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The Relationship Between Stomatal Opening and Photosynthetic Activity of the Mesophyll in *Commelina Communis* L.

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To investigate the influence of the mesophyll cells on stomatal opening in response to white light, the segments of isolated epidermis were transferred on partly exposed mesophyll cells of a leaf and stomatal apertures were measured. Transferring the isolated epidermis on partly exposed mesophyll cells of a leaf caused a marked increase on stomatal apertures while stomata in isolated epidermis incubated in MES buffer hardly opened. Mesophyll infiltration with photosynthetic inhibitors (DCMU, DCCD, NaN_3) was performed to elucidate the correlation between stomatal apertures and the degree of photosynthetic activity. It was found that transferring the isolated epidermis on partly exposed mesophyll cells of a leaf caused an increase of stomatal apertures depending on the degree of photosynthetic activities. In NaN_3 infiltrated leaf discs, transferring the fresh isolated epidermis on partly exposed mesophyll cells of a leaf showed no significant effect, but a slight increase on stomatal apertures. Isolated epidermis alone did not respond to the light properly, but if it was closely contacted with mesophyll cells, the stomata regained the ability of the light response. Therefore, it could be suggested that stomatal apertures were related with the degree of photosynthetic activity in the mesophyll cells.

Key Words : *Commelina communis,* Guard cells, Mesophyll cells, Stomatal opening

1. Introduction

Extensive studies have shown that stomatal guard cells have independent sensory transduction pathways for environmental factors such as light fluence and CO_2 concentrations[1~3]. So far, how these signals are sensed and how they are transduced into driving the ion fluxes that control stomatal movements is not fully understood.

Recently, the signal transduction chain for a blue light photoreceptor in guard cells has been suggested as the carotenoid pigment zeaxantin[4,5]. Phototropin has also been postulated as a blue light photoreceptor in guard cells[6].

Chloroplasts are a characteristic feature of guard cells and are present in most (but not all) species, and they show photosynthetic electron transport[7,8]. Although guard cell chloroplasts are generally smaller, less numerous, and have fewer grana than mesophyll chloroplasts[7,9], photophosphorylation, on a chlorophyll basis, have been reported as high as 80% of that in the mesophyll cells[10]. Although guard cells have much lower chlorophyll contents than mesophyll cells (25- to 100-fold lower), they are also considerably smaller (approximately 10-fold smaller)[7], Zeiger (1990)[11] cites considerable evidence in favour of the red light receptor being in the guard cell and that the chloroplasts in the guard cell can carry out photosynthesis. Recent studies have implicated photosynthetic carbon fixation in the guard cell chloroplast as a site of sensing[2,4].

However, the role of guard cell chloroplasts in CO_2 fixation is still a matter of debate[7,12~17]. Many investigators attribute the observed correlation between photosynthesis and stomatal conductance[12,18~20]. Outlaw (1989)[18] has reviewed the evidence for CO_2 fixation by guard cells but dismisses most of it on the ground of flawed techniques.

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Although the understanding of the sensory transduction chain for the red light is still in the mystery, it is clear that red light photoreceptor could be situated in the guard cell itself or the mesophyll cells. Lee and Bowling (1995)[12] reported that the basic role of stomata is regulation of 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. 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.

It is difficult to demonstrate if there is a specific influence of the mesophyll on stomatal activity when using whole leaves as it is possible to attribute correlations between photosynthesis and stomatal conductance to parallel response of guard cells and mesophyll cells to the same parallel stimulus. On the other hand, the use of isolated epidermis alone could mean that any mesophyll effect is eliminated and thus missed by the investigators.

The main aim of this study was to investigate the role of the mesophyll cell on stomatal opening. In order to understand the influence of the mesophyll on the stomatal opening, the behaviour of isolated leaf epidermis of *Commelina* is compared with that of the intact leaf. In addition, it was carried out how mesophyll cells affected on stomatal opening when they were attached to isolated epidermis. Lastly, mesophyll cells were infiltrated with photosynthetic inhibitors to reduce the photosynthetic activity and then isolated epidermis was transferred on partly exposed mesophyll cells to examine the relationship between stomatal apertures and the degree of photosynthetic activity.

2. Materials and methods

2.1. Plant material and growth conditions

Commelina communis L. was grown from seeds in a growth chamber (minimum temperature of 20°C during the day and 15°C at night) under light regime of 16 h day and 8 h night (photon flux density was 300 μmol $m^{-2}s^{-1}$ by mercury lamps). At all stages of development, the plants were kept free of water stress by periodic watering and Phostrogen (plant food: 1g/L) was furnished to the plants twice a week. Three weeks old healthy plants were used as materials.

2.2. Measurements of stomatal apertures

Fully expanded leaves were detached and laid, abaxial surface uppermost, in plastic Petri dishes lined with wet filter paper. The dishes were placed in the dark for 1 h before the experiments in order to close the stomata. Strips of lamina between the major parallel veins on either side of the midrib were removed by cutting with a razor blade on a glass slide. A cut was made through the upper epidermis at one end of the lamina strips, taking care not to cut the lower epidermis. When the tissue was inverted, the 'tab' of lamina formed could be lifted with forceps and pulled back for a few mm and the lower epidermis could be readily separated from the mesophyll by pulling gently on the tab. A 90° peeling angle was used which represents a compromise between high cell mortality at obtuse angles[21]. 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 needles fitted in the lids. 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 2.0 M KOH solution. For experiments involving a light response, samples were placed under a mercury vapour lamp (300 μmol $m^{-2}s^{-1}$) at 22±2°C.

For experiments with the intact leaf, the 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 apertures, a microscope (Olympus, Japan) with a camera lucida (x 200 magnification) was connected to a monitor video and a printer calibrated by an ocular micrometer disc. After incubation, isolated epidermis were mounted under the microscope. Stomatal apertures screened in the video could be photographed directly within a minute and then stomatal apertures were accurately measured with a scaler.

2.3. Infiltration of the mesophyll cells with photosynthetic inhibitors

Fig. 1b describes how mesophyll cells were in

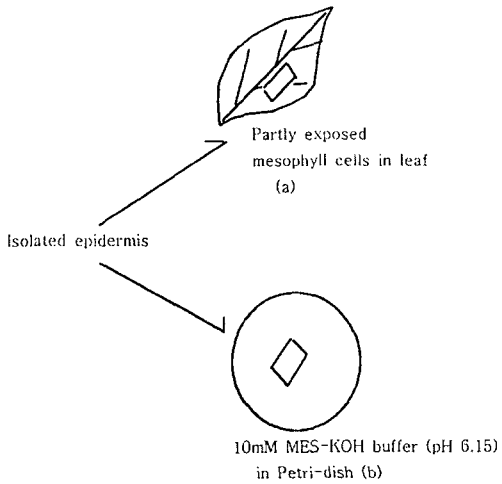

Fig. 1a. The segments of isolated epidermis were transferred on partly exposed mesophyll cell of a leaf in distilled water (a) or 10 mM MES-KOH buffer (pH 6.15; b). The behaviour of the stomata in the isolated epidermis alone and isolated epidermis transferred on the partly exposed mesophyll cells in a leaf has been compared by the measurements of stomatal apertures according to the incubation time.

filtrated with DCMU (3, 4-dichlorophenyl)-1, 1-dimethylurea, Sigma, U.K.), DCCD (dicyclohexylcarboimmide, Sigma, U.K), and NaN_3 (Sigma, U.K) in *Commelina communis*. The mature leaves (second bifoliate leaf) were excised and their petioles immediately dipped into a solution containing 0.5 mM $CaCl_2$ and 0.25 mM $MgSO_4$ buffered at pH 5.9 with 5 mM sodium citrate/10 mM sodium with photosynthetic inhibitors or standard medium. DCMU and DCCD were dissolved in 80% ethanol for a stock solution. The leaves were kept in a growth chamber (minimum temperature of 20°C during the day and 15°C at night) under a regime of 18 hours day and 6 hours night by mercury lamps (300 μmol $m^{-2}s^{-1}$). This infiltration lasted 48 hours and its efficiency was checked by measuring the rates of uptake for medium. The volume of uptake of the medium was around 0.9 mL for 24 h.

2.4. Measurements of photochemical efficiency of PSII

Chlorophyll fluorescence was measured with a PAM-2000 fluorometer (Walz, Germany) after dark-adaptation for 1 h. Minimal fluorescence (Fo), with all open reaction centers, was obtained by measuring the amount of modulated light sufficiently low enough (<0.1 μmol $m^{-2}s^{-1}$) not to induce any significant variable fluorescence. Chlorophyll fluorescences (Fm), with all closed PSII reaction centers, were determined by applying 0.8-s saturation pulse at 600~2100 μmol $m^{-2}s^{-1}$ to dark-adapted leaves. Variable fluorescence (Fv) was equal to Fm minus Fo, hence, the photochemical efficiency of PSII was defined as Fv/Fm[22].

3. Results and discussion

Fig. 2 shows a comparison between the behaviour of the stomata in the intact leaf and isolated epidermis. On transfer to the light, the stomata in the intact leaf,

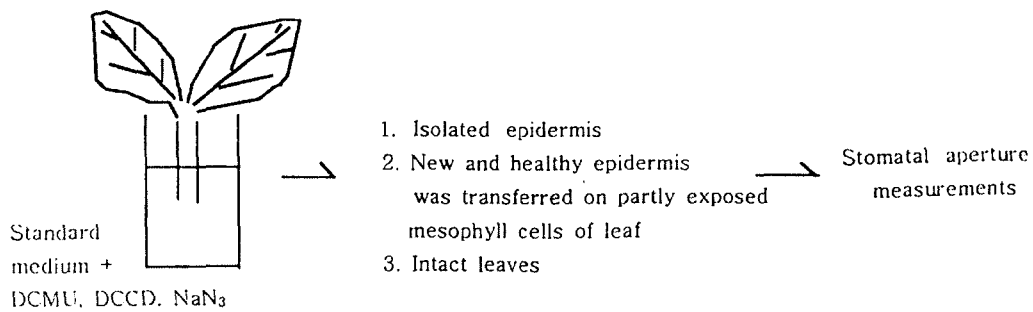

Fig. 1 b. The leaves of *Commelina communis* were infiltrated with photosynthetic inhibitors (DCMU, DCCD, NaN_3) for 48 h. After infiltration with inhibitors, three different experiments for stomatal aperture measurements were performed. Firstly, the segments of isolated epidermis were peeled out and incubated in 10 mM MES-KOH buffer (pH 6.15). Secondly, isolated epidermis was transferred on the partly exposed mesophyll cells of leaf infiltrated with inhibitors. Thirdly, intact leaves infiltrated with inhibitors were cut into segments and laid, abaxial surface uppermost in distilled water. After that the behaviour of stomata in three different materials has been compared by the measurements of stomatal apertures according to the incubation time.

floated in water in an enclosed Petri dish, opened to a maximum aperture of about 11.5 μm after 80 minutes. Stomata in the isolated epidermis, floated on MES buffer without KCl, hardly opened in the light. There was also a linear relationship between time and stomatal apertures under the illumination in intact leaves, but in isolated epidermis, stomatal apertures were kept under 2 μm. In the case of the intact leaf, stomatal aperture was 4.7 μm at 10 min, but in isolated epidermis, the starting point for stomatal opening was not shown and stomatal aperture was only 0.9 μm at 10 min. This kind of similar experiment was already reported and the main point of the results indicates that stomata in isolated epidermis behave differently, both quantitatively and qualitatively, from those in the intact leaf. Stomata in the intact leaf are very sensitive to environmental factors such as light, CO_2 and osmotic stress, but stomata in the isolated epidermis are less sensitive to these factors than those in the intact leaf[12].

In this study, to investigate the role of the mesophyll, the new and healthy isolated epidermis was transferred on partly exposed mesophyll cells of a leaf. After that, the changes of stomatal opening in isolated epidermis were examined how the mesophyll could affect stomatal apertures (Fig. 1a and 3). Transferring the isolated epidermis on partly exposed mesophyll cells of a leaf significantly increased on

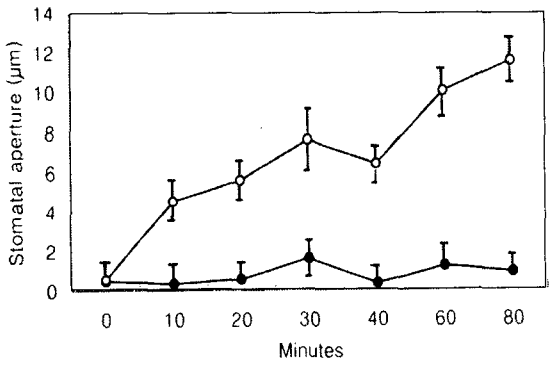

Fig. 2. Stomatal opening of intact leaves and isolated epidermis in *Commelina*. Leaves were kept in the dark for 1 h and exposed to light for 80 min. Each point is the means ± standard errors (n=3 for each variety) and 60 stomatal apertures were measured. Intact leaves (open circles) in distilled water, and isolated epidermis (closed circles) in 10 mM MES-KOH buffer.

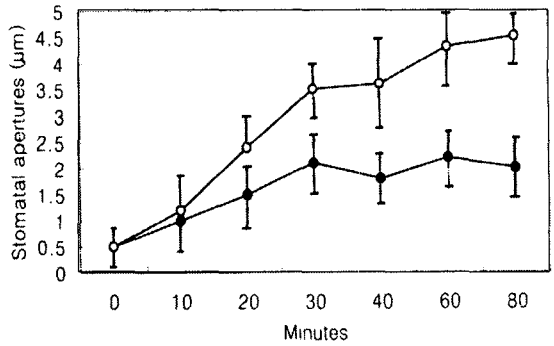

Fig. 3. The effects of transferring the isolated epidermis to partly exposed mesophyll cells of a leaf on stomatal apertures. Each point is the means ± standard errors (n=3 for each variety) and 60 stomatal apertures were measured. Transferred isolated epidermis to the partly exposed mesophyll cells of a leaf (open circles), and isolated epidermis (closed circles) in 10 mM MES-KOH buffer.

stomatal opening in the light while stomata in the isolated epidermis incubated in MES buffer were hardly opened. Stomata in the isolated epidermis might have the ability to open itself. Stomatal aperture in the isolated epidermis was 0.5 μm at zero time, but opened to 2 μm at 30 min. However, Fig. 2 showed that there was no change of stomatal apertures according to the time. Likewise, the results of the stomatal aperture measurements in the isolated epidermis were varied time to time, but the variance was not significant.

Mesophyll infiltration with photosynthetic inhibitors was performed to elucidate the correlation between stomatal apertures and the degree of photosynthetic activity (Table 1 and Fig. 4). Infiltration of the mesophyll cells with DCMU caused about 6 % decrease of the chlorophyll fluorescence parameter (Fv/Fm) in the whole range of light intensity (Table 1). The saturation point of light intensity for the ratio of Fv/Fm was 1200 μmol $m^{-2}s^{-1}$. In the increased light intensity such as 1800 and 2100 μmol $m^{-2}s^{-1}$, the ratio of Fv/Fm was almost same or even lower than that of 1200 μmol $m^{-2}s^{-1}$. The ratio of Fv/Fm is proportional to the activity of photosynthetic reaction center and particularly is related with photosystem II[23]. It was found that infiltration of the mesophyll cells with DCCD showed no significant difference of Fv/Fm in response to light. However, in NaN_3 infiltrated leaf

Table 1. The effects of DCMU, DCCD, and NaN_3 on the quantum yield of Photosystem II, Fv/Fm. The concentrations of photosynthetic inhibitors used here were all 1 mM. Each result is the means ± standard errors (n=3 for each variety) and three of the plants for an each experiment were measured

Light intensity (μmol $m^{-2}s^{-1}$)	Control	DCMU	DCCD	NaN_3
600	0.721±0.05	0.678±0.05	0.733±0.06	0.344±0.02
1200	0.765±0.07	0.688±0.03	0.755±0.05	0.345±0.03
1800	0.766±0.04	0.683±0.04	0.757±0.06	0.347±0.03
2100	0.755±0.03	0.687±0.06	0.747±0.02	0.337±0.02

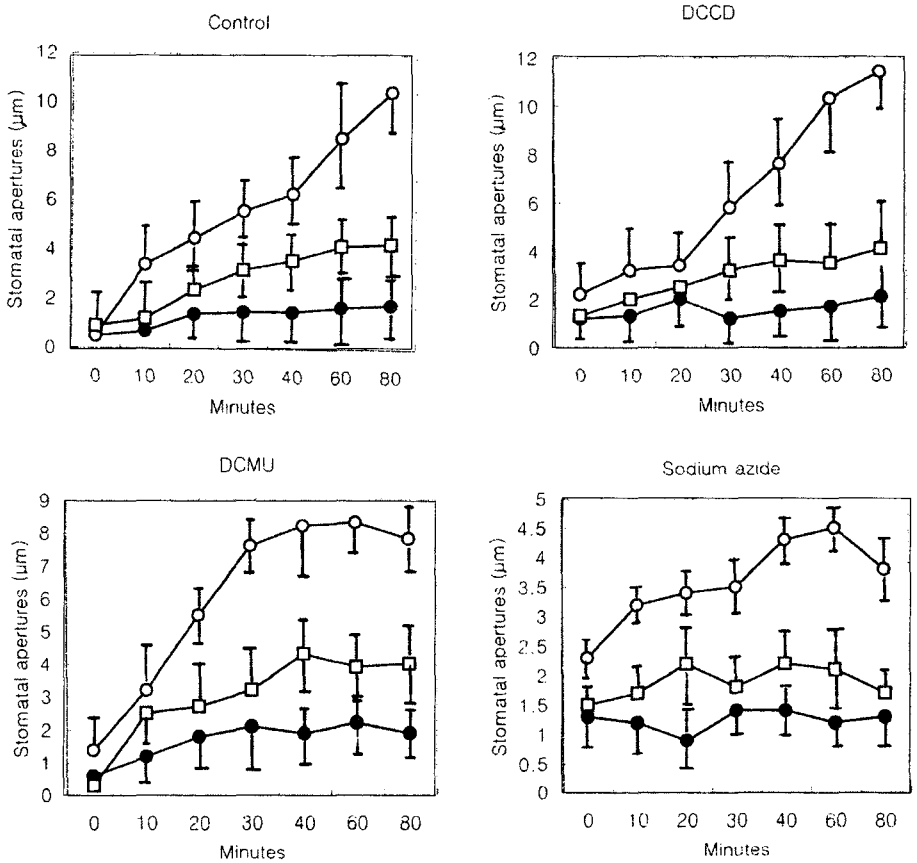

Fig. 4. The effects of mesophyll cells infiltrated with photosynthetic inhibitors on stomatal opening in *Commelina*. The processes of these experiments were explained in detail in Fig. 1b. Each point is the means ± standard errors (n=3 for each variety) and 60 stomatal apertures were measured. Intact leaves (open circles), transferred isolated epidermis to the partly exposed mesophyll cells of a leaf (open squares, and isolated epidermis (closed circles) in 10 mM MES-KOH buffer.

discs, Fv/Fm ratio was reduced to 55%. Even if the site of action of NaN_3 is not known, it has been reported that NaN_3 inhibits Hill reaction[24]. The mesophyll cells infiltrated with NaN_3 was a very important clue as NaN_3 inhibited almost half of the photosynthetic activity compared to the control. Therefore, if the stomatal apertures were measured with new and healthy isolated epidermis transferred on partly exposed mesophyll cells of a leaf infiltrated with NaN_3, the evidence of mesophyll cell participation on the stomatal opening in response to light will be very crucial.

Infiltration of the mesophyll cells with DCMU caused 26% decrease of stomatal apertures in intact

leaves after 80 min. in response to light (Fig. 1b, 4). In this study, DCCD did not affect on stomatal aperture in intact leaves. In NaN_3 infiltrated leaf discs, stomatal opening in the intact leaves was seriously decreased and there was a much reduced stomatal apertures of 55% contrast to the control. Stomata in the isolated epidermis hardly open in whole leaf discs infiltrated with all photosynthetic inhibitors.

It was found that transferring the isolated epidermis on partly exposed mesophyll cells of a leaf caused a marked increase in stomatal apertures depending on the degree of photosynthetic activities. In NaN_3 infiltrated leaf discs, transferring the isolated epidermis to partly exposed mesophyll cells of a leaf showed no significant effect, but a slight increase on stomatal apertures.

This is the first study using the method which the new and healthy isolated epidermis was transferred on partly exposed mesophyll cells of a leaf. The design of this study was referred to Went's experiment as he showed that the active growth promoting substances, auxin, can diffuse into a gelatin block. Stomata in isolated epidermis was not sensitive to the light, but the sleeping stomata in isolated epidermis was awaken when they were closely contacted with the mesophyll cells and responded to the environmental factor such as light[12]. Transferring the isolated epidermis on partly exposed mesophyll cells of a leaf increased stomatal apertures as much as almost two times. However, when the photosynthetic activity of the mesophyll cells were seriously inhibited, transferring the isolated epidermis on the mesophyll cells did not work for an increase of stomatal apertures. In NaN_3 infiltrated leaf discs, Fv/Fm ratio was reduced to 55% and transferring the isolated epidermis on partly exposed mesophyll cells of a leaf showed no significant effect, but a slight increase on stomatal apertures indicating that mesophyll cells were essential to increase the stomatal opening in the isolated epidermis (Fig. 4).

There is no clue how the mesophyll affect on stomatal opening, even though Lee and Bowling (1995)[12] reported that stomatal opening could be mediated by an electrical signal or a chemical propagated from the mesophyll. However, these factors have not yet been identified.

A renewed interests in guard cell carbon metabolism involving sugars was stimulated by two reports[13]. First, Gotow *et al.* (1988)[25] reported that sugar phosphates are formed by photosynthesis in guard cells of broad bean (*Vicia faba* L.). Second, Tallman and Zeiger (1988)[26], also working with *V. faba*, reported that red light causes an increase in stomatal aperture size on epidermal peels and a decrease in guard cell Ψ_s without either an increase in guard cell K^+ concentration or a decrease in guard cell starch content[27,28]. Under other conditions, they found that stomata also open without an increase in guard cell K^+ concentration but with a loss of guard cell starch loss. They indicated that their data are not consistent with K^+ being the universal guard cell osmoticum, and they suggested, as additional osmotica, internal sugars arising from the photosynthetic carbon reduction pathway or starch breakdown.

Lu *et al.* (1997)[13] suggested a hypothesis which sucrose in guard cell wall is a physiological signal that integrates of transpiration, photosynthesis, and translocation. It is generally believed that most evaporation of transpiration stream occurs in or near the guard cell wall[29~33]. In any case, sucrose would accumulate at the distal point in the pathway, the guard cell wall, because of the chromatography effect[29]. The accumulation of sucrose in the guard cell wall would increase with two factors: (a) the sucrose concentration in the apoplast and (b) the rate of transpiration. The first factor, sucrose concentration, is the net effect of sucrose release from mesophyll and efficiency of transport from the leaf[34~36].

Reckman *et al.* (1990)[37] reported that whereas in *P. sativum*, the chlorophyll content of guard cells corresponds to about 1/80 that of mesophyll cells, their Rubisco activity (0.12 pmol $cell^{-1}$ h^{-1}) is just about 1/300 that of ordinary mesophyll cells. The rate of production of hexose through the photosynthetic pentose-phosphate cycle in guard cells could not deliver more than 2% of the flow of osmotic material required for stomatal opening. Pulse labeling, movement of solutes from labeled mesophyll into epidermis placed thereon, and the much greater rate of accumulation of $^{14}CO_2$-fixation products in attached compared with isolated epidermis, all indicate that there is fairly rapid exchange of soluble metabolites between the mesophyll and epidermis[38~42]. These metabolites include glucose, sucrose, sugar phosphate, malate, glycine, serine, and the ethanol-insoluble HCl-hydro-

lysable fraction.

Lu *et al.* (1997)[13] reported that guard cells import mesophyll-drived sucrose from the apoplast. They found low ^{14}C contents in guard cells during the first 20 min after labeling, which eliminated guard cell photosynthetic carbon reduction pathway as a substantial source of the increase in sucrose concentration in the guard cell apoplast. At 40 min after labeling, the sucrose specific radioactivity in the guard cell apoplast was high, which eliminated starch breakdown as the substantial source of the increase in sucrose concentration in the guard cell apoplast.

Stomatal opening in response to light is fairly rapid. On trasnfer to the light, the stomata in the intact leaf opened about 3.4 μm within 5 min[43]. However, it took at least 20 min for the arrival of sucrose to guard cell apoplast from the mesophyll cells[13]. At 20 min, stomatal aperture was already about 6 μm that was half of the maximum stomatal aperture (Fig. 2). Trejo *et al.* (1993)[44] have found that rapid metabolism of Abscisic acid (ABA) in the mesophyll can have a controlling influence on the ABA in both the mesophyll and the epidermis. This is a novel view that the mesophyll can influence hormonal concentrations of guard cells through the metabolism. Although the movement of such metabolites (glucose, sucrose, and sugar phosphate) and hormone from the mesophyll to guard cell seems to be quite clear, it should be clarified how they could affect on stomatal control.

Anyhow, the concept of the white light effect being mediated through the mesophyll is an attractive one as it explains how photosynthesis can exert some control over stomatal conductance and it helps us to understand how stomata lacking chloroplasts such as those in the orchid *Paphiopedilum* can respond to light[12,45,46]. Therefore, it could be suggested that stomatal apertures is closely related with the degree of the photosynthetic activity of the mesophyll cells.

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