fricker *et al.* 1991; Grantz and Schwartz 1988; Lee and Bowling 1992; Zeiger and Hepler 1973). Talbot and Zeiger (1998) reported that both K^+ and sucrose are primary guard cell osmotica, and that the use of these two solutes is separated into two distinct phases in which one or the other constitutes the dominant osmoticum. In the intact leaf, opening at the beginning of a day cycle is supported by K^+. In the second half of the daily cycle, K^+ content in guard cells decreases dramatically and sucrose becomes the dominant solute. Gerhard *et al.* (1999) also reported that sucrose could replace potassium and malate as the osmoticum for the maintenance of stomatal opening. Their reports invoke two aspects. The first is that the impotance of sucrose as a osmotica of a day cycle on stomatal opening in the intact condition of the plant implies that we need to reexamine the starch-sugar hypothesis which has been replaced by the present paradigm of guard cell osmoregulation by K^+ and its counterions. The second aspect is that if sucrose is the important osmotica on stomatal opening in the intact leaf, where the sucrose come from? Does the sucrose come from the mesophyll cells which are the most active site of the photosynthesis or from guard cell photosynthesis? Outlaw (1989), in his review, disputes the notion of the operation of the Calvin cycle in guard cells. Some researchers reported that guard cells have low levels of Rubisco activity (Gotow *et al.* 1988; Reckmann *et al.* 1990). More interesting reports come from studies of lady's slipper orchid, *Paphiopedilum*. In the case of *Paphiopedilum* (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 provides evidence that guard cell chloroplasts may not be necessary as a 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 *Paphiopedilum* opened normally in light. This raises the possibility that red photo-receptor may be located in the chloroplasts of the mesophyll.

Weyers and Meidner (1990) pointed out that the use of isolated epidermal strips has been a valuable technique central to many advances in our understanding of the stomatal mechanism. In case of guard cell protoplasts, the response to light is without the mechanical interaction between guard cells and neighbouring cells. Therefore, light response on isolated epidermal strips will be different from that of guard cell protoplasts.

We know that stomata *in vivo* usually open in the light and close in the darkness. In addition, guard cell apoplastic pH changes in response to light could be expected. However, there are no attempts of parallel measurements of stomatal apertures and apoplastic pH on guard cells from isolated epidermal strips and intact leaves. Therefore, this study was carried out to investigate the different effects of light and dark on stomatal apertures and apoplastic pH in isolated epidermal strips and intact leaf of *Commelina communis*.

Materials and Methods

Stomatal Aperture Measurements

The experiments were carried out on the abaxial surface of leaves of *Commelina communis* L. The plants were grown from seeds in mixture of vermiculite and Peat & Loam potting compost with Hyponex in Growth Chamber ($22 \pm 2°C$) with supplementary lighting from natrium lamps (400 μmole $m^{-2} s^{-1}$) to give a photoperiod of 16 h. At all stages of development the plants were kept free from water stress by periodic watering.

Fully expanded leaves were obtained by the method of Lee and Bowling (1992). The strips were cut into segments and incubated in 10 cm diameter plastic Petri dishes containing an appropriate medium, into which air was bubbled through hypodermic neddles fitted in the lids. Either normal air (containing approximately 350 μmol mol^{-1} CO_2) or CO_2-free air was given by means of a pump. CO_2-free air was obtained by passing air through a cylinder of soda lime and 2000 mol m^{-3} KOH solution. The incubation medium consisted of MES

buffer (10 mol m^{-3}) at pH 6.15 to which KCl at 50 mol m^{-3} was added. For experiments involving a light response, samples were placed under a mercury vapour lamp (200 μmole $m^{-2} s^{-1}$) at 22±2°C.

For experiments with the intact leaf, was cut into segments and laid, abaxial surface uppermost in plastic Petri dishes lined with filter paper moistened with distilled water. After various intervals, intact segments were transferred into liquid paraffin and epidermal strips were peeled.

To measure stomatal aperture, a microscope (Olympus) with a camera lucida (×400 magnification) was connected to a monitor video and the screen was calibrated by an ocular micrometer disc. After time intervals, epidermal strips were mounted under the microscope. Stomata screened in the monitor could be measured directly with a calibrated scaler. This system was more convenient and accurate for measuring the stomatal apertures than direct microscopic measurements.

Micro-electrode determination of apoplastic pH

Direct measurement of apoplastic pH was carried out using pH sensitive micro-elctrodes. Glass capillaries (2.0 mm o.d., 1.6 mm i.d. with internal filament, Clark Electromedical Instruments, Pangbourne, Berks., U.K.) were pulled on a C.F.P. vertical puller (C.F. Palmer, Searle Bioscience, Sheerness, Kent, U.K.) to give micropipettes of tip diameter 0.5~1.0 μm. These were baked at 200°C, initially for 1 h to dry the glass and them for a further 1h in silane vapour (2% dimethyldichlorosilane in 1, 1, 1-trichloroethane: BDH, Poole, dorset, U.K.). On cooling, the micropipette tips were back-filled with a proton cocktail (1.22 ml tridodecylamime, 5.93 ml 2-nitrophenyl octyl ether, 0.07 g Na tetraphenylborate) and the stem filled with 3000 mol m^{-3} KCl (Bowling, 1989). The calibration of pH sensitive micro-electrode was carried out with standard pH buffer solution (pH 4, 5, 6, 7, 9.2). The slope of the calibration curve between pH 9.0 was always greater than 40 mV per pH unit. Reference electrodes were made by pulling micropipettes as before and filling with KCl (3000 mol m^{-3}). The pH and reference micro-electrodes were connected via Ag/AgCl half-cells to a high impedance electrometer (437 Electrometer, Pitman Instruments, Weybridge, England).

The samples of isolated epidermal strips for pH measurements were prepared using the same method described for stomatal aperture experiments. The pH was measured by lowering both micro-electrodes on to the same cell as close together as possible without touching (5~10 μm apart) using Zeiss micromanipulators (C-J, Jena). pH was read over 1 min. The entire process of stripping, mounting and measuring apoplastic pH took less than 2 min. Each strip was used for one measurement on one cell only. Micro-electrodes were calibrated before and after readings. The pH of the suspension medium did not vary over the course on the measurement. Light intensity used in this experiments was 550 μmole $m^{-2} s^{-1}$, provided by a Lux 150, 4-Port projector.

Results

Figure 1 shows a comparison between the behaviour of the stomata in the intact leaf and isolated epidermal strips. On transfer to the light, the stomata in the intact leaf, floated in water in an enclosed Petri dish, opened to a maximum aperture of about 12 μm after about 70 min. Stomata in the isolated epidermal strips, floated on 50 mol m^{-3} KCl, opened slowly to a maximum aperture of about 5 μm.

The results in Fig. 1 suggest that the opening of stomata in isolated epidermal strips was inferior to that in the intact leaf. There was also a relationship between time and stomatal aperture under the illumination in intact leaves and in each disc the stomatal apertures were quite close to the mean as could be seen in the standard errors of the mean (±s.e.m.). However, in isolated epidermal strips there was a wide variation of stomatal aperture within the samples.

It is well documented that proton efflux is a necessary precursor of stomatal opening. It is interesting to see whether apoplastic pH of the intact leaf and isolated epidermal strips will be changed or not. These results will give us a clue for a stomatal mechanism of the intact leaf and isolated epidermal strips in responses to light. Accordingly, apoplastic pH changes across the stomatal complex were measured both in the intact leaf and isolated epidermal strips over the same time period

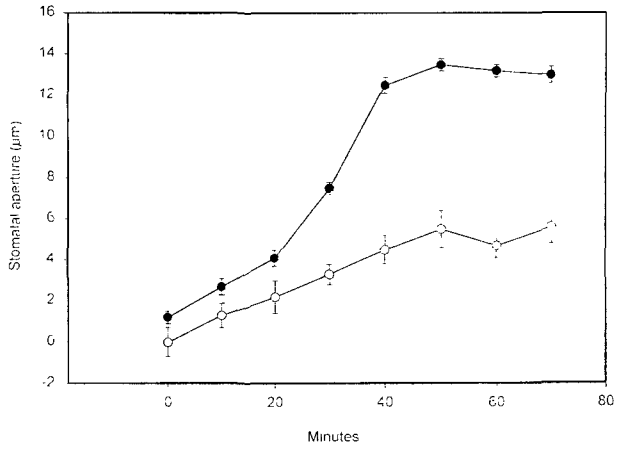

Fig. 1. Opening of stomata of *Commelina communis* in intact leaves and isolated epidermal strips. Leaves were kept in the dark for 1 h, exposed to light for 70 min. Each point is the mean of two replicate experiments and 80 stomatal apertures were measured. Closed circles indicate intact leaves in distilled water and open circles indicate isolated epidermal strips in 10 mol m^{-3} MES-KOH buffer (pH 6.15, 50 mol m^{-3} KCl).

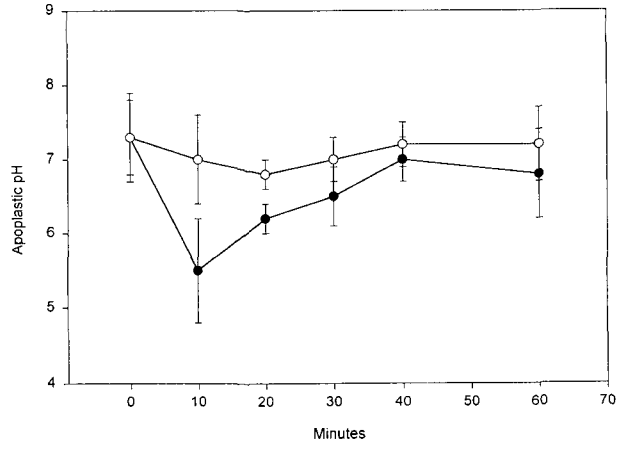

Fig. 2. The effect of light on the change of guard cell apoplastic pH both of intact leaf and isolated epidermal strips. Each point is the mean (±s.e.m.) of three replicate experiments. Isolated epidermal strips were incubated in 10 mol m^{-3} MES-KOH buffer (pH 6.15, 50 mol m^{-3} KCl). Closed circles indicate intact leaves and open circles indicate isolated epidermal strips.

using micro-electrodes (Fig. 2). There was a big difference in guard cell apoplastic pH in response to light between the intact leaf and isolated epidermal strips. The guard cell wall of intact leaf was acidified by pH 1.9

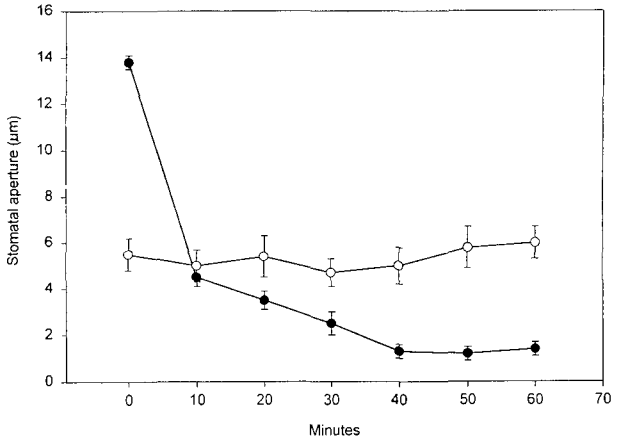

Fig. 3. Closing of stomata of *Commelina communis* in intact leaves and isolated epidermal strips. When stomata were open, the leaf sections were returned to the dark. Each point is the mean of two replicate experiments and 80 stomatal apertures were measured. Closed circles indicate intact leaves in distilled water and open circles indicate isolated epidermal strips in 10 mol m^{-3} MES-KOH buffer (pH 6.15, 50 mol m^{-3} KCl).

units, falling from pH 7.3 to pH 5.4 in the first 10 min. in the light. After 40 min. in the light, apoplastic pH in the intact leaf had reached a uniform level approximately between pH 7.0 and 6.8.

On the contrary, apoplastic pH of isolated epidermal strips changed slowly from pH 7.3 to pH 6.9 at 20 min. and after that it maintained a uniform level of around pH 7.0~pH 7.3 in the light. As we can see in Fig. 1, the response of isolated epidermal strips by light was much smaller than that of the intact leaf. However, it can be seen that there was a slow acidification in isolated epidermal strip in response to light. This acidification in isolated epidermal strips by light could be enough to motivate K^+ influx to guard cells? The results shown in Fig. 1 indicate that acidification in isolated epidermal strips by light could not be enough to trigger stomatal opening.

Stomatal closing is a fairly rapid response. Therefore, it was necessary to investigate how both of the isolated epidermal strips and intact leaf could respond to darkness. Fig. 3 shows the effect of darkness on stomatal aperture in isolated epidermal strips and intact leaf. Both samples were incubated in distiled water or in 10 mol m^{-3} MES-KOH buffer (pH 6.15, 50 mol m^{-3} KCl)

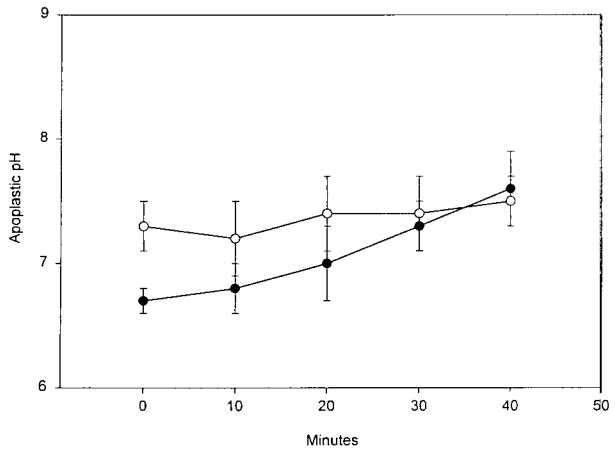

Fig. 4. The effect of darkness on the change of guard cell apoplastic pH both of intact leaf and isolated epidermal strips. Each point is the mean (±s.e.m.) of three replicate experiments. Isolated epidermal strips were incubated in 10 mol m^{-3} MES-KOH buffer (pH 6.15, 50 mol m^{-3} KCl). Closed circles indicate intact leaves and open circles indicate isolated epidermal strips.

respectively for 1.5 h at 25±2°C under photon density of 200 μmole m^{-2} s^{-1}. When the stomata were open, the leaves were returned to the dark. The stomata in the intact leaf closed fairly rapidly, in contrast to those in the intact leaf stomata in the isolated epidermal strips, floated on 50 mol m^{-3} KCl, failed to close.

As stomata usually close rapidly, it was exepected that the apoplastic pH changes in darkness will be more clear that in the light. Fig. 4 shows the effect of dark on the change of guard cell apoplastic pH both of the intact leaf and the isolated epidermal strips. On transfer to the dark after incubation for 1.5 h in the light there was a slow increase in apoplastic pH and the level observed before the experiment was regained after around 40 min. When the isolated epidermal strips was transferred to the dark, guard cell wall pH maintained a uniform level of around pH 7.2~7.4.

Discussion

The use of isolated epidermal strips has been a valuable technique central to many advances in our understanding of the stomatal mechanism. However, attempts to study stomatal behaviour on isolated pieces of epidermis have met with various degree of success and this led to doubts about the value of the technique. For instance, a fully hydrated epidermis is likely to have a higher turgor pressure than the epidermis of intact leaves (Willmer & Mansfield 1969), so the natural changes of turgor between guard and subsidiary cells can be inhibited. In case of isolated epidermal strips, the response to light is without the metabolic interac-tion between guard cells and mesophyll cells. Therefore, light response on stomata *in vivo* will be different from that of isolated epidermal strips.

In previous reports stomata opened reaching up to 12~15 μm in isolated epidermal strips (MacRobbie & Lettau 1980; MacRobbie 1984). It has been found in this study that stomatal aperture in the light was around 5 μm. However, this is not exceptional. Willmer and Mansfield (1969) concluded that epidermal strips of *Commelina* were suitable for studies of stomatal responses to light and CO_2 so long as they were incubated in a suitable medium. They used a medium containing 66 mol m^{-3} KCl but observed apertures of only 4~6 μm after exposing the epidermal strips for 1h to light. Agbariah and Roth-Bejerano (1990) investigated the effect of light on stomata of *Commelina* using epidermal strips incubated in 100 mol m^{-3} KCl. They observed maximum stomatal apertures of 5~6 μm.

The results show that under the conditions of this experiments stomata in isolated epidermal strips from *Commelina* leaves behave differently, both quantitatively and qualitatively, from those in the intact leaf. Stomata in isolated epidermal strips did not respond clearly to light. They opened to a maximum aperture of around 5 μm and, in contrast to those in the intact leaf, failed to close in darkness. Stomata in the intact opened wide in the light and closed rapidly on transfer to the dark. In contrast, stomata in isolated epidermal strips were less sensitive to light and dark. There have been many supporting reports the different response between the intact leaf and the isolated epidermal strips. Some of the reports also demonstrated that the responses of guard cells in isolated epidermal strips to environmental stimuli were less sensitive than those in the intact leaf. 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. Grantz and Schwartz (1988) reported that isolated epidermal strips may show rather different stomatal responses from those in the in the intact leaf. Travis and Mansfield (1979) found that stomatal responses to light and CO_2 in isolated epidermal strips 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 pore width in isolated epidermal strips of *Commelina communis* using a liquid flow porometer and observed that there was no response by the stomata to light.

In order to know stomatal mechanism in isolated epidermis and intact leave it was necessary to measure apoplastic pH. It was found that apoplastic pH was in the range of 5.5~7.5 in all guard cells investigated. These results were similar with those of Bowling and Edwards (1984) in which they found that pH values of the apoplast of the cells of stomatal complex ranged from pH 6.1 to pH 8.3.

The most important point of this study was the parallel measurements of stomatal apertures and apoplastic pH on guard cells from isolated epidermal strips and intact leaves. Separate measurements in either system are well known. In intact leaves, it can be seen that there was a faster opening responses in the light. The maximum stomatal aperture was about 12 μm. Stomata in the intact leaf closed rapidly on transfer to darkness.

The guard cell wall of intact leaf was acidified by pH 1.9 units, falling from pH 7.3 to pH 5.4 in the first 10 min. in the light. After 40 min. in the light, apoplastic pH in the intact leaf had reached a uniform level approximately between pH 7.0 and 6.8. These results are very similar to those obtained by Edwards *et al.* (1988) for *Commelina communis*. They reported that previously darkened leaves exposed to light showed quenching of fluorescence in the apoplast surrounding the guard cells up to 20 min. On transfer to the dark after incubation for 1.5 h in the light there was a slow increase in apoplastic pH and the level observed before the experiment was regained after around 40 min. These results indicate that proton efflux or influx was a necessary precursor of stomatal opening or stomatal closing.

In conclusion, stomata in the intact leaf opened wide in the light and closed in the dark. They also showed very sensitive apoplastic pH changes in response to light and darkness. In contrast, stomata in isolated epidermal strips responded neither to light nor darkness. Therefore, this study give us an important evidence that the mechanism of stomatal opening and closing from isolated epidermal strips and intact leaves may be different.

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