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ORIGINAL ARTICLE

The Electrophysiology Application on Guard Cells to See the Influence of Carbon Dioxide

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Abstract

The effect of CO_2 on the opening of stomata in the intact leaf of *Commelina communis* has been investigated. Full opening of stomatal apertures(around 18 μm) was achieved in the intact leaf by addition of CO_2(900 μmol mol^{-1}). At 90 minutes, the stomatal apertures of leaves treated with CO_2 free air were reduced. In contrast, stomata opened most widely with the treatment of CO_2 air at 90 minutes. The effects of light, CO_2 air and CO_2 free air on the change of membrane potential difference(PD) were measured. Fast hyperpolarization of guard cell membrane PD was recorded reaching up to -12 mV in response to light. If CO_2 free air was given firstly, there was no response. When light was given after CO_2 free air, the light effect was very clear. At the onset of CO_2 air, the PD showed a dramatic hyperpolarization to about -25 mV. Changes in the pH of apoplast in intact leaves in response to CO_2 air were observed. CO_2 air caused a change of 0.4 pH unit. Therefore, it can be hypothesized that CO_2 flowing could stimulate proton efflux which is a necessary precursor of stomatal opening.

Key words : CO_2, *Commelina communis*, Stomatal aperture, Membrane potential difference

1. Introduction

Atmospheric CO_2 increases affect not only plant development, phenology, and morphology, but also photosynthesis and growth of plants(Kim, 2012). Thus, CO_2 increase may affect stomatal traits as well. Over the last 50 years, CO_2 concentration increased by 80 μmol mol^{-1}. During this period stomatal density and index of both C3 and C4 plants were declined, while epidermal cell density and stomatal size were increased in more recent years. However, C3 and C4 plants exhibited quite different patterns. During the last 50 years, reduction in stomatal density was greater in C3 plants than in C4 plants(55.6% and 32.9%, respectively) with an opposite pattern in stomatal size (9.3% and 18.3%, respectively)(kim, 2012). Despite the importance of stomata in photosynthesis, little studies have been conducted how CO_2 affect on stomatal mechanism as it is very difficult to see the real effects whether it is CO_2 response or light response. Furthermore, stomata will react differently according to the degrees of CO_2 concentration. The possible significance of endogenous rhythms should not be overlooked as well. The basic role of the stomata is to regulate transpiration and photosynthesis. Therefore, an understanding of the response to CO_2 is critical to discuss to how plant sense the signal(Kim and Lee, 2007).

Environmental factors such as light or CO_2 could 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

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stomatal movements are still controversial. After it have been reported that blue light modulates guard cell osmoregulation via its activation of proton pump via the stimulation of the synthesis of organic solutes, many papers about the candidates of blue light photoreceptors were published(Taiz and Zeiger, 2007; Lee and Bowling, 1992, 1993; Kim and Lee, 2007; Lee, 2010, 2013). The observation of light responses in epidermal strips(Kuiper, 1964; Hsiao et al., 1973; Ogawa et al., 1978) and in isolated guard cell protoplasts(Zeiger, 1983) provide evidence that stomatal responses to light can be separated from those to change in Ci (intercellular 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 5 to 8-fold higher in the light than in the dark. They also reported that, by analogy to the role of guard cell photoreceptors in the light responses, CO_2 sensing by a metabolic reaction in guard cells can be expected to be the first step of sensory transduction process regulating the quantitative modulation of stomatal apertures by CO_2. The CO_2 sensor for stomatal action is located in the epidermis, presumably in guard cells(Pallaghy, 1968), the inner lateral walls of which are permeable to CO_2(Meidner and Mansfield, 1965). 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, gymonosperms, dicots, monocots, C3 plants, C4 plants and CAM(crassulcean acid metabolism) plants. It can be assumed that stomatal response to CO_2 is a general phenomenon(Morison, 1987).

Therefore, to investigate how CO_2 affect on stomatal opening, a series of experiments in which stomatal aperture, membrane potential difference, and apoplastic pH measurements in condition of CO_2 air and CO_2 free air were carried out.

2. Materials and Method

2.1. Light microscopy

Lady`s slipper orchid, *Paphiopedilum insigna* var. sanderae and *Commelina communis* were used for microscopic studies. The lady`s slipper orchid was brought from the Royal Botanic Gardens, Edinburgh in March 1992 by Dr. Tong. *Commelina communis* was gifted from Dr. T. A. Mansfield of the university of Lancaster about 20 years ago. Freshly stripped epidermal peels were washed with distilled water and placed on slides for microscopic examination. Photomicrographs were taken within 30 min. of stripping. Photomicrographs have been included to illustrate a species which have chloroplasts(Plate 1). To determine the presence of chlorophyll in the cells fresh epidermal samples were examined under a Zeiss epifluorescence microscope with blue dark ground, UV fluorescence illumination from a mercury vapour light source. The microscope was equipped with a B229 excitation filter, a 510 nm beam splitter, and a G245/247 barrier filter(Zeiss, Jena). Colour photographs were recorded on Fujichrome 1600 ASA or Kodachrome 800/1600 ASA film. Ilford XP1 film(ASA 50) was used for black and white photography.

2.2. Stomatal aperture measurements

The experiments were carried out on the abaxial

surface of leaves of *Commelina communis*. The plants were potted in John Innes No. 2 compost supplemented with Phostrogen and watered every morning. They were grown in a glass house(minimum temperature of 20 °C during the day and 15 °C at night) under a light regime of 18 hours day and 6 hours night(natural daylight supplemented by high pressure mercury lighting(150 μmole $m^{-2}s^{-1}$). The intact leaves were cut into segments and incubated in a 10cm diameter specially designed plastic Petri dish containing a distilled water, into which air was bubbled through hypodermic needles fitted in the lid. The plastic Petri-dish was specially designed. Water current blocker with 2-3 holes was installed to decrease water current in the medium and to block the spread of small water droplets on the intact leaf disks because of air flow. CO_2 air(900 μmol mol^{-1}) from a cylinder was passed into the plastic Petri dish. CO_2 free air was obtained by passing air through a cylinder of soda lime and 2M KOH solution. For experiments involving a light response, samples were placed under a mercury vapour lamp(200 μmole $m^{-2}s^{-1}$) at 22±2 °C.

The leaf was cut into segments and laid, abaxial surface uppermost in plastic Petri dishes with distilled water. After various intervals intact segments were transferred into liquid paraffin and epidermal strips were peeled. The epidermis was peeled away from the mesophyll by pulling gently on the tab. A 90° peeling angle was used. The peeling angle of 90° represents a compromise between high cell mortality at obtuse angles and excessive contamination with mesophyll at acute angles(Weyer and Travis, 1981). Stomatal apertures of epidermal strips from the intact leaves were measured under a microscope with a calibrated ocular micrometer disc. Measurements of 20 stomata took 2 min., and a strict timetable was employed during experiment. Each experiment was repeated three times and started approximately at the same time in every morning.

2.3. Micro-electrode determination of membrane PD and apoplastic pH.

The measurements were conducted on the abaxial surface of leaves of *Commelina communis* L. Three or four week old fully expanded leaves were excised early in the morning and floated on distilled water in the dark for 1 hour to ensure that stomata were tightly closed. After 1 h., the leaf was put, abaxial side uppermost, on a slide which was placed in a chamber with two holes provided for the air stream. The slide will be inclined at 30^{o} to facilitate micro-electrode implantation. The cut end of the leaf was dipped in distilled water. The chamber was installed on the stage of a streomicroscope and the leaf surface was viewed at a magnification of x 100 with the microscope lamp giving 100 μmole $m^{-2}s^{-1}$.

Micro-electrodes for electrical potential difference measurements were pulled out from glass capillaries (2.0 mm o.d., 1.6 mm I.d.) with internal filament, Clark Electromedical Instrument(Pangbourne, Berks, U.K.), filled with 3 M KCl solution and connected by way of a Ag/AgCl half cell to a voltage follower and a chart recorder. The circuit was completed by a Ag/AgCl reference electrode dipping into a bathing solution. Microelectrodes were inserted into the guard cells using a micromanipulator(Research Instruments TVC 300).

When a steady reading for the PD had been obtained, a stream of CO_2 air(900 μmole $m^{-2}s^{-1}$ from a cylinder was passed into the box chamber. CO_2 free air was obtained by passing air through a cylinder of soda lime and 2M KOH solution. Light intensity used in the measurements was 550 μmole $m^{-2}s^{-1}$, provided by a Lux 150-4 port projector.

The samples of intact leaves for apoplstic pH measurements were prepared using the same method described for the PD experiments. The pH was measured by lowing both micro-electrode on to the same cell as close together as possible without touching(5～10 μm apart) using Zeiss micromanipulators

(C-J, Jena). For the measurements the pH, micropipette tips were back-filled with a proton cocktail(1.22 ml tridodecylamine, 5.93 ml 2-nitrophenyl octyl ether, 0.07 g Na tetraphenylborate) and the stem filled with 3 M KCl(Bowing, 1989). The calibration of pH sensitive micro-electrode was carried out with standard pH buffer solutions(pH 4, 5, 6, 7, 9.2). The slope of the calibration curve between pH 4.0 and pH 9.0 was always greater than 40 mV per pH unit.

3. Results and Discussion

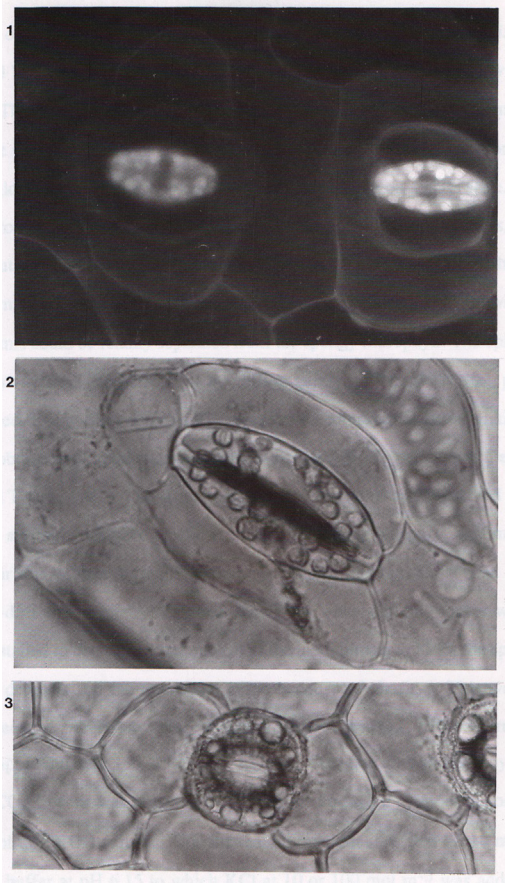

Plate 1. 1, Micrograph of the stomata of *Commelina communis* taken under the fluorescent microscope to highlight the chloroplasts in the guard cells(x315). 2, Micrograph of the stoma of *C. communis*(x315). 3, Micrograph of the stomata of *Paphiopedium insigna* var. sanderae showing no chloroplasts in guard cells(x315).

Plate 1 shows micrographs of the stomata of *Commelina* and *Paphiopedilum*. Chlorophyll in guard cell chloroplasts in *Commelina* emitted a strong red fluorescence(Plate 1-1). The fluorescent *Commelina* chloroplasts appear as white bodies in the micrograph. In contrast to those of *Commelina*, the leaves of the orchid *Papiopedilum* have no chloroplasts on their abaxial and adaxial surface(Plate 1-3). There were no subsidiary cells in *Papiopedilum*. The guard cells of the abaxial surface contain colourless plastids.

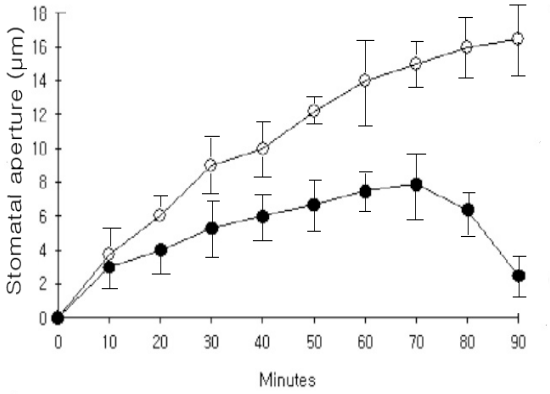

Fig. 1. The effect of CO_2 on stomatal aperture of intact leaves in *Commelina communis*. Leaves were kept in the dark, then exposed to light for 90min. Intact leaf disks were incubated in a chamber which allowed CO_2 air and CO_2 free air to be passed over them. Each point is the mean(±s.e.m.) from three replicate experiments and 60 stomatal apertures were measured. Open circles, light+CO_2 air; closed circles, light+CO_2 free air.

Light is the most important environmental factor stimulating stomatal opening. Stomata usually open when leaves transferred from the darkness to the light. Stomatal opening response was quite fast as it was shown in Fig. 1. On transfer to the light+CO_2 air, the stomata in the intact leaf, floated in distilled water in an enclosed Petri dish, opened about 6 μm at 20 min. Stomata treated with light+CO_2 free air opened to 4 μm at the same time. The stomata(light+CO_2 air) opened to a maximum aperture of around 16 μm at 90

min. However, stomata incubated in light+CO_2 free air opened to a maximum aperture about 8 μm at 70 min. and then closed to 3 μm at 90 min. Full opening of stomatal apertures in the intact leaf was achieved by the addition of CO_2, while the removal of CO_2 caused stomatal opening reduction. In simple qualitative terms, stomatal conductance in *Eucalyptus*, *Commelina*, and *Triticum* leaves was sensitive to C_i at low quantum flux densities(0-250 μmole m^{-2} s^{-1}), where the largest changes in Ci normally occur. At higher quantum flux densities(250-2,000 μmole m^{-2} s^{-1}), conductance was more sensitive to CO_2, but in these ranges Ci did not decrease further with increasing light in normal atmospheric CO_2 concentrations. Quantitatively, the analyses of studies indicate that the response of stomata to the changes in CO_2 is indeed clear(Farquhar et al., 1978; Raschke, 1978; Wong et al., 1978; Sharkey and Raschke, 1981; Ramos and Hall, 1983). Stomatal responses to CO_2 were investigated in *Arabidopsis thaliana* wild type and ABA insensitive mutants(*abi1-1* and *abi1-2*) (Laymarie et al., 1999). Although stomata from *abi1* and *abi2* mutant plants failed to respond to ABA and were originally wide open, they still remained sensitive to illumination and atmospheric CO_2. Results obtained in bioassays as well as in whole plant experiments strengthen the conclusion that stomata from *abi1* and *abi2* remain sensitive to CO_2, suggesting that these mutations differently affect ABA and CO_2 sensing. The specific, rapid effect of CO_2 in stomatal movements could be mainly mediated by changes in membrane permeability. An amplified acidification of guard cell cytoplasm and a concomitant decrease in the pmf (proton motive force), or the use of HCO^{3-} as a counter ion for K^+, occurred by CO_2 could affect long-term response(Lee and Bowling, 1995). The result in Fig. 1 suggested that the opening of stomata treated with light+CO_2 free air was inferior to that treated with light+CO_2 air. Therefore, it can be proposed that a certain amount of CO_2 stimulate stomatal opening. However, light itself has a capacity to induce stomatal opening and it is still in controversialism how CO_2 itself can contribute to stomatal opening.

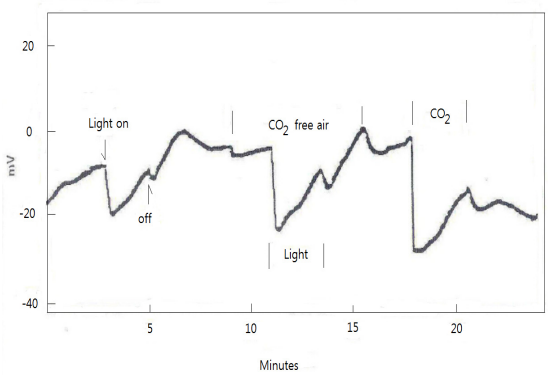

Fig. 2. The effect of white light and CO_2 on the change of membrane PD of the guard cell in a closed stomata in the intact leaf of *Commelina communis*.

To test how CO_2 affect on the stomatal mechanism, electrophysiology technology was applied. When salts diffuse across a membrane, an electrical membrane potential(voltage) can develop. Proton transport is a major determinant of membrane potential. 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. 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(Rachke and Humble, 1973). Therefore, when stomata open, protons are first pumped out from the guard cell, resulting in hyperpolarization of the plasmalemma PD.

The effects of light, CO_2 air and CO_2 free air on the change of membrane potential difference were investigated (Fig. 2). Fast hyperpolarization of guard cell membrane PD was recorded reaching up to -12 mV in response to light. The saturation point of hyperpolarization was reached with a lag time between 1 and 6 seconds. If CO_2 free air was given firstly, there was no response. when light was given after CO_2 free air, the light

effect was very clear. At the onset of CO_2 air, the PD showed a dramatic hyperpolarization to about -25 mV. The effect of CO_2 air on the PD was greater and faster than that of light.

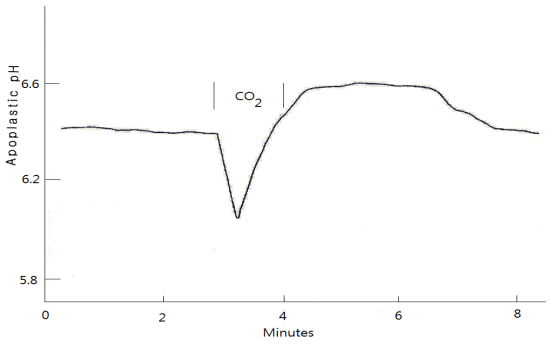

Fig. 3. The effect of CO_2 on the change of apoplastic pH of a closed guard cell in intact leaf of *Commelina communis*.

Cell walls provide plant cells with a substantial degree of volume homeostasis relative to the large changes in water potential that they experience as the every day consequence of the transpirational water losses associated with photosynthesis. The outward, active transport of H^+ across the plasma membrane creates gradients of apoplastic pH and cytosol pH. It was thought that membrane PD changes in response to CO_2 could be result of proton efflux from the guard cell membrane. Accordingly, apoplastic pH changes across the stomatal complex was measured in the intact leaf using pH micro-electrode. CO_2 air caused a abrupt change to about 0.4 pH unit(Fig. 3). The guard cell wall acidified by 0.4 pH units, falling from pH 6.4 to pH 6.0 within 30 sec.

Melis and Zeiger(1982) have put forward a hypothesis that CO_2 modulates phosphorylation. This hypothesis predicts that CO_2 concentrations, inhibition of photophosphorylation, and stomata closure would be positively correlated. Shaish et al.(1989) suggested that CO_2 may regulate ATP levels in guard cells via its effect on oxidative phosphorylation which may be he additional mechanism that operates in darkness. However, CO_2 effect on membrane hyperpolarization was very abrupt and dramatic and it was proved that its effect was not related to energy. Therefore, it can be hypothesized that CO_2 flowing could stimulate proton efflux which is a necessary precursor of stomatal opening.

4. Conclusions

How CO_2 affect stomatal mechanism is very controversial as it is very difficult to see the real effects whether it is CO_2 response or light response. Changes in membrane potential in response to CO_2 are a known feature of many plant cells, and their demonstration in stomatal guard cells has a special significance as a plausible electrical correlation with an electrogenic mechanism driving the large ion fluxes associated with stomatal movements. The specific, rapid effect of CO_2 in stomatal movements could be mediated by changes in membrane permeability. Therefore, the changes in membrane permeability in guard cells will be dominant when the leaf is exposed to certain mount of CO_2. The hypothesis which is widely accepted to explain 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. It was reported that CO_2 induced a great hyperpolarization. Proton transport is a major determinant of the membrane potential. Therefore, CO_2 affect the change of the membrane permeability to proton efflux which is a necessary precursor of stomatal opening.

Actually, there is a supporting report which is directly connected with membrane hyperpolarization in response to CO_2 in green plants, namely, photoinduced ion fluxes in green plant tissue are probably associated pumps regulated by photosynthesis(Higinbotham, 1973).

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