

The Stomata of the Fern *Adiantum capillus-veneris* Do Not Respond to CO₂ in the Dark and Open by Photosynthesis in Guard Cells¹[OA]

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The stomata of the fern *Adiantum capillus-veneris* lack a blue light-specific opening response but open in response to red light. We investigated this light response of *Adiantum* stomata and found that the light wavelength dependence of stomatal opening matched that of photosynthesis. The simultaneous application of red (2 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and far-red (50 $\mu\text{mol m}^{-2} \text{s}^{-1}$) light synergistically induced stomatal opening, but application of only one of these wavelengths was ineffective. *Adiantum* stomata did not respond to CO₂ in the dark; the stomata neither opened under a low intercellular CO₂ concentration nor closed under high intercellular CO₂ concentration. Stomata in *Arabidopsis* (*Arabidopsis thaliana*), which were used as a control, showed clear sensitivity to CO₂. In *Adiantum*, stomatal conductance showed much higher light sensitivity when the light was applied to the lower leaf surface, where stomata exist, than when it was applied to the upper surface. This suggests that guard cells likely sensed the light required for stomatal opening. In the epidermal fragments, red light induced both stomatal opening and K⁺ accumulation in guard cells, and both of these responses were inhibited by a photosynthetic inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The stomatal opening was completely inhibited by CsCl, a K⁺ channel blocker. In intact fern leaves, red light-induced stomatal opening was also suppressed by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. These results indicate that *Adiantum* stomata lack sensitivity to CO₂ in the dark and that stomatal opening is driven by photosynthetic electron transport in guard cell chloroplasts, probably via K⁺ uptake.

Stomata are small adjustable pores located on the surface of leaves. They allow the exchange of CO₂ and water between the leaf interior and the atmosphere. Through the regulation of stomatal apertures, plants obtain the CO₂ required for photosynthesis and prevent excessive water loss through transpiration. Stomata are found in mosses, ferns, and higher plants, and several works have shown their morphological and/or functional diversity (Willmer and Fricker, 1996; Franks and Farquhar, 2007). Such differences may have arisen as the plants evolved to adapt to different environmental factors. While several environmental stimuli, such as light, CO₂, humidity, and temperature, have been shown to regulate stomatal movements in higher plants (Willmer and Fricker, 1996), little is known about how these stimuli affect the stomata of ferns.

In higher plants, stomata open in response to light through at least two light signaling cascades, so-called blue light-specific and red light-induced responses (Shimazaki et al., 2007). Because the blue light-specific

response is mediated by guard cells, this response is observed in both the detached epidermis and guard cell protoplasts (Zeiger and Hepler, 1977; Shimazaki et al., 1986). The response is initiated by blue light absorption by blue light receptors, phototropins, followed by the activation of the plasma membrane H⁺-ATPase via the signal transduction cascade, which is not yet fully understood (Kinoshita and Shimazaki, 1999; Kinoshita et al., 2001; Shimazaki et al., 2007). The blue light-specific response is largely enhanced by background red light irradiation (Ogawa et al., 1978), and blue light at low intensity is effective in the presence of red light.

In contrast, a high intensity of red light is required for inducing stomatal opening, and the light wavelength dependence matches photosynthetically active radiation. The action spectrum for stomatal opening resembles that for leaf photosynthesis (Sharkey and Raschke, 1981). In accord with these results, some studies have indicated that a photosynthetic electron transport inhibitor, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU), inhibits red light-induced stomatal opening (Sharkey and Raschke, 1981; Schwartz and Zeiger, 1984; Tominaga et al., 2001; Olsen et al., 2002). Mesophyll and guard cells have photosynthetically active chloroplasts (Zeiger et al., 1981; Shimazaki et al., 1982; Shimazaki and Zeiger, 1985; Cardon and Berry, 1992; Wu and Assmann, 1993; Lawson et al., 2002), and their photosynthesis has been suggested to induce stomatal opening in intact plants.

Because stomata open in response to a low concentration of ambient CO₂ (Ca) and close in response

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to elevated levels of CO₂ (Morison, 1987; Assmann, 1999; Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005), it is generally accepted that guard cells sense intercellular CO₂ concentration (C_i; Mott, 1988). This suggests that the decrease in C_i brought about by mesophyll photosynthesis induces the stomatal opening. Furthermore, it has been suggested that the ratio of C_i to C_a is kept constant under various conditions of the leaves (Wong et al., 1979), although the ratio is not always conserved in the plants (von Caemmerer et al., 2004; Baroli et al., 2008). In accord with these, red light applied to a large area of the leaf, including mesophyll cells, triggered stomatal opening, probably due to mesophyll photosynthesis. However, the responses were not found when a localized beam of red light was applied only to individual guard cells, and thus C_i was proposed to have a role as an intermediate in the stomatal response (Roelfsema et al., 2002). Moreover, red light did not induce stomatal opening in albino leaf patches (Roelfsema et al., 2006).

By contrast, several lines of evidence have suggested that guard cell chloroplasts have a direct role in stomatal opening. Red light induces stomatal opening in isolated epidermis (Sharkey and Raschke, 1981; Schwartz and Zeiger, 1984; Olsen et al., 2002). Red light is suggested to activate the H⁺ pump in the plasma membrane of guard cells (Serrano et al., 1988), although the pump activation has not been reproduced (Roelfsema et al., 2001; Taylor and Assmann, 2001). A recent report indicates that cocklebur stomata open in response to red light independent of reduced C_i (Messinger et al., 2006). However, it is unclear whether or not guard cell chloroplasts are responsible for stomatal opening in intact plant leaves. Furthermore, phytochrome has been suggested to be involved in stomatal response to red light (Talbot et al., 2003).

Adiantum capillus-veneris belongs to the Leptosporangiopsida, which are newly diversified fern species that grow in shadow beneath angiosperms (Schneider et al., 2004), and usually makes its habitat beneath the canopy, where the ambient light is weak and rich in far-red. The stomata are found only on the lower surface of leaves and lack subsidiary cells, and their guard cells contain densely arranged chloroplasts. Recently, we reported that the stomata of the fern *A. capillus-veneris* lacked a blue light-specific opening response but did open in response to red light (Doi et al., 2006). A lack of blue light-specific response has commonly been seen in other species of Leptosporangiopsida. We also showed that phy3 (neochrome 1; Suetsugu et al., 2005), a chimeric protein of phototropin and phytochrome, did not work as a photoreceptor for red light-induced stomatal opening in *Adiantum*. Therefore, the red light-induced stomatal opening in *Adiantum* could be explained by the photosynthetic activity and/or by the response via phytochrome. Here, we investigated the stomatal responses of *Adiantum* to light and CO₂ in the dark. We demonstrated that the guard cells are insensitive to CO₂ and that the guard cell chloroplasts are responsible

for stomatal opening in response to light in this plant species.

RESULTS

Stomatal Opening in Response to Photosynthetically Active Radiation

Stomatal responses to light were investigated in intact *Adiantum* leaves using gas exchange techniques (Fig. 1). As previously reported, the application of photosynthetically saturating red light at 600 μmol m⁻² s⁻¹ induced a fast increase in stomatal conductance (Fig. 1A; Doi et al., 2006). The same intensity of blue light (600 μmol m⁻² s⁻¹) brought about a similar increase in conductance. The initial rates of conductance increase by red and blue light were 0.061 ± 0.011 and 0.063 ± 0.009 (means of five measurements ± SE) mmol m⁻² s⁻², respectively. When the light was turned off, the stomatal conductance decreased very slowly, but the photosynthetic CO₂ fixation ceased instantly. The C_i decreased immediately upon light irradiation but did not return to the original levels after the light was turned off, probably due to the inability of the stomata to close rapidly. Neither a brief pulse of red light nor a brief pulse of blue light induced stomatal opening; rather, continuous irradiation was required for the opening (data not shown).

To characterize the mechanisms underlying the stomatal responses to light in *Adiantum*, we determined the light dependence of the conductance increase using red, green, and blue light (Fig. 1B). The rates of stomatal conductance increase occurred at around 5 μmol m⁻² s⁻¹. The response was saturated at 80 μmol m⁻² s⁻¹, at which wavelength mesophyll photosynthesis was not saturated (see Fig. 4). Similarly to red light, both blue and green light increased stomatal conductance at low intensity. In contrast, far-red light did not induce any increase in the conductance up to 300 μmol m⁻² s⁻¹. A linear regression was obtained for each light wavelength that fell in the linear response range (Fig. 1B). The slopes of the linear regression lines in blue, green, and red light were 0.043 ± 0.003, 0.042 ± 0.003, and 0.048 ± 0.006 (average of three measurements ± SE), respectively. The lines were parallel to each other. Using these data, we determined the effectiveness of each light wavelength (Table I). Blue, red, and green light were effective at inducing an increase in conductance, with blue light being the most effective and green light the least, and this pattern of efficacy was in accord with the hypothesis that the photosynthetic pigments acted as photoreceptors for the responses (Taiz and Zeiger, 2006).

Synergistic Effect of Red and Far-Red Light on Stomatal Opening

We showed that phy3, a chimeric protein of phototropin and phytochrome, did not function as a photoreceptor for red light-induced stomatal opening in

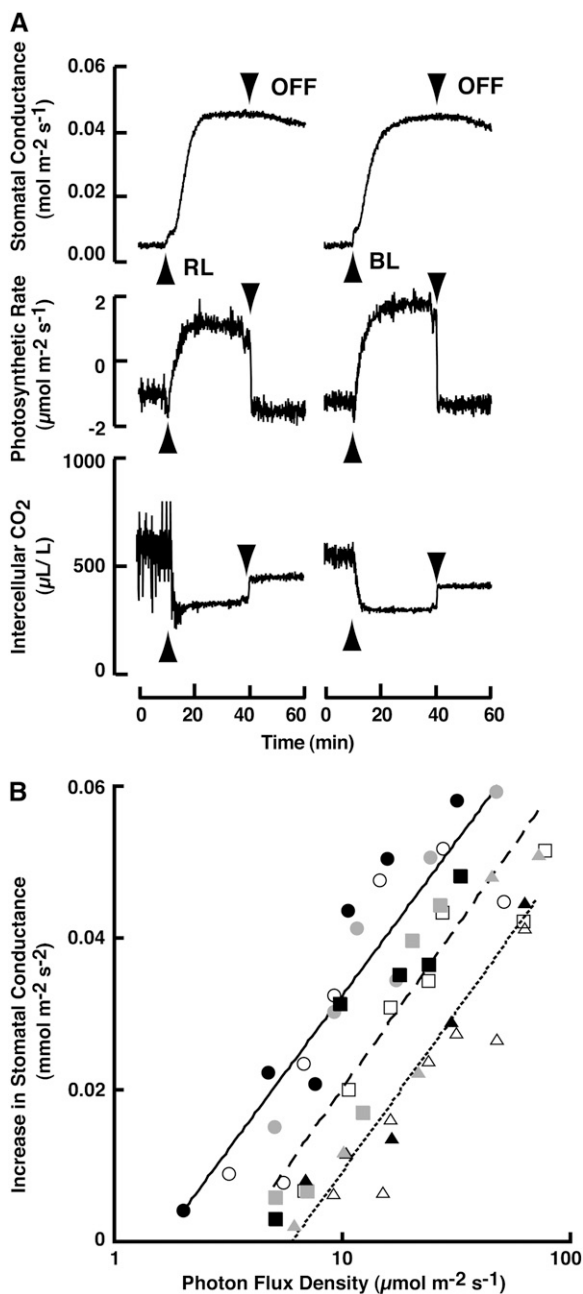


Figure 1. Stomatal responses to light in intact leaves of *A. capillus-veneris*. A, Stomatal conductance, photosynthetic rate, and Ci in response to red (left) and blue (right) light. Each light at an intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$ was applied to the upper surface of a leaf as indicated by the upward-pointing arrowhead, and turned off as indicated by the downward-pointing arrowhead. B, PFD response curves for stomatal opening. For each type of light, opening rates were measured at five to seven PFDs with separate leaves of the same plant. Circles, Blue light; squares, red light; triangles, green light. Black, gray, and white symbols represent the separate experiments that were conducted on the different days, respectively. Solid, broken, and dotted lines represent the regression curves for blue, red, and green lights, respectively.

Adiantum (Doi et al., 2006). However, *Adiantum* has at least three phytochromes besides phy3 (Wada et al., 1997). To determine whether or not these phytochromes are responsible for red light-induced stomatal opening in *Adiantum*, we investigated the effect of far-red light on stomatal opening. Red light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ applied to the upper surface of leaves induced stomatal opening (Fig. 2A, gray line). When this red light was applied together with far-red light at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$, the added far-red light increased stomatal conductance by 15% (Fig. 2A, black line).

The remarkable synergistic effect between red and far-red light on stomatal opening was found in *Adiantum* when the light intensities were decreased. When far-red light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ was applied to the leaf, no increase in stomatal conductance was found (Fig. 2B). However, when red light at $2 \mu\text{mol m}^{-2} \text{s}^{-1}$, which alone could not induce any detectable stomatal opening (Fig. 2C), was superimposed on the far-red light, the conductance increased markedly (Fig. 2B). Furthermore, when far-red light at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ was applied to leaves superimposed on the preceding red light at $2 \mu\text{mol m}^{-2} \text{s}^{-1}$, a similarly large increase in conductance was observed (Fig. 2C). Because neither red light nor far-red light alone increased stomatal conductance, the combined effects of red and far-red light on stomatal conductance were synergistic. In a trial in which the leaves were preirradiated with red or far-red light, this pretreatment had no effects on the subsequent stomatal response elicited by far-red or red light, respectively (data not shown). As shown in Figure 2A, far-red light did not suppress red light-induced stomatal opening but rather enhanced it. These results suggest that light induces stomatal opening through photosynthesis and that phytochromes are not involved in the response of *Adiantum*. The synergistic effect between red and far-red light on stomatal opening may be attributable to the so-called enhancement effect (the Emerson effect) in photosynthesis, where PSI and PSII operate in series (Taiz and Zeiger, 2006).

Lack of Stomatal Response to Ci in *Adiantum* under Darkness

It is likely that red light induces stomatal opening through the reduction of Ci in *Adiantum* leaves, because a high Ci causes stomatal closure via the activation of anion channels in guard cells of higher plants (Hanstein and Felle, 2002; Roelfsema et al., 2002; Roelfsema and Hedrich, 2005). We previously reported that the Ci was 250 to $275 \mu\text{L L}^{-1}$ under saturated red light in *Adiantum* leaves in which photosynthetic CO₂ fixation proceeded (Fig. 1A; Doi et al., 2006) when Ci was $350 \mu\text{L L}^{-1}$. If light-induced stomatal opening occurred via the reduction of Ci brought about by mesophyll photosynthesis, the stomata in *Adiantum* should open at a Ci below $250 \mu\text{L L}^{-1}$. To test this, we investigated the stomatal responses under different Ci values, which we manipulated by changing Ci in the dark. First, we

Table 1. PFD required for achieving a 50% increase in the rate of stomatal conductance increase

Values are averages of three measurements \pm SE.

	Blue Light	Green Light	Red Light
PFD	10.4 \pm 1.2	37.5 \pm 5.7	19.2 \pm 2.3

verified the stomatal response to Ca using *Arabidopsis* leaves as a control (Fig. 3A). In *Arabidopsis*, stomatal conductance was $0.0114 \pm 0.0005 \text{ mol m}^{-2} \text{ s}^{-1}$ (average of three measurements \pm SE) when a leaf was exposed to Ca at $350 \mu\text{L L}^{-1}$ in the dark, which was close to the atmospheric concentration of CO_2 . When Ca was decreased to $0 \mu\text{L L}^{-1}$, stomatal conductance increased substantially, peaking at $0.1213 \pm 0.007 \text{ mol m}^{-2} \text{ s}^{-1}$ (average of three measurements \pm SE), and it decreased to a new steady state when Ca was increased from 0 to $1,200 \mu\text{L L}^{-1}$. The C_i values were determined to be 85.4 ± 0.7 , 455.6 ± 3.4 , and $1,200.0 \pm 6.3 \mu\text{L L}^{-1}$ (average of three measurements \pm SE) at Ca of 0, 350, and $1,200 \mu\text{L L}^{-1}$, respectively. Thus, *Arabidopsis* stomata had sensitivity to CO_2 ; they opened when Ca was reduced below atmospheric concentration in the dark and closed under high Ca. Surprisingly, stomata in the *Adiantum* leaves did not open when Ca was decreased from $350 \mu\text{L L}^{-1}$ to $0 \mu\text{L L}^{-1}$ (Fig. 3B) and did not close when Ca was increased from $0 \mu\text{L L}^{-1}$ to $1,200 \mu\text{L L}^{-1}$. These results indicate that stomata in *Adiantum* leaves are not sensitive to CO_2 in the dark. However, it was suspected that the tight closing of the stomata prevented any changes in C_i despite the drastic changes in Ca. To test this possibility, we changed the Ca over a wide range and determined both C_i and stomatal conductance in *Adiantum* leaves (Fig. 3C). The C_i changed with the changes of Ca and showed a linear correlation with Ca, whereas stomatal conductance did not change in the Ca range of 0 to $1,200 \mu\text{L L}^{-1}$. C_i was $<200 \mu\text{L L}^{-1}$ and $>1,000 \mu\text{L L}^{-1}$ under darkness when leaves were exposed to CO_2 -free air and air having Ca of $1,200 \mu\text{L L}^{-1}$, respectively. The C_i under the CO_2 -free air was lower than that of the *Adiantum* leaves under light, in which stomata opened (Fig. 1A).

Stomatal Opening Is Dependent on Guard Cell Photosynthesis

As described above, photosynthetic active radiation increased stomatal conductance in the fern *Adiantum*. Because a decrease in C_i did not induce stomatal opening (Fig. 3), photosynthesis in guard cells seems to drive stomatal opening in *Adiantum*. To examine the role of chloroplasts in guard cells, we utilized the fact that guard cells are localized exclusively to the lower surface of *Adiantum* leaves. If guard cell chloroplasts are responsible for stomatal opening, it would be expected that light applied to the lower leaf surface would be much more effective at increasing stomatal

conductance than light applied to the upper surface. And indeed, when red light was applied to the lower surface, stomatal conductance increased at a light intensity of $<5 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and continued to increase with increasing light intensity, reaching a maximum at about $20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (Fig. 4). By contrast, no conductance increase was found at $5 \mu\text{mol m}^{-2} \text{ s}^{-1}$ when red light was applied to the upper surface. The half-saturation for the conductance increase was $20 \mu\text{mol m}^{-2} \text{ s}^{-1}$ under this condition. Despite the difference in the requirement of light intensity for stomatal opening, the maximum rates of conductance increase were the same irrespective of the difference in the irradiated leaf surfaces (Fig. 4). We also measured the photosynthetic CO_2 fixation of *Adiantum* leaves. The CO_2 fixation rate increased with photon flux density (PFD) and became saturated above $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The light dependence of the CO_2 fixation did not differ irrespective of the irradiation direction. Interestingly, there was a clear difference in the PFD dependence between the conductance increase and the photosynthetic CO_2 fixation (Fig. 4). These results

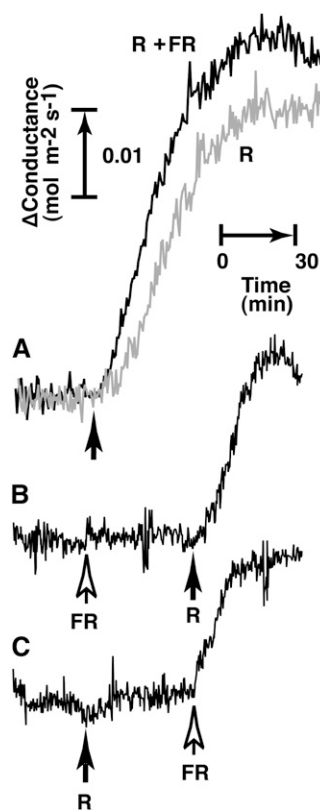


Figure 2. Stomatal responses to red and far-red light in *Adiantum*. A, Increase in stomatal conductance by red (R; $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$) plus far-red (FR; $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$) light (black line) or by red light alone (R; gray line). Lights were applied to the upper surface of the leaf as indicated by the upward-pointing arrow. B and C, Increase in stomatal conductance by a sequential application of red (R; $2 \mu\text{mol m}^{-2} \text{ s}^{-1}$) and far-red (FR; $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$) light. Red and far-red lights were applied as indicated by the black and white arrows, respectively.

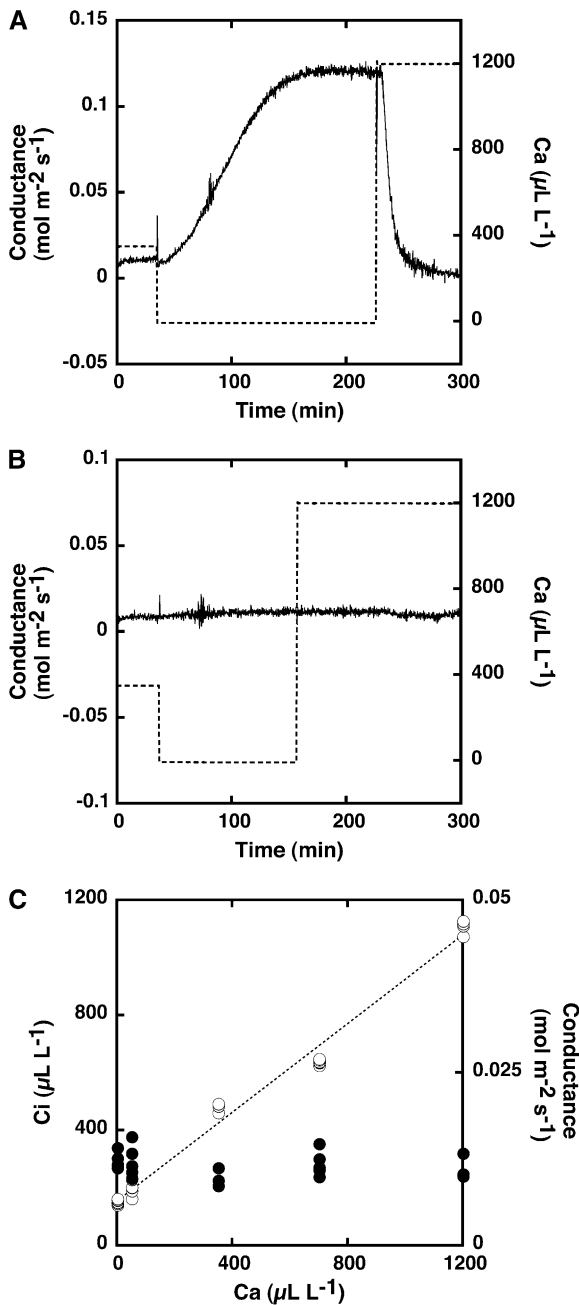


Figure 3. Stomatal responses to Ca in the dark. A, Stomatal conductance in intact leaves of *Arabidopsis*. B, Stomatal conductance in intact leaves of *A. capillus-veneris*. Leaves were held in a leaf chamber for 1 h under darkness at a CO₂ concentration of 350 μL L⁻¹ and then exposed to low (0) and high (1,200 μL L⁻¹) concentrations of CO₂ by changing the concentration in the chamber. Dotted lines show the CO₂ concentration in the chamber. C, Relationship between Ca and Ci (white circles) and stomatal conductance (black circles) in the range of 0 to 1,200 μL L⁻¹ Ca. The dotted line represents the linear relationship between Ca and Ci.

indicate that stomatal opening in *Adiantum* is most likely mediated by guard cell chloroplasts and is not dependent on the decreased Ci via mesophyll CO₂ fixation.

Inhibition of Stomatal Opening by a Photosynthetic Electron Transport Inhibitor

To test whether or not light-dependent stomatal opening is driven by photosynthesis of guard cell chloroplasts, we determined stomatal responses using well-sonicated epidermal fragments devoid of mesophyll and epidermal cells (Fig. 5A). Stomata opened when the epidermis was irradiated by red light at 30 μmol m⁻² s⁻¹ for 3 h. The red light-induced stomatal opening was completely inhibited by a photosynthetic electron transport inhibitor, DCMU, at 20 μM. We further tested the effect of DCMU on the increase in stomatal conductance by red light in intact *Adiantum* leaves (Fig. 5B). Stomatal opening and photosynthetic CO₂ fixation were similarly inhibited by DCMU.

Accumulation of K⁺ in Guard Cells

We determined the amount of K⁺ in guard cells of isolated epidermis to elucidate the underlying mechanisms of the light response (Fig. 6). Small amounts of K⁺ were detected in guard cells of the epidermal fragments prepared from leaves that had been kept in the dark for 12 h. When the fragments were irradiated by red light for 2 h at 30 μmol m⁻² s⁻¹, substantial amounts of K⁺ were found in the guard cells (Fig. 6B). The light-induced K⁺ accumulation was abolished by 20 μM DCMU, suggesting that K⁺ uptake was driven by photosynthetic electron transport in guard cells of *Adiantum*.

To test whether or not K⁺ is the major osmoticum in *Adiantum* guard cells, we investigated the effect of Cs⁺, a known K⁺ channel blocker in plant cells (Ichida et al., 1997; Bei and Luan, 1998), on light-induced stomatal opening. Red light-induced stomatal opening was completely inhibited by Cs⁺ at 10 mM (Fig. 7). These results indicate that K⁺ uptake is essential for the stomatal opening in epidermal fragments.

DISCUSSION

Lack of CO₂ Sensitivity in *Adiantum*

Although there are differences in the CO₂ sensitivity of stomatal movement among plant species and/or their growth conditions, stomatal responses to Ca have been found in more than 50 higher plant species (Morison, 1985, 1987). High concentrations of CO₂ stimulate stomatal closure, probably due to activation of anion channels in the plasma membrane, and low concentrations of CO₂ induce stomatal opening by inactivating the channels (Brearley et al., 1997; Hanstein and Felle, 2002; Roelfsema et al., 2002; Roelfsema and Hedrich, 2005). The guard cells in higher plants are equipped with a mechanism to sense CO₂ and may directly respond to CO₂, and we confirmed that *Arabidopsis* stomata responded to Ca in the dark (Fig. 3). In this study, we found that *Adiantum* stomata lacked the sensitivity to CO₂ in the dark. The

Adiantum stomata neither opened at low concentrations nor closed at high concentrations of CO₂ (Fig. 3). The fern *Adiantum* likely impairs the CO₂ sensor and/or the components in the CO₂ signaling cascade, such as anion channels, found in higher plant guard cells (Hashimoto et al., 2006; Marten et al., 2007). The lack of CO₂ sensitivity in *Adiantum* stomata would result in the extremely slow stomatal closure upon darkness, as indicated in this study (Fig. 1A). It is interesting to note the very recent findings that *SLOWANION CHANNEL-ASSOCIATED1* is essential for stomatal closure in response to CO₂, and the impairment of this gene greatly decreased stomatal closure upon darkness (Negi et al., 2008; Vahisalu et al., 2008). *Adiantum* is a plant whose stomata lack CO₂ sensitivity, and conditional CO₂-insensitive plant has been reported (Raschke et al., 1976; Hashimoto et al., 2006).

Guard Cell Chloroplasts Mediate Stomatal Opening in *Adiantum* Leaves

Stomatal response to light has been much less examined in ferns than in other plants despite the importance and prevalence of ferns in the terrestrial ecosystem, which includes over 10,000 living fern species. Recently, we demonstrated that the stomata of Leptosporangiopsida ferns lack a blue light-specific opening response (Doi et al., 2006). Phototropins do not function for stomatal opening as blue light receptors in the ferns, whereas the stomata open by red light. Thus, photosynthesis and/or phytochromes seem to be responsible for the light-induced stomatal opening in the ferns. The efficiency of light from the blue-to-red region at inducing stomatal opening indicates that photosynthesis is

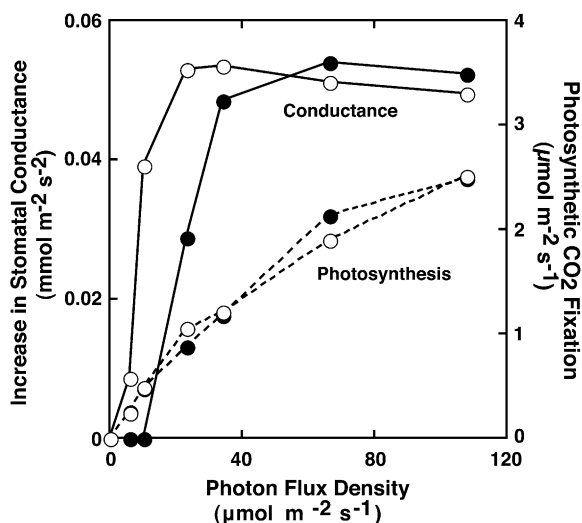


Figure 4. Dependencies of PFD for stomatal conductance increase and photosynthetic CO₂ fixation. Solid and dotted lines represent the increase in stomatal conductance and photosynthetic CO₂ fixation, respectively. Red light was irradiated onto the upper (black circles) or lower side (white circles) of the leaf.

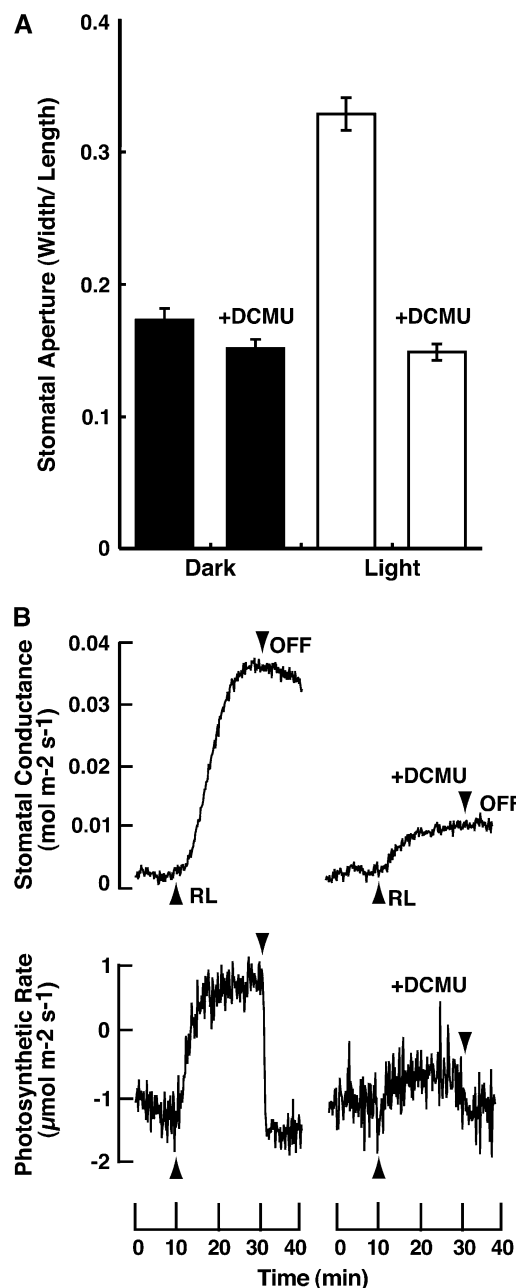


Figure 5. Inhibition of stomatal opening by DCMU in epidermal fragments and intact leaves of *Adiantum*. *Adiantum* plants were kept in the dark overnight. A, The epidermal fragments were obtained from the dark-adapted plants, and were kept in the dark for 1 h and irradiated by red light at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 2 h. The sizes of stomatal apertures of 60 to 70 stomata were determined microscopically. Values are the means \pm SE. DCMU was administered to the epidermal fragments at 20 μM in 0.02% ethanol. B, An *Adiantum* plant was irradiated by red light at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from the upper side of the leaf, and stomatal conductance and photosynthetic CO₂ fixation were determined. DCMU at 50 μM in 0.05% ethanol was administered to *Adiantum* leaves via the petiole under a fluorescent light at 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 h, and then the leaves were left in the dark for 3 h before the measurement.

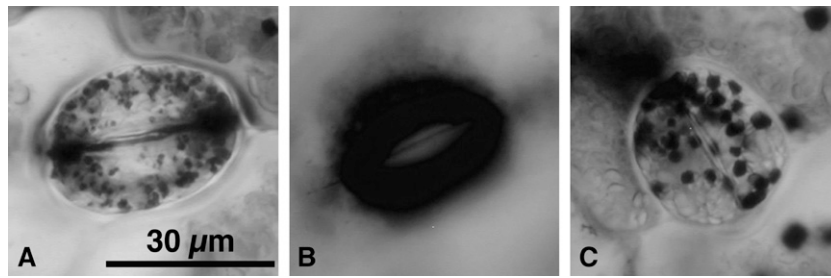


Figure 6. K^+ accumulation in guard cells of *Adiantum* epidermis. Epidermal fragments prepared from the dark-adapted leaves were kept in the dark for 1 h in 5 mM MES, 50 mM KCl, and 0.1 mM $CaCl_2$, pH 6.5. The fragments were then irradiated by red light at $30 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 2 h in the same solution. K^+ was stained with sodium hexanitrocobaltate (III). A, Kept in darkness; B, irradiated by red light; C, irradiated by red light in the presence of 20 μM DCMU. Scale bar in A = 30 μm .

most likely responsible for the response. The remarkable synergistic effect between red and far-red light on stomatal opening also suggests the essential role of linear photosynthetic electron transport.

In higher plants, mesophyll photosynthesis decreases C_i , and the decreased C_i most likely induces stomatal opening (Roelfsema and Hedrich, 2005; Vavasseur and Raghavendra, 2005). As a typical case, stomata in *Arabidopsis* open under low C_i in the dark (Fig. 3A). We therefore suspected that the decrease in C_i induced by mesophyll photosynthesis brought about stomatal opening in the fern. However, this was not the case, because the stomata in the fern did not open in response to decreased C_i (Fig. 3, B and C). This result suggests that guard cell chloroplasts may have an important role in stomatal opening. In accord with this idea, the light was much more effective when it was applied to the lower epidermis than to the upper one and the guard cells existed exclusively in the lower epidermis in *Adiantum*. We next utilized epidermal fragments devoid of mesophyll and epidermal cells, in which guard cell chloroplasts are the sole photosynthetic machinery, and investigated the stomatal response. We found that the stomata opened in response to red light, and this opening was completely inhibited by DCMU (Fig. 5A). Furthermore, we applied DCMU to intact *Adiantum* leaves via the petiole and inspected the stomatal opening in response to red light (Fig. 5B). The red light-induced stomatal opening was inhibited by DCMU, supporting the functional importance of guard cell chloroplasts in the stomatal opening of intact leaves.

Adiantum guard cells have large and dense chloroplasts. This enables the chloroplasts to capture the light efficiently for guard cell photosynthesis in their habitat, where the ambient light is weak and enriched in far-red light. To meet the demand of CO_2 for photosynthesis in mesophyll cells, *Adiantum* stomata are required to open in response to low-intensity light. In accord with this, we found that the stomata in *Adiantum* opened at the maximum rate in response to a weak red light of $20 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 4), which is almost the same value as the compensation point of *Arabidopsis* photosynthesis (Takemiya et al., 2005).

K^+ Is the Osmotica in Stomatal Response in *Adiantum*

We found that the stomatal opening was driven by guard cell chloroplasts in *Adiantum* epidermis (Fig. 5A) and was completely inhibited by DCMU. K^+ accumulated in guard cells concomitantly with stomatal opening (Fig. 6), and such K^+ accumulation was suppressed by DCMU and CsCl. These results suggested that K^+ worked as the main osmotica for stomatal opening and that K^+ was taken up via the K^+ channels in the fern guard cells. Furthermore, we found that the stomatal opening in intact *Adiantum* leaves was inhibited by DCMU (Fig. 5B), implying that photosynthetic electron transport by guard cell chloroplasts provided energy for K^+ uptake.

However, whether or not K^+ is the major osmotica in stomatal opening of intact *Adiantum* has yet to be determined. The mechanism by which guard cells accumulate K^+ remains unknown in *Adiantum*. According to the established mechanisms operating in higher plants, K^+ accumulation is driven by the increase in membrane

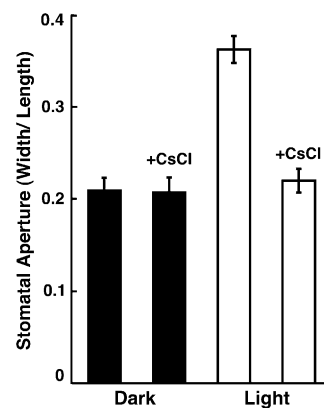


Figure 7. Inhibition of stomatal opening by CsCl in epidermal fragments of *Adiantum*. Epidermal fragments were prepared and irradiated by red light as in Figure 5. CsCl was added at 10 mM to the epidermal fragments, which were then kept in the dark for 1 h. Red light was irradiated onto the epidermal fragments for 2 h. The sizes of stomatal apertures of 50 to 60 stomata were determined by microscopic examination. Values are the means \pm SE.

hyperpolarization that is brought about by activation of the H⁺-ATPase and deactivation of the anion channels in the plasma membrane (Schroeder et al., 2001; Marten et al., 2007; Merlot et al., 2007; Shimazaki et al., 2007). H⁺-ATPase activation in the fern may be the principal mechanism generating the driving force for the uptake of K⁺. In accord with this, we showed the presence of fusicoccin-activated H⁺-ATPase in the fern guard cells (Doi et al., 2006). However, the activation mechanism of the H⁺-ATPase by photosynthetic electron transport in the fern remains unclear. The photosynthesis-induced membrane hyperpolarization by H⁺-ATPase has been reported in green algae *Chara* (Takeshige et al., 1992) and in aquatic angiosperm *Vallisneria* leaves (Harada et al., 2002). In *Vallisneria*, the H⁺-ATPase is activated by a decrease in the K_m for ATP through photosynthetic active radiation and is suppressed by DCMU. In *Vicia* guard cells, provision of ATP from guard cell chloroplasts to the cytoplasm stimulates the H⁺-ATPase in the plasma membrane (Tominaga et al., 2001). Further investigation is needed to address the mechanism of K⁺ uptake by guard cells in *Adiantum*.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The wild type of *Adiantum capillus-veneris* was kindly provided by Professor Masamitsu Wada (National Institute for Basic Biology, Okazaki, Japan). *Adiantum* was grown in a growth room under a white fluorescent lamp (30 μmol m⁻² s⁻¹) on a 12-h-light/-dark cycle at 24°C.

Gas Exchange Measurements

Photosynthetic CO₂ fixation and stomatal conductance were measured using a gas exchange system (LI-6400; LI-COR) equipped with an *Arabidopsis thaliana* chamber (LI-COR) as previously described (Doi et al., 2004). Red light was provided from a tungsten lamp (MHF-G150LR; Moritex) by passing the light through a red cutoff filter (2-61; Corning) or from an LED Lamp Drive unit (SS-001; Shimadec) equipped with a red LED (WBS-Φ-30-16R, λ = 656 ± 18 nm). Blue light was provided by a metal halide lamp (LS-M250; Moritex) by passing the light through a blue filter (5-60; Corning) or an LED Lamp Drive unit equipped with a blue LED (WBS-Φ-30-16B, λ = 467 ± 19 nm). Green light was provided from a tungsten lamp by passing the light through a green interference filter (λ = 500 ± 26 nm; Optical Coatings Japan). Far-red light was provided from an LED Lamp Drive unit equipped with a far-red LED (WBS 720; λ = 723 ± 12 nm). PFD was measured using an LI-2500 light meter with an LI190SA quantum sensor (LI-COR) and an SKR 110 sensor (Skye Instruments). To administer DCMU to *Adiantum* leaves, we cut off the leaf with its petiole and placed the petiole in water under darkness. The next morning, the leaf was transferred to 0.05% ethanol solution that contained 50 μM DCMU and was kept under fluorescent light at 30 μmol m⁻² s⁻¹ for 1 h. Then, the *Adiantum* leaf was returned to the darkness for 3 h and used for the measurement.

Preparation of Epidermal Fragments and Determination of Stomatal Aperture

Adiantum plants were kept in the dark for 16 h before the preparation of epidermal fragments to induce stomatal closing. Ten to 15 leaves were harvested and blended three times in cold water using a Waring blender for 30 s at full speed. The blender contents were filtered through a 200-μm nylon mesh, and the retained samples were washed with 5 mM MES, 50 mM KCl, and 0.1 mM CaCl₂, pH 6.5. The resultant epidermal fragments were floated in the

same solution in petri dishes and kept in the dark for 1 h. The epidermal fragments were irradiated for 2 h, then 1% (w/v) fluorescein diacetate in acetone was added at 0.1 μg mL⁻¹ to determine the viability of the guard cells. Micrographs of over 50 viable stomata were obtained using a Nikon fluorescence microscope equipped with a CCD camera. The stomatal aperture surrounded by viable guard cells was defined as the width to length ratio of the stomatal pores.

Cytochemical Detection of K⁺ in Guard Cells

Accumulation of K⁺ by guard cells was determined with sodium hexanitrocobaltate (III) staining as described previously (Green et al., 1990; Willmer and Fricker, 1996).

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