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Properties of Proton Pumping in Response to Blue Light and Fusicoccin in Guard Cell Protoplasts Isolated from Adaxial Epidermis of *Vicia* Leaves[1]

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Guard cell protoplasts (GCPs) were isolated from the adaxial epidermis of *Vicia* leaves. The properties of isolated adaxial GCPs (ad GCPs) were compared with those of abaxial GCPs (ab GCPs) with respect to H^+-pumping activity. A saturating pulse of blue light (200 μmol m^{-2} s^{-1}, 30 s) induced H^+ pumping in both ad GCPs and ab GCPs under red light. The maximum rate of blue-light-dependent H^+ pumping was slightly higher in ad GCPs than in ab GCPs, but the magnitude of H^+ pumping in ad GCPs was 68% of that in ab GCPs. H^+ pumping was responsive to the second pulse, and the rate and magnitude of the pumping increased with the time between two pulses. The periods required to achieve 50% of the maximum rate were 12 and 22 min for ad GCPs and ab GCPs, respectively. The rates of blue-light-dependent H^+ pumping were saturable, with half-saturation at 630 μmol m^{-2} (21 μmol m^{-2} s^{-1}, 30 s) for ad GCPs and 105 μmol m^{-2} (3.5 μmol m^{-2} s^{-1}, 30s) for ab GCPs. In contrast, fusicoccin, an activator of the plasma membrane H^+-ATPase, induced H^+ pumping with a slightly higher rate in ad GCPs than in ab GCPs. Both types of protoplast swelled similarly in response to fusicoccin. These results suggest that ad GCPs have almost the same activity for H^+ pumping as ab GCPs, whereas ad GCPs require a larger number of photons to activate the H^+ pump than ab GCPs.

Most herbaceous plant species have amphistomatous leaves, bearing stomata on both surfaces. The adaxial epidermis of the leaves is exposed to direct radiation, whereas the abaxial epidermis is shaded and receives only the light transmitted through the leaf and reflected from the surroundings. Thus, the abaxial epidermis receives light of lower intensity and different wavelengths than the incident light (Turner, 1970; Pospísilová and Solárová, 1980; Pemadasa, 1981). Considerable differences in stomatal opening in response to light have been found between adaxial and abaxial stomata; generally, abaxial stomata open at lower values of a PFD than do the adaxial stomata (Pospísilová and Solárová, 1980). However, the mechanism for this differing behavior is still unsolved.

Investigations of the different photosensitivities of adaxial and abaxial stomata, as reported for *Commelina communis*, *Vicia faba*, and most other amphistomatous leaves, have been conducted with stomata on the intact leaf (Turner, 1970; Kassam, 1973; Davies, 1977; Nagarajah, 1978; Yera et al., 1986), and enzymatically isolated (Lu et al., 1993) or manually detached (Travis and Mansfield, 1981; Pemadasa, 1982) epidermal peels. However, still little is known about the physiological and biochemical properties of adaxial stomata. One of the reasons is the difficulty in isolating physiologically active GCPs from the adaxial epidermis of leaves.

It is well established that guard cells extrude H^+, take up K^+ and Cl^-, and produce malate^{2-} during stomatal opening (Zeiger, 1983; Hedrich and Schroeder, 1989; Assmann, 1993). K^+ uptake generally proceeds via a chemiosmotic mechanism (Zeiger, 1983), whereby H^+ pumping hyperpolarizes the membrane potential (Assmann et al., 1985; Shimazaki et al., 1986), thus creating an electrical gradient (inside negative) required for passive K^+ uptake through the K^+-selective channels (Schroeder et al., 1987). The H^+ pump is activated by blue as well as red light in guard cells (Serrano et al., 1988). The blue-light-induced H^+ pumping from guard cells seems to be mediated by plasma membrane H^+-ATPase (Assmann et al., 1985; Shimazaki et al., 1986; Amodeo et al., 1992), although participation of the plasma membrane redox chain (Gautier et al., 1992; Vani and Raghavendra, 1992) cannot be excluded. Comparative study of the properties of H^+ pumping in isolated ad GCPs and ab GCPs is therefore valuable for understanding the mechanisms for the different sensitivities to light of adaxial and abaxial stomata.

We report a method for preparing highly purified GCPs from the adaxial epidermis, capable of carrying out physiological studies of the adaxial stomata of *Vicia* leaves in vitro. The properties of the isolated ad GCPs were compared with those of ab GCPs with respect to the blue-light-dependent H^+ pumping.

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Abbreviations: ab GCP, abaxial guard cell protoplast; ad GCP, adaxial guard cell protoplast; FC, fusicoccin; FDA, fluorescein diacetate; GCP, guard cell protoplast; PFD, photon flux density.

MATERIALS AND METHODS

Plant Material

Seeds of *Vicia faba* L. (Ryosai Issun) were germinated on vermiculite in a controlled environment growth chamber (25°C, 12 h of light/12 h of dark) for 1 week, and the seedlings were cultured hydroponically in a greenhouse at 20 ± 2°C under sunlight as previously described (Shimazaki et al., 1992).

Isolation of ad GCPs and ab GCPs

The ad GCPs and ab GCPs were isolated separately, but simultaneously, from two surfaces of the same leaves unless otherwise stated. Third to fourth leaves of 4- to 8-week-old plants were collected before sunset and used for protoplast isolation. Adaxial epidermal tissues were separated mechanically using the method of Kruse et al. (1989) with slight modifications by homogenizing leaves, whose abaxial epidermis had been removed. Midveins and leaf edges were removed from the leaves, and the tissue was sectioned into small pieces with ceramic scissors and homogenized in a Waring Blendor at 7000 rpm for about 3 min. In each homogenization procedure, a chilled homogenizing solution (30 mL) contained 10% Ficoll, 5 mM $CaCl_2$, 0.1% PVP K-30, and the tissue of six leaves. Twenty-four leaves were used for each experiment. Epidermal strips were collected through a stainless steel net by several rinses of the homogenate with tap water.

To remove mesophyll cells attached to epidermal strips, the strips were digested in a medium (pH 5.4) containing 4% cellulase Onozuka R-10 (Yakult Pharmaceutical Industry Co., Tokyo, Japan), 0.25 M mannitol, 1 mM $CaCl_2$, 0.1% PVP K-30, and 0.5% BSA at 20°C with shaking (60 excursions/min) for 1 h. After the digestion, epidermal strips were retrieved on a nylon net (200-μm mesh size) and rinsed thoroughly with a solution of 0.35 M mannitol and 1 mM $CaCl_2$. Epidermal strips were digested again in medium (pH 5.4) containing 1.5% cellulase Onozuka R-10, 0.35 M mannitol, 1 mM $CaCl_2$, 0.5% BSA, and 20 μg/mL chloramphenicol without shaking at 20°C for 12 h. If tissue was shaken at even a low speed, a large number of cell fragments were produced. Released protoplasts were passed through a 25-μm nylon net and collected by centrifugation (110*g*, 7 min). The protoplasts were rinsed three times with 0.4 M mannitol and 1 mM $CaCl_2$ and were layered on top of a discontinuous Percoll gradient consisting of the following: 2 mL each of 0, 25, 45, and 90% (v/v). Each layer contained 0.4 M mannitol, 1 mM $CaCl_2$, and 5 mM Mes-NaOH (pH 6.2). After the gradients had been centrifuged at 400*g* for 5 min, ad GCPs were collected from the 45/90% Percoll interface and washed twice with a solution of 0.4 M mannitol and 1 mM $CaCl_2$. The ab GCPs were isolated using the method described previously (Shimazaki et al., 1992) except that 1 mM rather than 2.5 mM $CaCl_2$ was used in the second digestion medium. The abaxial epidermal tissues were manually peeled. The ab GCPs were obtained by two-step digestion with gentle shaking. However, a Percoll gradient centrifugation was not required to purify ab GCPs. The first digestion medium did not contain PVP K-30 and BSA. Other procedures were the same as those of ad GCPs. Isolated ad GCPs and ab GCPs were stored in 0.4 M mannitol and 1 mM $CaCl_2$ on ice until used in the dark.

Viability and Purity of Protoplast Preparations

Viability of GCPs was judged by the FDA test (Kruse et al., 1989). FDA-treated cells were observed using an inverted microscope (Optiphot; Nikon Inc., Tokyo, Japan) equipped with an epifluorescence optic unit. Chl fluorescence was viewed with the same microscope.

Assessment of ad GCP Yield and Diameter

Protoplast yields were estimated by counting cell numbers in a constant volume taken from protoplast preparations. Protoplast diameters were measured using a light microscope with an eyepiece micrometer under dim light.

Measurements of Blue-Light-Dependent and FC-Stimulated H^+ Pumping

Blue-light-dependent H^+ pumping by GCPs was measured as the pH decrease in the medium, using a dual-beam protocol at 24°C as described previously (Shimazaki et al., 1986). Blue light was applied as a short pulse (30 s) at the indicated PFD 1 h after the onset of red-light (600 μmol m^{-2} s^{-1}) irradiation. PFD was adjusted with neutral density filters. The reaction mixture (1.0 mL) consisted of 0.125 mM Mes-NaOH (pH 6.2), 0.4 M mannitol, 1 mM $CaCl_2$, 10 mM KCl, and GCPs (20 μg of protein). FC-stimulated H^+ pumping was measured in the same reaction mixture under irradiation by red light. FC was added at 10 μM. All preparations were preincubated for 10 min in the dark before red-light irradiation.

Measurement of Protoplast Swelling by FC

GCPs were stirred gently with 10 μM FC in a temperature-controlled room (24°C) under dim light. The incubation medium contained 0.4 M mannitol, 1 mM $CaCl_2$, 10 mM KCl, 10 mM Mes-NaOH (pH 6.2), and GCPs (30 μg protein/mL). The swelling of GCPs by FC was determined by measurement of the diameter using the assumption that the protoplast was a perfect sphere. Measurements were repeated three times on separate days and expressed as mean volumes ± SE.

Protein Determination

The amount of protein was determined by the method of Bradford (1976) using BSA as a standard.

RESULTS

Separation and Purification of ad GCPs

The GCPs isolated from adaxial *Vicia* epidermis are shown in Figure 1. The protoplasts were spherical and contained chloroplasts (Fig. 1A). Inspection of the microscopy fields showed that ad GCP yields averaged 3×10^4

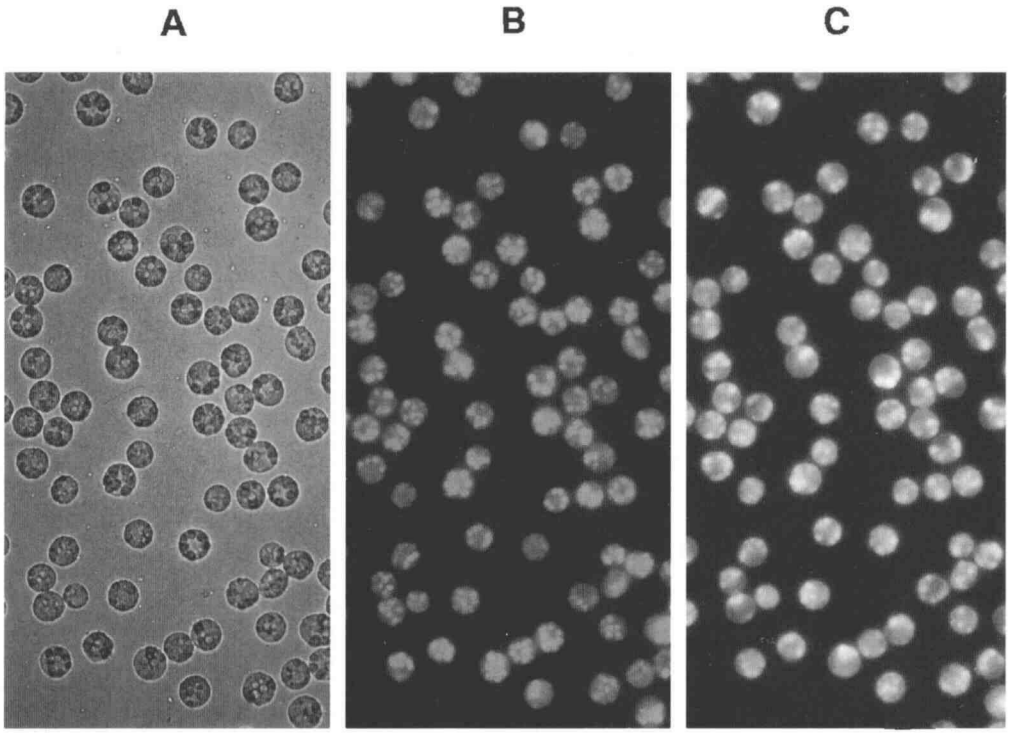

Figure 1. Isolated GCPs from adaxial epidermis of *Vicia* leaves in the same field of vision. A, Light micrograph; B, red Chl fluorescence; C, fluorescence staining with FDA. GCPs were suspended in 0.4 M mannitol and 1 mM $CaCl_2$.

protoplasts per leaflet. Red Chl fluorescence was observed only in GCPs in the same field of vision (Fig. 1B). The typical preparations contained less than 0.04% broken mesophyll fragments on a cell basis; no epidermal and mesophyll protoplasts were found. Isolated ad GCPs hydrolyzed FDA (Fig. 1C), suggesting that ad GCPs were viable. The mean value ± SE for the diameter of ad GCPs was 16.3 ± 1.4 μm and became 15.6 ± 0.7 μm after discontinuous Percoll density gradient centrifugation, probably because of the preferential removal of some large protoplasts. The mean value for ab GCPs was 16.9 ± 1.8 μm ($n = 50$; F test, 0.01 level of significance). The size difference between the two protoplast types was consistent with an intrinsic difference in the sizes of guard cells on opposing surfaces of intact leaves.

Properties of Blue-Light-Dependent H^+ Pumping in ad GCPs and ab GCPs

A typical response of H^+ pumping in ad GCPs and ab GCPs of *Vicia* leaves to a pulse of saturating blue light (200 μmol m^{-2} s^{-1}, 30 s) in the presence of red light (600 μmol m^{-2} s^{-1}) is shown in Figure 2. Continuous red-light irradiation was used to saturate photosynthesis in guard cell chloroplasts. Photosynthetic activity to red light was estimated by DCMU-sensitive CO_2 uptake calculated from the rate of alkalization (Shimazaki and Zeiger, 1987). The red-light-induced alkalization was saturated at 600 μmol m^{-2} s^{-1} in both ad GCPs and ab GCPs, and the half-saturating PFD was 60 μmol m^{-2} s^{-1} for ad GCPs and 22 μmol m^{-2} s^{-1} for ab GCPs (data not shown). A pulse of blue light

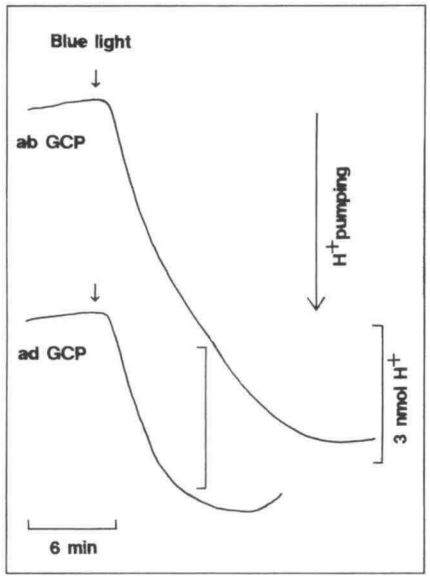

Figure 2. Typical blue-light-dependent H^+ pumping in ab GCPs and ad GCPs from *Vicia* leaves under background red light (600 μmol m^{-2} s^{-1}). A pulse of blue light (200 μmol m^{-2} s^{-1}, 30 s) was given at the time indicated by arrows. The amount of acid equivalents was determined by addition of 10 nmol of H^+ at the end of each

applied 1 h after the onset of red-light irradiation induced H^+ pumping with a similar maximum rate in both ad GCPs and ab GCPs, whereas the magnitude of H^+ pumping in ad GCPs was 68% of that in ab GCPs on a protein basis (Table I). The maximum rate of pumping occurred approximately 2 min after the pulse in both ad GCPs and ab GCPs. The smaller H^+ pumping in ad GCPs was due to the shorter period for H^+ pumping induced by a pulse; pumping was sustained for 10.1 min in ad GCPs and 15.9 min in ab GCPs. This difference between ad GCPs and ab GCPs was also observed in the range of PFD from 12 to 400 μmol m^{-2} s^{-1} (data not shown).

The protoplasts, once illuminated with a single pulse, responded to another pulse (Fig. 3A). Responsiveness to the second pulse increased progressively with the time interval between the two pulses. The time required for the half-maximal rate was 12 min for ad GCPs and 22 min for ab GCPs (Fig. 3B). This indicates that the capacity to respond to the second pulse was restored faster in ad GCPs than in ab GCPs. When the second pulse was given to ad GCPs 7.5 min after the first one, marked H^+ pumping was induced (Fig. 3A[d]). The rate was about 26% of the control. In contrast, the second pulse 7.5 min after the first one induced only slight H^+ pumping in ab GCPs (Fig. 3A[c]).

A smaller magnitude and a shorter period of H^+ pumping in response to a blue-light pulse and faster restoration of responsiveness to the second pulse were found in ad GCPs (Fig. 3; Table I). These differences could be due to the supply of background red light, because PFD required for the saturation of photosynthesis by the red light was larger in ad GCPs than in ab GCPs, although photosynthesis was saturated in both GCPs in our experimental conditions (600 μmol m^{-2} s^{-1}). We compared the blue-light-dependent H^+ pumping of ad GCPs under background red light at 600 μmol m^{-2} s^{-1} with that at 900 μmol m^{-2} s^{-1} (Table II). However, no significant difference was observed in the rate, magnitude, period, and responsiveness, suggesting that background red light used in this study is sufficient to induce the blue-light-dependent H^+ pumping in ad GCPs.

Light saturation of H^+ pumping due to a pulse of blue light was quantified by measuring the rate in both ad GCPs and ab GCPs under background red light. As shown in Figure 4, the rate of H^+ pumping in both ad GCPs and ab GCPs increased steeply with increasing PFD at lower ranges and progressively at higher ones. However, H^+ pumping was much more sensitive to blue light in ab GCPs than in ad GCPs; rates of blue-light-dependent H^+ pumping were saturated at 200 and 80 μmol m^{-2} s^{-1} for ad GCPs and ab GCPs, respectively (Fig. 4A). The PFD values for half-saturation were 21 μmol m^{-2} s^{-1} for ad GCPs and 3.5 μmol m^{-2} s^{-1} for ab GCPs when the pulse duration was 30 s (Fig. 4B). The dependence in the magnitude of H^+ pumping to blue light is shown in Figure 5. H^+ pumping was considerably more sensitive in ab GCPs than in ad GCPs at low PFD, in particular. These results are consistent with the fact that stomata opened to wider apertures at a faster rate in abaxial epidermis than in adaxial epidermis at lower values of PFD (Pemadasa, 1981).

H^+ Pumping Capacity by FC in ad GCPs and ab GCPs

The sensitivity of H^+ pumping in response to blue light differed between ad GCPs and ab GCPs (Fig. 4). However, ad GCPs and ab GCPs may be expected to have similar H^+-pumping activities because the maximum rate of blue-light-dependent H^+ pumping had almost the same values in both protoplasts. To test this possibility, the activities of H^+ pumping in ad GCPs and ab GCPs were determined using a fungal toxin, FC, which is an activator of H^+-ATPase in the plasma membrane. FC at 10 μM induced ad GCPs to pump H^+ into the suspending medium at a rate as high as, or somewhat higher than, ab GCPs. This suggests that there is no large difference in H^+-pumping activity between ad GCPs and ab GCPs (Fig. 6).

FC has also been shown to induce swelling of GCPs by the activation of plasma membrane H^+-ATPase (Schnabl et al., 1978; Zeiger, 1983; Assmann and Schwartz, 1992). There was no volume change in ad GCPs and ab GCPs during the preincubation for 35 min in dim light before the addition of FC. Those protoplasts that were stimulated by 10 μM FC gradually increased in volume with a similar response (Fig. 7). Five minutes after the addition of FC, there was an increase in volume of 11.1% for ad GCPs and 10.5% for ab GCPs. At 65 min the volume of ad GCPs and ab GCPs had increased by 46 and 39%, respectively. These swelling responses in ad GCPs and ab GCPs were consistent with the

Table I. *Properties of H^+ pumping in response to a pulse of blue light in the presence of background red light in ab GCPs and ad GCPs from Vicia leaves*

Three separate experiments were conducted for each protoplast preparation. Values are means ± SE. Other details are as in Figure 2.

Type of Protoplasts	H^+ Pumping		
	Rate[a]	Magnitude[b]	Time[c]
	nmol H^+ μg^{-1} protein h^{-1}	*nmol H^+ μg^{-1} protein $pulse^{-1}$*	*min*
ab GCP	2.85 (± 0.09)	0.336 (± 0.045)	15.9 (± 1.3)
ad GCP	2.98 (± 0.15)	0.227 (± 0.013)	10.1 (± 1.1)

[a] Rate represents the maximum rate of H^+ pumping, which occurred approximately 2 min after the pulse stimulation. [b] Magnitude represents the maximum amounts of H^+ pumped into the suspending medium. [c] Time indicates the period of sustained H^+ pumping in response to a pulse of saturating blue light.

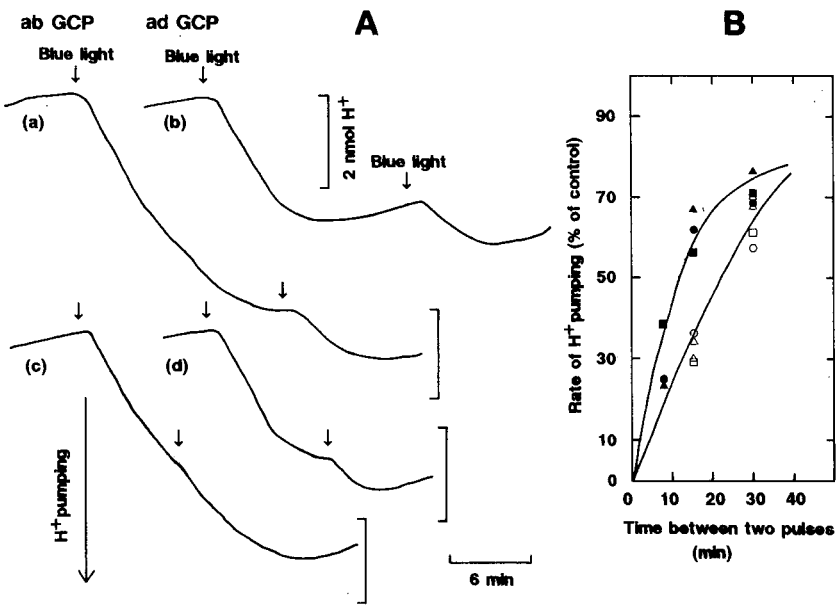

Figure 3. H^+ pumping in response to two consecutive, saturating blue-light pulses in ab GCPs and ad GCPs from *Vicia* leaves. A, Time intervals between two pulses were 15 min (a and b) and 7.5 min (c and d). B, Increase in the rate of H^+ pumping as a function of time between two pulses of blue light. The rates in response to the second pulse are expressed as percentages of those obtained with the first pulse. Identical symbols correspond to the same protoplast batch. Open and closed symbols are for ab GCPs and ad GCPs, respectively. Other experimental conditions are as in Figure 2.

results of H^+-pumping activities stimulated by FC. This also suggests that ad GCPs had almost the same H^+-pumping activity as ab GCPs.

DISCUSSION

We isolated GCPs from adaxial epidermis of *Vicia* leaves, and the protoplasts were used to investigate the causes of different photosensitivities between adaxial and abaxial stomata. The isolated ad GCPs were 96% of the diameter of ab GCPs. The smaller size of the former reflects the size of intact guard cells of *Vicia* leaves; the length of adaxial guard cells is 95% of that in abaxial ones (Yera et al., 1986).

H^+ pumping was induced more efficiently by a pulse of blue light in ab GCPs than in ad GCPs under background red light. The half-saturating PFD required for blue-light-dependent H^+ pumping in ab GCPs was less than 17% of that in ad GCPs (Fig. 4). In contrast, the maximum rates of H^+ pumping in response to saturating blue light and the rates of FC-induced H^+ pumping showed almost the same values in ad GCPs and ab GCPs. These results suggest that the H^+-ATPase may have nearly the same density in the plasma membrane of ad GCPs and ab GCPs, based on the assumption that FC fully activates the H^+-ATPase(s). Therefore, the different sensitivities to blue light found in ad GCPs and ab GCPs seem to be related largely to their differences in signal-sensing processes for activation of the plasma membrane H^+-ATPase. A possible explanation for the higher sensitivity in ab GCPs than ad GCPs is that a higher concentration of the photoreceptor exists in ab GCPs than in ad GCPs based on the assumption that properties of signal transduction components are the same in both GCPs, because photochemical (light) reaction limits the whole processes of light-signaling under low PFD.

In agreement with the similar activities of H^+ pumping in ad GCPs and ab GCPs (Fig. 6), both GCPs swelled similarly when FC was added (Fig. 7). Since H^+-pumping activity, the capacity for K^+ uptake through K^+-selective channels, and other metabolic processes such as malate formation are involved in the swelling response (Hedrich and Schroeder, 1989; Assmann, 1993), this result under-

Table II. *Blue-light-dependent H^+ pumping under different PFDs of background red light in ad GCPs of Vicia leaves*

Mean values ± SE from triplicate experiments are presented. Other details are as in Figure 2.

PFD[a] of Red Light	H^+ Pumping				
	First pulse			Second pulse[b]	
	Rate	Magnitude	Time	Rate	Responsiveness[c]
$\mu mol\ m^{-2}\ s^{-1}$	$nmol\ H^+\ \mu g^{-1}$ protein h^{-1}	$nmol\ H^+\ \mu g^{-1}$ protein $pulse^{-1}$	min	$nmol\ H^+\ \mu g^{-1}$ protein h^{-1}	%
600	2.93 (±0.21)	0.169 (±0.024)	8.92 (±0.48)	1.82 (±0.19)	62.1
900	3.03 (±0.29)	0.184 (±0.030)	8.85 (±0.53)	1.79 (±0.39)	59.1

[a] The applied period of PFD of red light was 1 h. [b] Time interval between two pulses of blue light was 15 min. [c] Responsiveness was expressed as the percentage of controls.

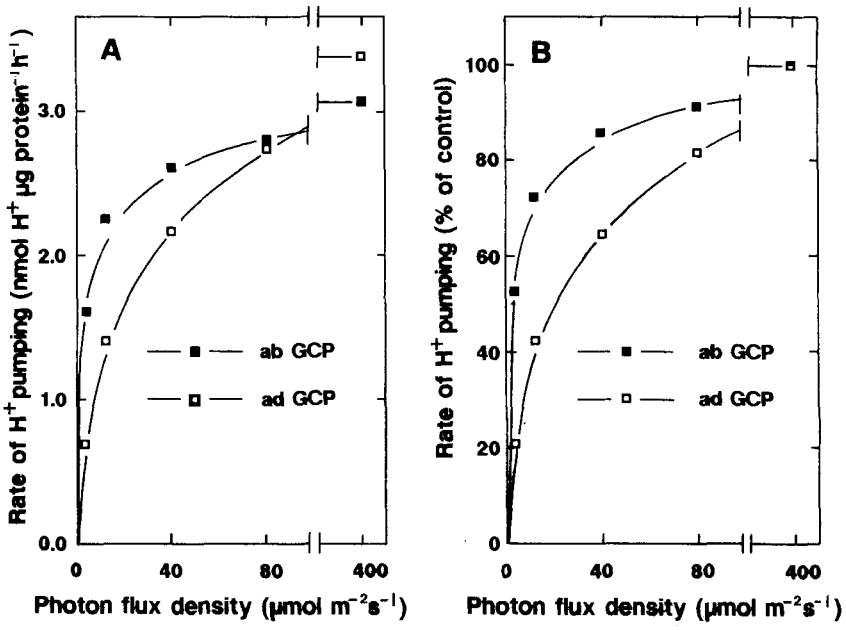

Figure 4. A, PFD dependency of the rate in blue-light-dependent H^+ pumping in ab GCPs and ad GCPs from *Vicia* leaves. B, The rates were normalized relative to those observed in response to blue light at 400 μmol m^{-2} s^{-1}. ab GCPs and ad GCPs were taken from the two surfaces on different leaves on separate days.

scores the distinct difference in the photosensitivity of H^+ pumping to blue light between ad GCPs and ab GCPs as shown above. The difference could explain at least partly the fact that the stomatal opening is more sensitive to light in the abaxial than the adaxial epidermis, as reported previously for stomata in the intact leaf and/or epidermal peels of *V. faba* (Kassam, 1973), *C. communis* (Travis and Mansfield, 1981; Pemadasa, 1982), and other amphistomatous species (Turner, 1970; Pospísilová and Solálová, 1980; Pemadasa, 1981).

Recent studies of the light sensitivity of adaxial and abaxial stomata of Pima cotton (*Gossypium barbadense*) indicated that adaxial stomata had a higher sensitivity to blue light than abaxial ones in peels obtained from both growth chamber and greenhouse (Lu et al., 1993). This is not consistent with the present findings in *V. faba*. Lu et al. (1993) also found that a larger amount of Chls and carotenoids were present in adaxial peels than in abaxial ones of Pima cotton. In contrast, the amount of Chls was found to be smaller in ad GCPs than in ab GCPs on a protein basis in *Vicia* (data not shown). Thus, a major reason for the discrepancy may be due to a difference in plant species.

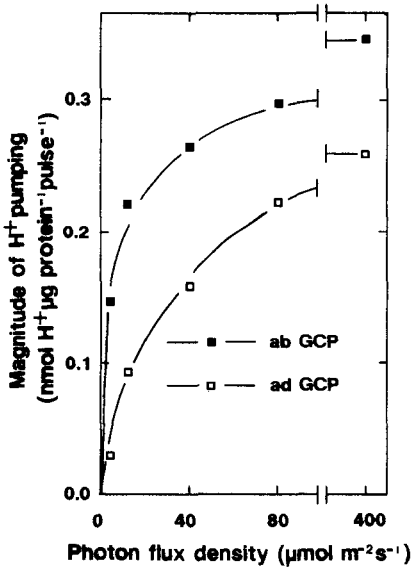

Figure 5. PFD dependency of the magnitude of H^+ pumping in response to blue light in ab GCPs and ad GCPs from *Vicia* leaves. Data are from Figure 4.

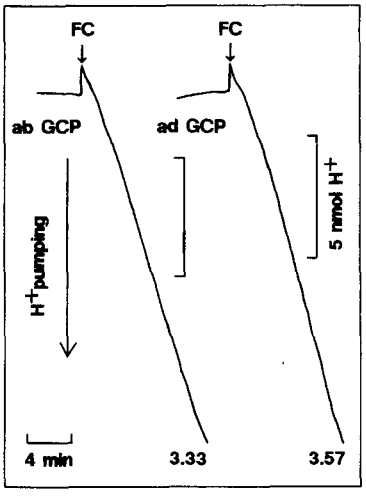

Figure 6. FC-induced H^+ pumping in ab GCPs and ad GCPs from *Vicia* leaves. FC was added at 10 μM to the protoplast suspension 1 h after the onset of red light. FC was dissolved in DMSO, and the final concentration of DMSO in the reaction mixture was 0.5%. Values are expressed as the rates of H^+ pumping (nmol H^+ μg^{-1} protein h^{-1}). Other details are as in Figure 2. The amount of acid equivalents was determined by the addition of 10 nmol of H^+ 10 min before the addition of FC.

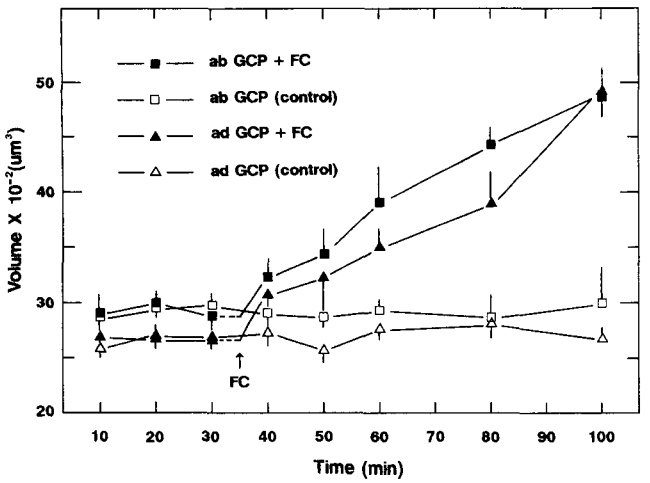

Figure 7. Time course of the swelling of ab GCPs and ad GCPs induced by FC. Data are mean volumes ± SE (vertical lines). In each measurement, 80 to 105 ab GCPs or 81 to 120 ad GCPs were used. FC (10 μM) was added at the time indicated by the arrow. The concentration of DMSO was 0.44%.

The H^+ pumping elicited by blue light was sustained longer in ab GCPs than in ad GCPs, with periods of 10.1 min for ad GCPs and 15.9 min for ab GCPs. The signal from the activated photoreceptor to H^+-ATPase seems to terminate faster in ad GCPs than in ab GCPs. The H^+ pumping could be induced by the second pulse of blue light separated by various time intervals from the first ones (Fig. 3). The responsiveness was restored faster in ad GCPs than in ab GCPs. This faster restoration may be related to the shorter period of H^+ pumping induced by the first pulse; when the plasma membrane H^+-ATPase has been activated, the H^+-ATPase cannot receive additional signals from the photoreceptor.

According to the kinetics model of the blue-light response of stomata presented by Iino et al. (1985) and Zeiger (1987), the phototransduction process includes a component that exists in two interconvertible forms, A and B. A is an inactive form that can be converted to B by the excitation of the blue-light photoreceptor. B is an active form that results in the activation of the proton pump. B is converted back to A in a thermal reaction. The complete process is represented by the reaction where k_e and k_d are rate constants for the light and dark (thermal) reactions, respectively. On the basis of this model, the rate of H^+ pumping in response to the second pulse of blue light is proportional to the amount of A that has been converted from B. A first pulse of saturating blue light converts A to B completely, and A is regenerated by conversion from B through thermal reaction after the first pulse. Thus, the faster restoration of the responsiveness to the second pulse in ad GCPs than in ab GCPs (Fig. 3B) indicates that k_d may be larger in ad GCPs than in ab GCPs.

We found a marked difference in light dependency of blue-light-dependent H^+ pumping between ad GCPs and ab GCPs from *V. faba*. However, we should note the possibility that such difference is artificially elicited by the protoplast isolation, although the difference explains well the responses in intact leaves of several plant species (Kassam, 1973; Pemadasa, 1982).

Evidence has accumulated in the literature concerning the blue-light photoreceptor in higher plants, but it is uncertain whether the blue-light response is mediated by a flavin (Ogawa et al., 1978; Leong and Briggs, 1982; Ahmad and Cashmore, 1993) or a zeaxanthin (Karlsson et al., 1992; Quiñones and Zeiger, 1994). Our data suggest that the blue-light photoreceptor is at a higher concentration in ab GCPs than in ad GCPs. Further studies of guard cells will provide data regarding the properties of the blue-light photoreceptor and the signal transduction pathway of external stimuli in plant cells.

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