Journal of Experimental Botany, Vol. 43, No. 252, pp. 951–957, July 1992

Effect of the Mesophyll on Stomatal Opening in *Commelina communis*

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Received 9 October 1991; Accepted 4 February 1992

ABSTRACT

The effect of a number of factors on the opening of stomata in the intact leaf and in the isolated leaf epidermis of *Commelina communis* has been investigated. Stomata in the intact leaf opened wide in the light and closed rapidly on transfer to the dark. They were also sensitive to CO_2. In contrast, stomata in isolated epidermis floated on an incubation solution containing 100 mol m^{-3} KCl responded neither to light nor CO_2. They opened as widely as those in the intact leaf when treated with fusicoccin.

Stomata in isolated epidermis opened almost as wide as those in the intact leaf when they were incubated with isolated mesophyll cells in the light. The solution in which the mesophyll cells were incubated was separated by centrifugation. The medium from cells previously incubated in the light caused the stomata in isolated epidermis to open but that from cells kept in the dark had no effect. A similar effect was observed when isolated chloroplasts were incubated with the isolated epidermis. However, the supernatant from the chloroplast suspension had no significant effect on stomatal opening.

These results indicate that the mesophyll plays an important role in stomatal opening in the light. The mesophyll appears to produce in the light, but not in the dark, a soluble compound which moves to the guard cells to bring about stomatal opening. The experiments with isolated chloroplasts suggest that this substance is a product of photosynthesis.

Key words: *Commelina communis*, stomata, light, mesophyll.

INTRODUCTION

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 evidence pointed to there being three possible mechanisms for the light response. Firstly, there is a direct response of stomata to light, initially demonstrated by the work of Heath and Russell (1954). Light appears to be absorbed by pigments in the guard cells as stomata in isolated epidermis and isolated guard cell protoplasts show light responses (Zeiger, Iino, Shimazaki, and Ogawa, 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 whilst 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 debate as to which one of these two effects plays the more important role in controlling stomata in the field.

Heath and Russell 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, Cowan, and Farquhar (1979) found that the diffusive conductance of the leaf epidermis to CO_2 transfer changed proportionately with the rate of assimilation. They considered that this suggested that the stomata responded to a metabolite of photosynthesis in the mesophyll. They were in effect proposing that photosynthesis was regulating stomatal

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aperture rather than the orthodox idea that stomata control photosynthesis.

Nelson and Mayo (1975) observed that the stomata of *Paphiopedilum* 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. More recently, Grantz and Schwartz (1988) found that guard cells of *Commelina communis* did not respond metabolically to osmotic stress in isolated epidermis. They concluded that their results were consistent with the view that signal metabolites from the mesophyll mediate stomatal responses. Fischer (1970) came to a similar conclusion from his experiments on water stress in leaves.

Weyers and Meidner (1990) point out that the use of isolated epidermis (epidermal strips) has been a valuable technique central to many advances in our understanding of the stomatal mechanism. However, the results of Grantz and Schwartz demonstrate that isolated epidermis may show rather different stomatal responses from those in the intact leaf. 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, Grantz, and Willmer (1991) measured stomatal pore width in the epidermis of *Commelina communis* using a liquid flow porometer and observed that there was no response by the stomata to light.

It is difficult to determine 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 responses of guard cells and mesophyll cells to the same stimulus. On the other hand, the use of isolated epidermis alone could mean that any mesophyll effect is eliminated and thus missed by the investigator. Therefore, in order to study the influence of the mesophyll on the stomata we have carried out a series of experiments in which the behaviour of the isolated leaf epidermis of *Commelina communis* is compared with that of the intact leaf.

MATERIALS AND METHODS

The experiments were carried out on the abaxial surface of leaves of *Commelina communis*. The plants were grown in John Innes No. 2 compost supplemented with Phostrogen. They were grown in a greenhouse (minimum temperature 15 °C) with supplementary lighting from mercury lamps (150 μE m^{-2} s^{-1}) between sunset and midnight.

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 spongy mesophyll. The epidermis was peeled away 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 and excessive contamination with mesophyll at acute angles (Weyers and Travis, 1981). 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. 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 2·0 mol dm^{-3} KOH solution. The incubation medium consisted of MES buffer (10 mol m^{-3}) at pH 6·15 to which KCl at 10 or 100 mol m^{-3} was added. For experiments involving a light response, samples were placed under a mercury vapour lamp (200 μE 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. Stomatal apertures of epidermal strips from both intact leaf and isolated epidermis were measured under a microscope with a calibrated ocular micrometer disc. Measurements of 20 stomata took 1 min, and a strict timetable was employed during experiments. Each experiment was repeated at least twice and started almost at the same time in the morning (9 am). In experiments with fusicoccin which was dissolved in ethanol, an equivalent amount of ethanol (0·1%) was added to the incubation media of the controls.

Isolation of mesophyll cells

Mesophyll cells were isolated according to the method based on Paul and Bassham (1977). Plants were selected in the early stages of maturation (3–6 weeks) for leaf material. The abaxial side of the epidermis was removed and the remainder cut into small sections with a razor blade. The samples were vacuum-infiltrated with 20 cm^3 digestion medium for 1 min. The vacuum was released slowly to prevent damage to cell ultrastructure. The digestion medium contained the following: pectinase (Sigma) (0·4%), sorbitol (350 mol m^{-3}), bovine albumin (0·1% w/v), succinic acid (20 mol m^{-3}, pH 5·7), KNO_3 (1·0 mol m^{-3}), KH_2PO_4 (0·5 mol m^{-3}), $MgSO_4$ (0·5 mol m^{-3}), and EDTA (1·0 mol m^{-3}).

Incubation in the digestion medium was carried out on a rotary shaker (80 rotations min^{-1}) for 90 min. The cells were filtered through a 100 μm mesh nylon screen, collected by centrifugation at 100 *g* for 1 min and washed three times with a suspension medium. The cell pellet was finally suspended in suspension medium to a chlorophyll concentration between 380–420 μg cm^{-3} before use in the experiments. Light intensity for incubation of mesophyll cells was 300 μE m^{-2} s^{-1}. Suspension medium contained HEPES–KOH (50 mol m^{-3}, pH 7·3), NaCl (10 mol m^{-3}), KCl (70 mol m^{-3}), EDTA (2·0 mol m^{-3}), and $MgSO_4$ (2·0 mol m^{-3}). The pH was adjusted to 7·3.

Isolation of chloroplasts

Chloroplasts were isolated by the method of Lee, Hong, Cho, Lee, and Kwon (1983). 3·0 g of young leaves were cut into small

pieces and then homogenized for 10 s at high speed with 30 cm^3 of extraction buffer (NaCl 10 mol m^{-3}, BSA 0·1%, sorbitol 330 mol m^{-3}, Tricine–KOH buffer 50 mol m^{-3} pH 7·9) by Waring blender. The chloroplasts were filtered through a 50 μm nylon screen and centrifuged at 300 *g* for 3 min. The pellet was discarded and the supernatant centrifuged again at 1300 *g* for 10 min. The chloroplast pellet was finally suspended to a chlorophyll concentration between 360 and 400 μg chl cm^{-3} with reaction buffer (NaCl 10 mol m^{-3}, KCl 2·0 mol m^{-3}, EDTA 2·0 mol m^{-3}, $MgCl_2$ 2·0 mol m^{-3} and HEPES–KOH buffer 50 mol m^{-3}, pH 7·3) and used for the experiment. Other conditions were the same as for the experiments with the mesophyll cells.

RESULTS

Figure 1 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, floated in water in an enclosed Petri dish, opened to a maximum aperture of about 16 μm after about 70 min. After 1·5 h, the leaves were returned to the dark and after a short delay the stomata closed fairly rapidly. Stomata in the isolated epidermis, floated on 100 mol m^{-3} KCl, opened slowly to a maximum aperture of about 5·0 μm and, in contrast to those in the intact leaf, failed to close again on return to darkness. Stomata in epidermal strips floated on 10 mol m^{-3} KCl hardly opened after 90 min in the light.

The results in Fig. 1 suggested that the opening of stomata in isolated epidermis was inferior to that in the intact leaf and was influenced by the concentration of KCl in the medium rather than by light. The opening response to light of stomata in epidermal strips floated on 100 mol m^{-3} KCl was investigated. Figure 2 shows the response of stomata in isolated epidermis transferred to MES buffer containing 100 mol m^{-3} KCl and left

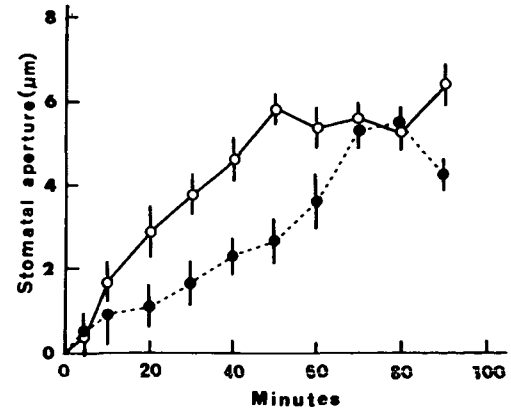

FIG. 2. The effect of light on stomatal aperture in isolated epidermis of *C. communis*. Samples were incubated in 10 mol m^{-3} MES–KOH buffer (pH 6·15) plus 100 mol m^{-3} KCl. Each point is the mean (±sem) from three replicate experiments and 60 stomatal apertures were measured. (○) Light; (●) dark.

either in the light or the dark. It can be seen that there was a faster opening response in the light at first but after 70 min there was no difference in stomatal aperture between the treatments. The maximum stomatal aperture was about 10 μm less than that attained in the intact leaf in the light (Fig. 1).

It was possible that the difference in the degree of stomatal opening observed in intact leaves and isolated epidermis could have been due to the concentration of CO_2. In the intact leaf in the light, photosynthesis in the mesophyll cells would have reduced the concentration of CO_2 in the sub-stomatal cavity thus encouraging stomatal opening. This reduction in CO_2 concentration would not have occurred around the guard cells of the stomata in the isolated epidermis and hence the stomatal opening

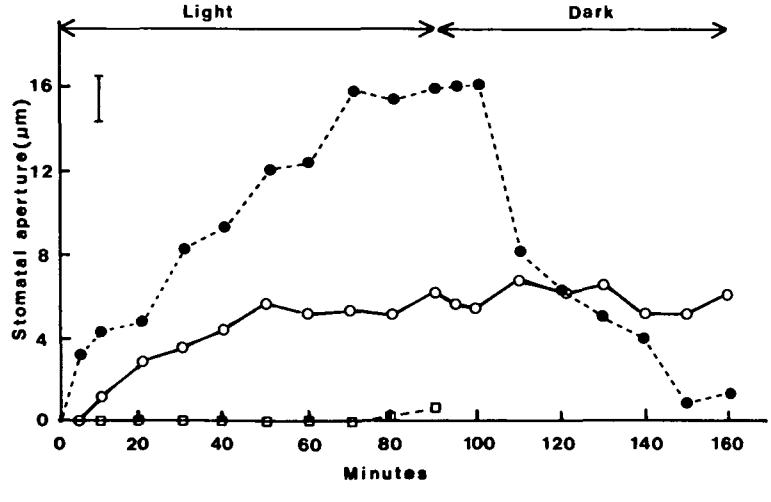

FIG. 1. Opening and closing of stomata of *Commelina communis* in intact leaves and isolated epidermis. Leaves were kept in the dark for 1 h, exposed to light for 90 min and then returned to the dark. Epidermis was taken from the leaf at the end of the dark period. Each point is the mean of two replicate experiments and 40 stomatal apertures were measured. Bar indicates maximum standard error (±0·89). (●) Intact leaves in distilled water; (○) isolated epidermis in 10 mol m^{-3} MES–KOH buffer (pH 6·15) plus 100 mol m^{-3} KCl; (□) isolated epidermis in buffer plus 10 mol m^{-3} KCl.

could have been inhibited. To test this possibility, stomatal opening in epidermal strips in the light was followed in normal air and CO_2-free air. The results of one experiment are shown in Fig. 3. In this experiment the general level of opening was greater than that shown by the epidermal strips in Figs 1 and 2. We found that after 70 min in the light stomatal apertures ranged from about 4 to 8 μm depending on the experiment. Nevertheless, the results show that the concentration of CO_2 had no effect on the degree of stomatal opening. In contrast, we found, as expected, that CO_2 inhibited stomatal opening in intact leaves (results not shown).

The above results showed that stomatal opening in the isolated epidermis of *Commelina* was not only insensitive to light but also unaffected by CO_2 in the medium containing 100 mol m^{-3} KCl. In order to show that there was no mechanical or osmotic impediment preventing the stomata in the isolated epidermis from opening as wide as those in the intact leaf the fungal toxin fusicoccin was used. FC is well known as a stimulator of potassium uptake and hydrogen efflux by the guard cells. It is one of the few compounds which are known to stimulate stomatal opening (Turner, 1973; Marrè, 1979). The effect of FC (0·1 mol m^{-3}) in both the light and the dark on the stomatal aperture is shown in Fig. 4. In the light, the stomata had opened to around 14 μm in the presence of fusicoccin after 90 min. This was almost as wide as the aperture attained in the intact leaf after this time (Fig. 1). Light had a small effect on opening when given in conjunction with FC. Incidentally, the controls in Fig. 4 indicate that there was no significant effect of light after 70 min, confirming the data in Fig. 2.

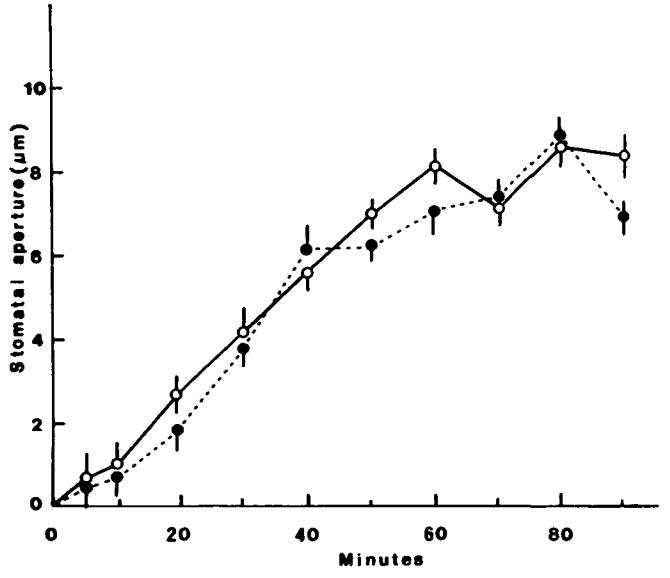

FIG. 3. The effect of CO_2 on stomatal aperture in isolated epidermis of *C. communis*. Leaves were kept in the dark then exposed to light for 90 min. Samples were incubated in 10 mol m^{-3} MES–KOH buffer (pH 6·15) containing 100 mol m^{-3} KCl. Each point is the mean (±sem) from three replicate experiments and 60 stomatal apertures were measured. (○) Light − CO_2; (●) light + CO_2.

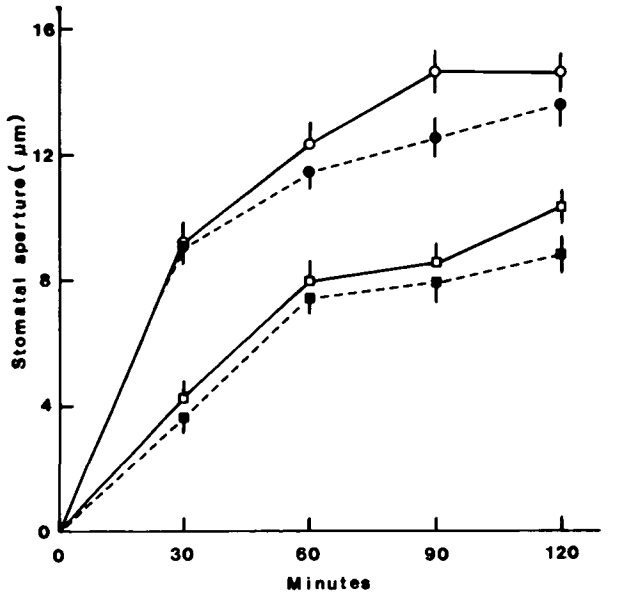

FIG. 4. The effect of fusicoccin (0·1 mol m^{-3}) on stomatal opening in isolated epidermis of *C. communis*. Samples were incubated in the same buffer as in Fig. 2. Each point is the mean (±sem) of three replicate experiments and 90 stomatal apertures were measured. (○) Light + fusicoccin; (●) dark + fusicoccin; (□) control light; (■) control dark.

The absence of an effect of light and CO_2 on the stomata in isolated epidermis and the evidence that they possessed the potential of those in the intact leaf to open wide, suggested that the mesophyll could be important in influencing stomatal opening in the intact leaf. It was possible that control by light and CO_2 was being exerted in the mesophyll. Experiments were carried out in which epidermal strips were incubated with and without isolated mesophyll cells to try to determine the effect of the mesophyll. The results of one experiment carried out in normal air are shown in Fig. 5. The presence of the mesophyll cells in the incubation solution significantly increased the degree of stomatal opening in the light. In the dark, opening was approximately the same as that for the controls without added mesophyll. A similar experiment, carried out in CO_2-free air showed no difference between the light and dark treatments after 90 min but both showed a considerable increase in aperture compared to the control (Fig. 6). Therefore, the presence of the mesophyll had restored the stimulatory effect of light and the inhibitory effect of CO_2.

Batches of isolated mesophyll cells were incubated either in the light or the dark for 1 h and then the cells were removed by centrifugation at 1000 *g* for 5 min. The resulting supernatant was completely free of cells and showed no absorption bands characteristic of chlorophyll. Figure 7 shows the effect of the mesophyll supernatant on the stomata in isolated epidermis. It can be seen that

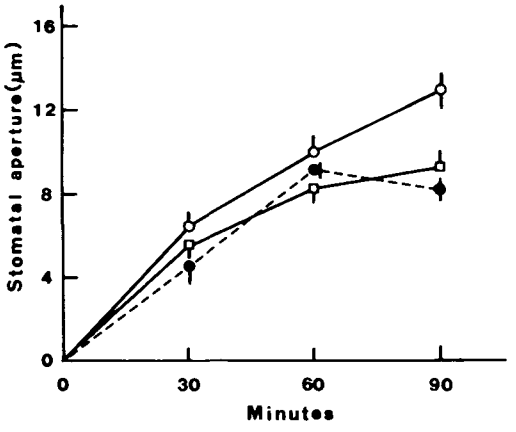

FIG. 5. The effect of mesophyll cells on stomatal opening in isolated epidermis of *C. communis*. The epidermis was incubated with mesophyll cells under normal air. Points represent means (±sem) based on 40 measurements. (○) Light + mesophyll cells; (●) dark + mesophyll cells; (□) control, light − mesophyll.

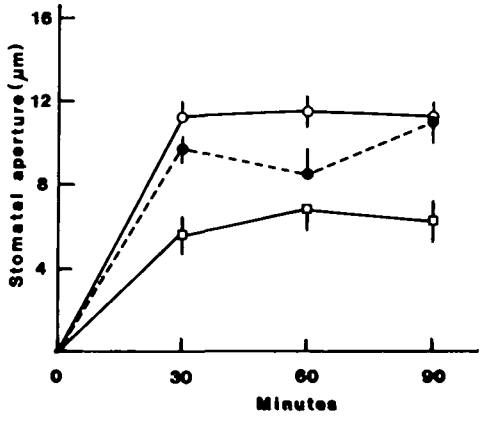

FIG. 6. The effects of mesophyll cells on stomatal opening in isolated epidermis of *C. communis*. The epidermis was incubated with mesophyll cells under CO_2-free air. Points represent means (±sem) based on 40 measurements. (○) Light + mesophyll cells; (●) dark + mesophyll cells; (□) control, light − mesophyll.

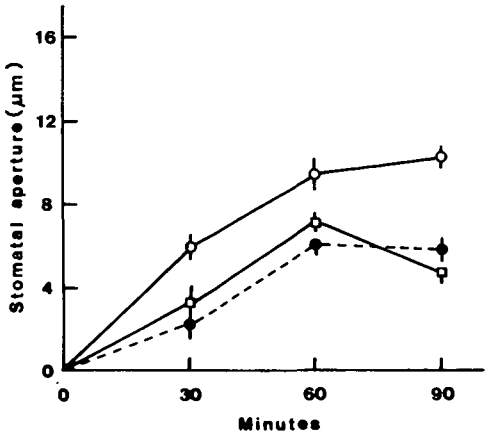

FIG. 7. The effect of the supernatant from mesophyll cells which were pretreated in light or dark on stomatal opening in isolated epidermis of *C. communis*. The epidermis was incubated in the supernatant under CO_2-free air. Each point is the mean (±sem) of three replicate experiments and 60 stomatal apertures were measured. (○) Light + supernatant; (●) dark + supernatant; (□) control, light − supernatant.

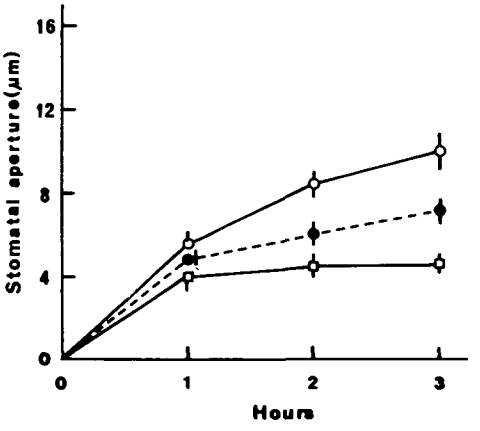

FIG. 8. The effect of chloroplasts on stomatal opening in isolated epidermis of *C. communis*. Each point is the mean (±sem) of three replicate experiments and 60 stomatal apertures were measured. (○) Light + chloroplasts; (●) dark + chloroplasts; (□) control, light − chloroplasts.

supernatant from cells previously incubated in the light caused a marked increase in stomatal aperture whilst supernatant from cells previously kept in the dark had no effect on the stomata. Thus, the stimulatory influence of the mesophyll cells in bringing about stomatal opening could be separated from the cells into solution.

Experiments were also carried out with isolated chloroplasts. These were similar to those involving isolated mesophyll. Figure 8 shows the effect of freshly isolated chloroplasts on stomata in isolated epidermis. There was a significant effect of chloroplasts incubated in the light on stomatal aperture although it was much slower to take effect than with the mesophyll. Chloroplasts in darkness also had a significant but lesser effect on aperture. The effect of supernatant from chloroplast suspensions was also investigated. However, as the results in Fig. 9 show, neither supernatant from chloroplasts incubated in the light nor in the dark produced any significant increase in stomatal aperture.

DISCUSSION

The results show that under the conditions of our experiments stomata in isolated epidermis from *Commelina* leaves behave differently, both quantitatively and qualitatively, from those in the intact leaf. Stomata in detached epidermis did not respond to light or CO_2 but appeared to open solely in response to the ambient concentration of KCl. They opened to a maximum aperture of 4–8 μm in 100 mol m^{-3} KCl but showed only a slight opening response in 10 mol m^{-3} KCl. This is in general agreement with the results of Travis and Mansfield (1979). They observed only a small effect of light and CO_2 at these

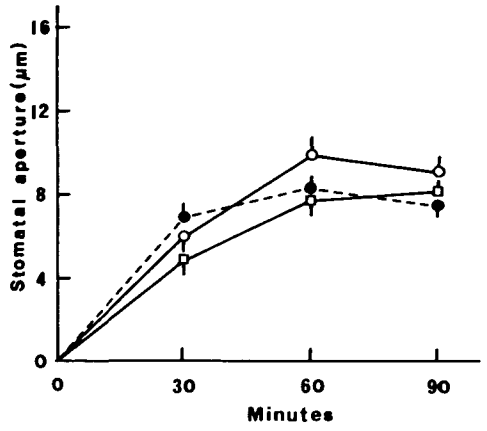

FIG. 9. The effect of the supernatant from chloroplasts which was pretreated by light or dark on stomatal opening in isolated epidermis of *C. communis*. Each point is the mean ±sem of four replicate experiments and 80 stomatal apertures were measured. (○) Light + supernatant; (●) dark + supernatant; (□) control, light − supernatant.

concentrations but a relatively large effect at 50 mol m^{-3} KCl. The stomata in the isolated epidermis also opened to a lesser degree than those in the intact leaf. However, removal of the epidermis did not appear to have damaged them as they opened in the presence of fusicoccin almost as wide as those in the intact leaf.

Freshly isolated mesophyll cells, when incubated with isolated epidermis, caused the stomata to open almost as wide as those in the intact leaf (Figs 5, 6). In CO_2-free air, opening was stimulated both in the light and in the dark but in normal air opening was only stimulated in the light. The presence of CO_2 appeared to inhibit opening in the dark. Therefore, addition of mesophyll cells to the isolated epidermis appeared to restore the sensitivity of the stomata to light and CO_2 indicating that the mesophyll was playing an important role in controlling stomatal aperture. This raised the question of the nature of the link between the epidermis and the mesophyll. Was it electrical or chemical? The results from experiments with supernatant isolated from the mesophyll such as those in Fig. 7 clearly indicate the latter. The cell-free extract produced the same effect on stomata in isolated epidermis as the mesophyll cells themselves.

Clearly, the mesophyll contained some compound which diffused out of the cells into the medium. This substance was present in freshly isolated mesophyll and exerted its effect on stomata whether the cells were subsequently incubated in the light or the dark (Fig. 6). However, if the mesophyll cells were incubated in the dark for 1 h and then separated from the medium there was no opening response, in contrast to the response evoked by the supernatant from mesophyll cells which had been exposed to light beforehand (Fig. 7). Therefore, the substance was produced by the mesophyll in the light and appeared to be stable. The stomata-opening factor appeared to be a product of the chloroplasts as a suspension of isolated chloroplasts caused a similar level of stomatal opening as the mesophyll (Fig. 8). This strongly suggested that it may be a product of photosynthesis.

The effect of the mesophyll factor on stomatal aperture was manifest because the effect of light on the stomata in epidermal strips incubated in 100 mol m^{-3} KCl was absent. We found stomatal apertures in the light of 5–8 μm. However, this was not exceptional. Willmer and Mansfield (1969) concluded that epidermal strips of *Commelina* are suitable for studies of stomatal responses to light and CO_2 provided they are 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 to 1 h of 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. Therefore, there is a fair amount of evidence that stomatal apertures in epidermal strips of *Commelina* fall far short of the apertures attained in the intact leaf.

For ease of description we tentatively suggest that the putative factor which promotes stomatal opening indicated by our results be called stomatin. We carried out a number of experiments to determine if stomatin could be identified with any of the common metabolites known to be involved in stomatal opening. We incubated isolated epidermis with D-glucose, sucrose, malic acid, and ATP, but found that none of these compounds stimulated stomatal opening. On the contrary, inhibition of stomatal opening was sometimes observed. Stomatin is likely to be present in very low concentrations in the supernatant from mesophyll cells and its concentration appeared to be so low in supernatant from chloroplasts as to evoke little or no response in the stomata (Fig. 9).

The evidence presented here for a particular compound produced by the mesophyll in the light which brings about stomatal opening in a manner similar to fusicoccin, helps to explain some of the earlier results which link the mesophyll and photosynthesis to stomatal regulation. Our results support the suggestion of Heath and Russell (1954) for an indirect effect of light on stomata and explain how photosynthesis could, under some conditions, be controlling the stomata as suggested by Wong *et al.* (1979). They also help us to understand how the stomata of *Paphiopedilum* can respond to light without guard cell chloroplasts. Abscisic acid has long been known as a substance produced at a distance from the stomata and which brings about closure. Here, we put forward the concept of a compound, 'stomatin', produced in the chloroplasts of the mesophyll which brings about stomatal opening.

ACKNOWLEDGEMENT

Joonsang Lee was a recipient of a Korean Government Overseas Scholarship during the course of this work.

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