Plant Physiol. (1996) 111: 433–440

# Inhibition of Blue Light-Dependent $H^+$ Pumping by Abscisic Acid in *Vicia* Guard-Cell Protoplasts

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**Blue-light (BL)-dependent $H^+$ pumping in guard-cell protoplasts (GCPs) from *Vicia faba* was inhibited by 65% in the presence of abscisic acid (ABA). The inhibition increased with the time after application of ABA and was concentration dependent with a saturating concentration of 1 μM at pH 6.2. The inhibition was nearly independent of the pH of the medium in the range 5.4 to 7.2 when ABA was applied at 10 μM, whereas it was dependent on pH when the ABA concentration was decreased. The protonated form of ABA was saturating at 40 nM in inhibiting BL-dependent $H^+$ pumping under various experimental conditions, whereas the dissociated form at 500 nM had no inhibitory effect on the pumping, suggesting that the protonated form of ABA is the form active in inhibiting the pumping. Fusicoccin (10 μM), an activator of plasma membrane $H^+$-ATPase, induced $H^+$ pumping from GCPs, and the rate of $H^+$ pumping was decreased to 70% by ABA. In contrast, ABA did not inhibit $H^+$ pumping in isolated microsome vesicles from GCPs. These results suggest that the inhibition of BL-dependent $H^+$ pumping by ABA in GCPs may be due to indirect inactivation of plasma membrane $H^+$-ATPase and/or inhibition of the BL-signaling pathway. The pump inhibition by ABA causes membrane depolarization and can be an initial step to induce stomatal closure and reduces the transpirational water loss under drought stress in the daytime.**

Stomatal opening is driven by an increase in the turgor of guard cells and depends on the uptake of $K^+$ and anions and the production of malate in guard cells (Zeiger et al., 1987; Hedrich and Schroeder, 1989; Assmann, 1993). Ion uptake is driven by $H^+$ pumping, which generates an inside-negative electrical potential across the plasma membrane, sustaining passive influx of $K^+$. Stomatal closing by ABA is driven by a decrease in guard-cell turgor as a result of effluxes of $K^+$ and anions from guard cells (MacRobbie, 1992). ABA-induced changes in the activity of ion channels have been investigated extensively in the guard-cell plasma membrane. Based on current results, the mechanism by which ABA induces stomatal closure involves membrane depolarization through an increase in $Ca^{2+}$ concentration and an increase of pH in the cytosol (Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Irving et al., 1992; McAinsh et al., 1992; Blatt and Armstrong, 1993; Lemtiri-Chlieh and MacRobbie, 1994; Ward et al., 1995), thereby finally activating the outward-rectifying anion and $K^+$ channels. This allows the sustained release of malate, $Cl^-$, and $K^+$ from the cells, opposes osmotic buildup, and thus causes stomatal closing. An initial membrane depolarization stimulated by ABA (Thiel et al., 1992) may be caused by $Ca^{2+}$ influx into the cytosol (Schroeder and Hagiwara, 1990) and/or inhibition of $H^+$-ATPase of plasma membrane (Raschke, 1987; Assmann, 1993).

Under conditions of water deficit, the levels of the phytohormone ABA are increased in the xylem through the transpiration stream or redistribution of ABA in leaves (Hartung and Davies, 1991). Elevated levels of ABA result in stomatal closure, reduce transpirational water loss of plants (Zeevaart and Creelman, 1988; Davies and Zhang, 1991), and thus provide drought resistance for plants under water stress. Since the most severe transpirational water loss occurs in the daytime, investigation of the effects of ABA on the light response of guard cells provides an ideal means of understanding the action mechanisms of ABA. In guard cells, plasma membrane $H^+$-ATPase is activated by blue and red light and pumps the $H^+$ out of cells, inducing the membrane hyperpolarization that elicits stomatal opening and opposes stomatal closure (Assmann et al., 1985; Shimazaki et al., 1986; Serrano et al., 1988). However, the effects of ABA on the light-activated processes of guard cells have been little studied (Gepstein et al., 1982; Ishikawa et al., 1983; Shimazaki et al., 1986). In the present study we showed that ABA inhibits the BL-dependent $H^+$ pumping, which may cause stomatal closure under drought stress in the daytime, and that ABAH is the active form inhibiting the pumping. A brief preliminary report of this result was reported (Shimazaki et al., 1986).

## MATERIALS AND METHODS

### Plant Material

Plants of *Vicia faba* L. (cv Ryosai Issun) were germinated on vermiculite and grown hydroponically in a greenhouse

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Abbreviations: $ABA^-$, dissociated form of ABA; ABAH, protonated form of ABA; ab GCP, abaxial guard-cell protoplast; ad GCP, adaxial guard-cell protoplast; BL, blue light; FC, fusicoccin; GCP, guard-cell protoplast.

at 20 ± 2°C under sunlight as described previously (Shimazaki et al., 1992).

### Preparation of GCPs

Young, fully expanded leaves were harvested from 4- to 8-week-old plants. GCPs were isolated enzymatically from abaxial epidermis according to the method of Shimazaki et al. (1992) with minor modifications. In this procedure, protoplast isolation was performed with 1.5% Cellulase R-10 (Yakult Pharmaceutical Industry, Tokyo, Japan) and 1 mM $CaCl_2$ in the digestion medium. Ad GCPs were isolated enzymatically from the adaxial epidermal layers according to the method of Goh et al. (1995). Adaxial epidermal tissues were separated mechanically from the leaves from which the abaxial epidermis had been removed. Isolated protoplasts were purified by discontinuous Percoll gradient centrifugation at 400*g* for 5 min. The isolated protoplasts were suspended and stored in the dark in a solution of 0.4 M mannitol containing 1 mM $CaCl_2$ and kept on ice until use.

### Measurements of $H^+$ Pumping in GCPs Induced by a BL Pulse and FC

BL-dependent $H^+$ pumping by GCPs was measured as the pH decrease in the medium (Shimazaki et al., 1992). BL (200 $\mu$mol $m^{-2}$ $s^{-1}$) was applied as a short pulse (30 s) 1 h after the onset of background red-light (600 $\mu$mol $m^{-2}$ $s^{-1}$) irradiation. BL was provided by a tungsten lamp (Sylvania EXR, 300 W) through a glass filter (Corning 5-60, Corning, NY). Red light was obtained from the lamp by filtering the light through a glass cutoff (Corning 2-61) and Cinemoid 5A filters. The basal reaction mixture (1.0 mL) consisted of 0.125 mM Mes-NaOH (pH 6.2), 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and GCPs (20 $\mu$g of protein) unless otherwise stated.

FC-induced $H^+$ pumping was measured in the same reaction mixture under irradiation with red light (600 $\mu$mol $m^{-2}$ $s^{-1}$). A stock solution of ABA was prepared to the desired pH with NaOH. All measurements were performed at 24°C. The preparations were preincubated in the reaction mixture for 10 min in the dark before red-light irradiation.

### Measurement of Photosynthetic Activity

Photosynthetic activity of GCP chloroplasts in response to red light (600 $\mu$mol $m^{-2}$ $s^{-1}$) was determined by $CO_2$ uptake calculated from the rate of alkalization using the Henderson-Hasselbach equation ($pK_a$ = 6.35, 24°C) (Shimazaki and Zeiger, 1987). The reaction mixture (1.0 mL) consisted of 0.125 mM Mes-NaOH (pH 6.2), 1 mM $CaCl_2$, 0.4 M mannitol, 10 mM KCl, 2 mM $NaHCO_3$, and GCPs (20 $\mu$g of protein). A stock solution of $NaHCO_3$ was prepared before use and the pH was adjusted to 6.2 with HCl. DCMU was added to a final concentration of 10 $\mu$M 10 min before illumination of red light. Red light was given at 10 min after preincubation of the protoplasts in the reaction mixture under darkness.

### Measurement of $H^+$ Pumping across the Plasma Membrane in Microsomal Vesicles

A membrane fraction that contained vanadate-sensitive $H^+$ transport activity was obtained by rupturing GCPs with a hand-held Teflon homogenizer in a solution of 0.1 M mannitol, 20 mM Mops-KOH (pH 7.5), 2 mM EGTA, 4 mM EDTA, and 20 $\mu$g/mL PMSF according to the method of Kinoshita et al. (1995). Crude microsomal vesicles obtained as a pellet were suspended in 0.25 M mannitol, 10 mM Mops-KOH (pH 7.0), and 1 mM EGTA and used immediately for assays.

Proton pumping across the membrane was monitored by fluorescence quenching of quinacrine with a fluorescence spectrophotometer (Kinoshita et al., 1995). Excitation and emission wavelengths were 424 and 500 nm, respectively. The reaction mixture (250 $\mu$L) contained membrane vesicles (10 $\mu$g of protein), 10 mM Mops-KOH (pH 7.0), 0.25 M mannitol, 5 mM $MgCl_2$, 1 mM EGTA, 50 mM $KNO_3$ (inhibitor of vacuolar ATPase), 5 $\mu$g/mL oligomycin (inhibitor of mitochondrial ATPase), and 1 $\mu$M quinacrine. The reaction was started by adding 2.5 $\mu$L of 200 mM ATP in 200 mM Mops-KOH (pH 7.0) at 22°C. Sodium orthovanadate (100 $\mu$M) and ABA (1 or 10 $\mu$M) were added prior to the addition of ATP.

### Protein Determination

Protein was determined by the method of Bradford (1976) for GCP preparations and by the method of Smith et al. (1985) for microsomal membrane preparations with BSA as a standard.

## RESULTS

### Inhibition of BL-Dependent $H^+$ Pumping by ABA in GCPs

The properties of BL-dependent $H^+$ pumping by GCPs have been well characterized elsewhere (Shimazaki et al., 1986, 1992; Zeiger et al., 1987; Mawson, 1993; Goh et al., 1995). Figure 1 shows typical inhibition of BL-dependent $H^+$ pumping by ABA in GCPs at pH 6.2. In this sample, the magnitude of the $H^+$ pumping was inhibited by 45% when ABA was added to a final concentration of 0.167 $\mu$M. Inhibition increased with the concentration of ABA and was saturated with 1 $\mu$M ABA at pH 6.2 (Fig. 2). BL-dependent $H^+$ pumping was not inhibited completely by 10 $\mu$M ABA, showing about 65% inhibition of the maximum response. Inhibition of BL-dependent $H^+$ pumping increased with the time of incubation of GCPs with ABA in both ab GCPs and ad GCPs (Fig. 3). The inhibition by ABA developed faster in ab GCPs than in ad GCPs. The extent of inhibition after 21 min was 60 and 35% for ab GCPs and ad GCPs, respectively. However, the inhibition had almost fully developed by that time in ab GCPs but was still increasing in ad GCPs.

GCPs were illuminated continuously with red light at a high fluence rate (600 $\mu$mol $m^{-2}$ $s^{-1}$) to saturate photosynthesis and to minimize any intrinsic photosynthetic response to BL. Inhibition of BL-dependent $H^+$ pumping by ABA could have resulted from a decrease in photosynthetic

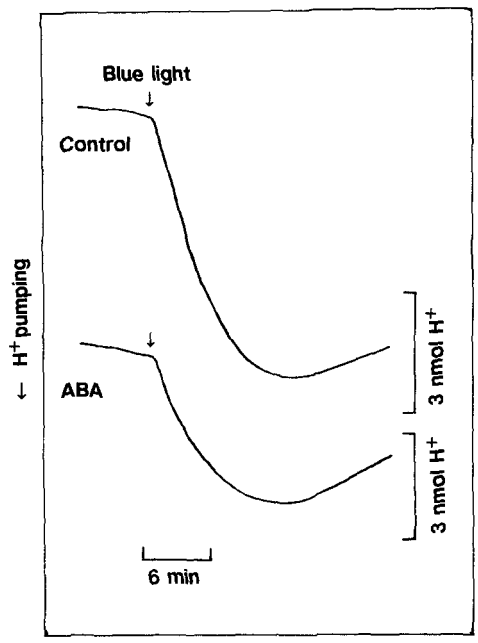


**Figure 1.** Effect of ABA on BL-dependent $H^+$ pumping in GCPs from *Vicia*. ABA was added to the protoplast suspension at a final concentration of 0.167 μM 21 min before the application of BL (30 s with 200 μmol $m^{-2}$ $s^{-1}$) under background red light (600 μmol $m^{-2}$ $s^{-1}$). The reaction mixture (1.0 mL) contained 0.125 mM Mes-NaOH (pH 6.2), 0.4 M mannitol, 10 mM KCl, 1 mM $CaCl_2$, and GCPs (20 μg of protein). ABA was dissolved in DMSO. The final concentration of DMSO in the reaction mixture was 0.5%. The amount of acid equivalents was determined by addition of 10 nmol of $H^+$ at the end of each experiment.

activity. We examined the effect of ABA on photosynthesis by GCP chloroplasts (Table I). No $CO_2$ uptake was observed in the presence of DCMU at 10 μM (data not shown). The result determined by the rate of DCMU-sensitive $CO_2$ uptake showed that there was no inhibition of the GCP photosynthesis by ABA.

### Effects of External pH on ABA Action

Inhibitory effects of ABA on BL-dependent $H^+$ pumping were determined under different pH conditions (Fig. 4). The magnitudes of BL-dependent $H^+$ pumping (in relation to controls) were enhanced slightly with the increase in external pH in this study. When ABA was applied at 10 μM, the extent of inhibition was similar over the pH range 5.4 to 7.2, with $H^+$ pumping being inhibited by about 60% (Fig. 4A). These similar inhibitory effects at different pH values are probably due to the use of a high concentration (10 μM) of ABA, at which concentration ABA may be saturating for the inhibition of $H^+$ pumping. When the concentration of ABA was decreased to 1 μM or less at both pH 5.4 and 7.2, the inhibition showed a prominent pH dependence (Fig. 4B). At 1 μM ABA, BL-dependent $H^+$ pumping was inhibited by 51% at pH 5.4 and 39% at pH 7.2. The BL-dependent $H^+$ pump was inhibited by 28% at pH 5.4; however, no inhibition was observed at pH 7.2 with 0.01 μM ABA. These results suggest that the inhibition of BL-dependent $H^+$ pumping by ABA may be brought about by ABAH in the medium.

We calculated ABAH from the data shown in Figures 2 and 4 on the basis of the $pK_a$ of ABA (Parry and Horgan, 1991) according to the Henderson-Hasselbach equation. ABAH was saturating at 40 nM in inhibiting $H^+$ pumping

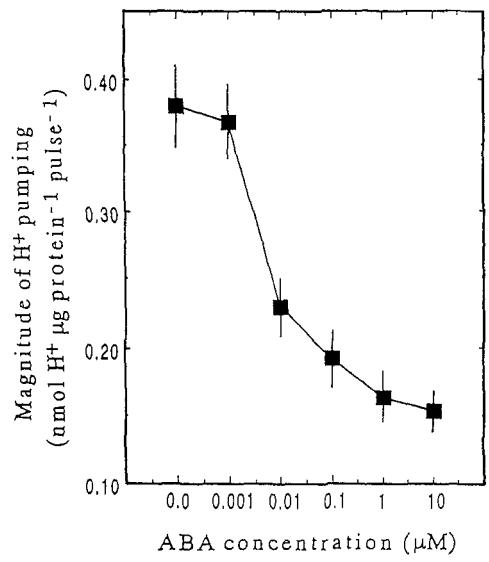


**Figure 2.** Concentration dependence of inhibition of BL-dependent $H^+$ pumping by ABA in *Vicia* GCPs. ABA was added to the protoplast suspension 21 min before application of BL under background red light. Each point represents the mean value ± SE of three replicates on separate days. Other experimental conditions were as in Figure 1.

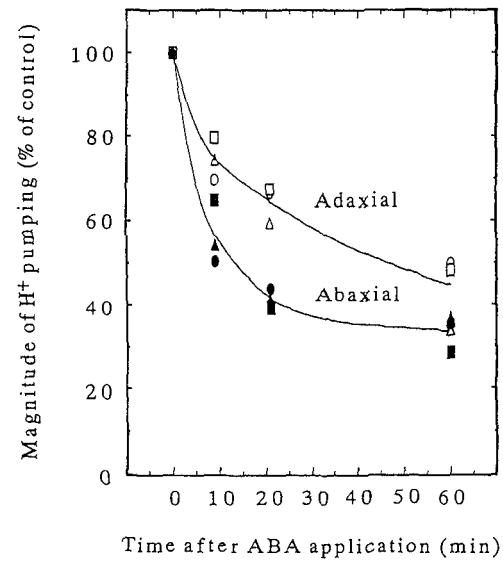


**Figure 3.** Time course of inhibition of BL-dependent $H^+$ pumping by ABA in *Vicia* GCPs. ABA was added to the protoplast suspension to give a final concentration of 10 μM. Identical symbols correspond to the values obtained from the same protoplast batches. Ad GCPs were prepared from *Vicia* plants according to the method by Goh et al. (1995). Other experimental conditions were as in Figure 1.

**Table I.** *Effect of ABA (10 μM) on red-light-dependent $CO_2$ uptake in Vicia GCPs*

Rates of $CO_2$ uptake were calculated from rates of alkalization under red light (600 μmol $m^{-2}$ $s^{-1}$) using the Henderson-Hasselbalch equation ($pK_a$ = 6.35, 24°C). The alkalization was completely inhibited by DCMU at 10 μM. Amount of alkaline equivalents was determined by the addition of 10 nmol of $H^+$ at the end of the experiment. Values represent the means ± SE of three replicates. All measurements are significant at the 0.1 level by Student's *t* test.

| Rate of $CO_2$ Uptake | |
|---|---|
| −ABA | +ABA |
| *nmol $CO_2$ $mg^{-1}$ protein $h^{-1}$* | |
| 1571.6 ± 137.5 | 1482.3 ± 128.5 |

by GCPs (Fig. 5), and the inhibition was apparently not affected by $ABA^-$. To show this, the effect of $ABA^-$ on $H^+$ pumping was investigated while keeping the concentration of ABAH at 2 nM under different pH conditions (Fig. 6). To achieve this, concentrations of total ABA ($ABA^-$ and ABAH) used were 0.01 μM for pH 5.4, 0.083 μM for pH 6.4, and 0.5 μM for pH 7.2. BL-dependent $H^+$ pumping was inhibited at pH 5.4 by 30% at 2 nM ABAH with 8 nM $ABA^-$ (Fig. 6A); however, an increase in $ABA^-$ concentration up to 500 nM without changing the ABAH concentration did not enhance inhibition (Fig. 6B). The results emphasize that the inhibition of BL-dependent $H^+$ pumping by ABA is strongly associated with the ABAH molecule in the medium.

### Effects of ABA on Plasma Membrane $H^+$ Pumps in Vivo and in Vitro

Plasma membrane $H^+$-ATPase has been postulated to be the part of the $H^+$ pump that is the terminal target of BL

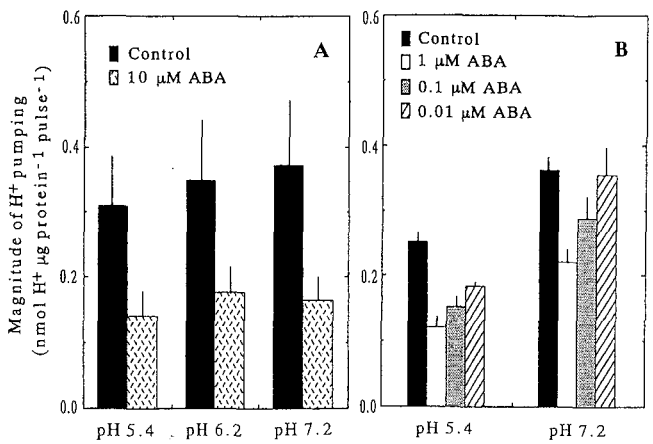


**Figure 4.** Effects of external pH on the inhibition of BL-dependent $H^+$ pumping by ABA in *Vicia* GCPs. ABA was added to the protoplast suspension 21 min before application of BL. The pH of the reaction mixture was adjusted to the indicated pH value with NaOH. Columns represent the mean values ± SE of four (A) and three (B) replicates on separate days. Other experimental conditions were the same as in Figure 1.

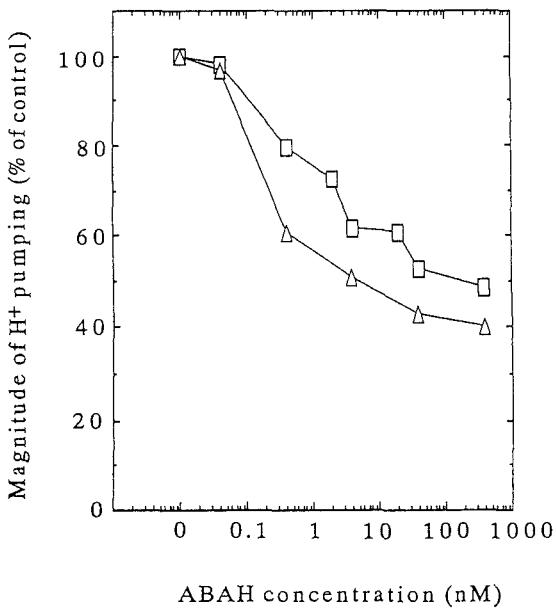


**Figure 5.** Effect of calculated ABAH on BL-dependent $H^+$ pumping from *Vicia* GCPs. The concentrations of ABAH under different experimental conditions were calculated from the Henderson-Hasselbalch equation (using $pK_a$ = 4.8 for ABA) from the data of Figures 2 (□) and 4 (△).

(Assmann et al., 1985; Shimazaki et al., 1986; Schwartz et al., 1991; Amodeo et al., 1992). A possible explanation for the inhibition of BL-dependent $H^+$ pumping by ABA is that it inhibits $H^+$-ATPase and/or BL signal transduction pathways. When GCPs were applied with FC, an activator of plasma membrane $H^+$-ATPase, $H^+$ pumping at a constant rate was induced (Shimazaki et al., 1993; Goh et al., 1995). If the $H^+$-ATPase is inhibited by ABA, inhibition of FC-dependent $H^+$ pumping by ABA would be expected. The rate of FC-dependent $H^+$ pumping in GCPs decreased by 30% when GCPs were incubated with 10 μM ABA for 21 min (Table II). Under the same experimental conditions, the rate of FC-induced $H^+$ pumping decreased by 15% in ad GCPs with ABA. These results suggest that ABA at least partially inhibited the plasma membrane $H^+$-ATPase in both ab GCPs and ad GCPs.

We then examined the effects of ABA on $H^+$ pumping in isolated microsomal vesicles from GCPs. The ATP-dependent $H^+$ pumping across the membranes determined by the fluorescence quenching of quinacrine showed strong inhibition by 100 μM vanadate, suggesting that the $H^+$ pumping was mediated by $H^+$-ATPase in the plasma membrane (Fig. 7). If ABA directly inhibits the $H^+$-ATPase, we can expect the pump inhibition by ABA in these membrane vesicles. However, the $H^+$ pumping was not affected by ABA.

## DISCUSSION

Guard cells respond to a wide variety of environmental signals by the opening and closing of stomata. Stomatal closure induced by water deficit has been shown to be

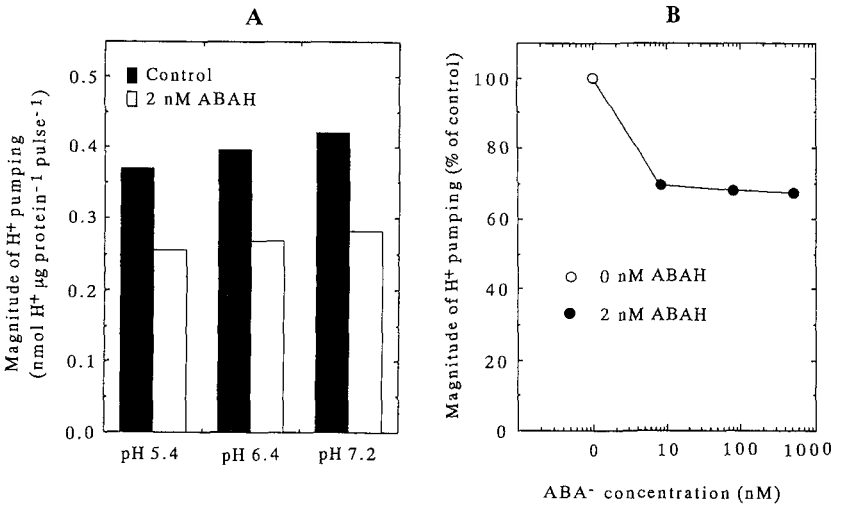


**Figure 6.** A, Effect of ABA on the BL-dependent $H^+$ pumping in GCPs from *Vicia* at different pHs when the concentration of ABAH was kept at 2 nM. Concentrations of total ABA used were 0.01 µM for pH 5.4, 0.083 µM for pH 6.4, and 0.5 µM for pH 7.2. The reaction mixture (1.0 mL) contained 2 nM ABAH and various concentrations of $ABA^-$ that depend on the pH of the medium. B, ●, Replotted from the data of Figure 6A. Concentrations of $ABA^-$ were calculated from the pH of the medium according to the Henderson-Hasselbalch equation. Experiments repeated on different occasions gave similar results. Other experimental conditions were the same as in Figure 1.

mediated by ABA, and the action mechanisms of ABA for stomatal closure have been investigated extensively with respect to the channel activities of the plasma membrane and the tonoplast of guard cells. However, only a few studies have investigated the effect of ABA on $H^+$ pumping in the plasma membrane, which opposes stomatal closure by hyperpolarizing the membrane potential, especially under light. In the present study, we showed that BL-dependent $H^+$ pumping in GCPs was inhibited by ABA (Fig. 1). Inhibition of the pump by ABA was not complete and the extent of maximum inhibition was 65%. Inhibition was saturated with ABA at 1 µM, and the concentration of ABA required to achieve 50% of the maximum inhibition was approximately 0.01 µM at pH 6.2 (Fig. 2). This value is close to that required for the inhibition of stomatal opening in *Commelina* (Willmer et al., 1978; McAinsh et al., 1990). In these reports, ABA concentrations required for 50% of the maximum inhibition of stomatal opening are approximately 0.01 µM at pH 6.0 and 6.15, respectively. The results suggest that the inhibition of BL-dependent $H^+$ pumping may be closely related to the inhibition of stomatal opening by ABA under light.

**Table II.** *Effect of ABA (10 µM) on the $H^+$ pumping induced by FC in Vicia GCPs*

Ad GCPs were prepared from adaxial epidermis of *Vicia* plants by the method of Goh et al. (1995). ABA was added 21 min before the application of BL. FC was added at 10 µM to GCP suspension (pH 6.2) 1 h after onset of red light. Values represent the means ± SE of three replicates. The amount of acid equivalents was determined by the addition of 10 nmol of $H^+$ before the addition of FC.

| Type of Protoplast | Rate of $H^+$ Pumping | |
|---|---|---|
| | −ABA | +ABA |
| | *nmol $H^+$ $\mu g^{-1}$ protein $h^{-1}$* | |
| ab GCPs | 2.53 ± 0.17[a] | 1.79 ± 0.09[a] |
| ad GCPs | 3.01 ± 0.24[b] | 2.53 ± 0.17[a] |

[a] and [b], Significant at the 0.05 level and 0.1 level, respectively, by Student's *t* test.

ABA triggers membrane depolarization, probably because of the activation of $Ca^{2+}$- and voltage-dependent anion channels, which is essential for the activation of $K^+$ efflux channels in the plasma membrane (Assmann, 1993; Ward et al., 1995). Activation of the anion channels depends on an increased concentration of cytosolic $Ca^{2+}$ and/or initial membrane depolarization, because guard cells have a highly negative membrane potential of −160 mV or lower and the anion efflux via the channels becomes substantial only when the membrane potential is depolarized to −100 mV or more positive values (Schroeder and Keller, 1992; Thiel et al., 1992). A mechanism capable of activating the anion channels is required. $Ca^{2+}$ influx into the cytosol induced by ABA is a candidate for this (Ward et al., 1995); however, it is uncertain whether $Ca^{2+}$ influx can induce sufficient membrane depolarization and ABA does not always induce $Ca^{2+}$ influx, although it always closes stomata (Gilroy et al., 1991; Allan et al., 1994). We therefore suggest that the inhibition of BL-dependent $H^+$ pumping by ABA may depolarize the membrane and activate the anion channels (Tazawa et al., 1987). From these observations, we conclude that inhibition of the BL-dependent $H^+$ pumping by ABA can be a driving force for stomatal closure in the daytime and prevents transpirational water loss from leaves under drought stress.

When the external concentrations of ABA were low (0.01-1 µM), inhibition increased with a decrease in the pH of the medium (Fig. 4B). Since the concentration of ABAH increases with the decrease in pH, this result suggests that ABAH is the form active in inhibiting BL-dependent $H^+$ pumping. ABAH was saturating at 40 nM in inhibiting BL-dependent $H^+$ pumping under various experimental conditions (Fig. 5). In contrast, there was no pH dependency of the inhibition from pH 5.4 to 7.2 when 10 µM ABA was applied to the GCPs (Fig. 4A). Under these experimental conditions, concentrations of ABAH were much higher than or equal to 40 nM and might be sufficient to inhibit the pump, eliminating the pH dependency of the inhibition. We

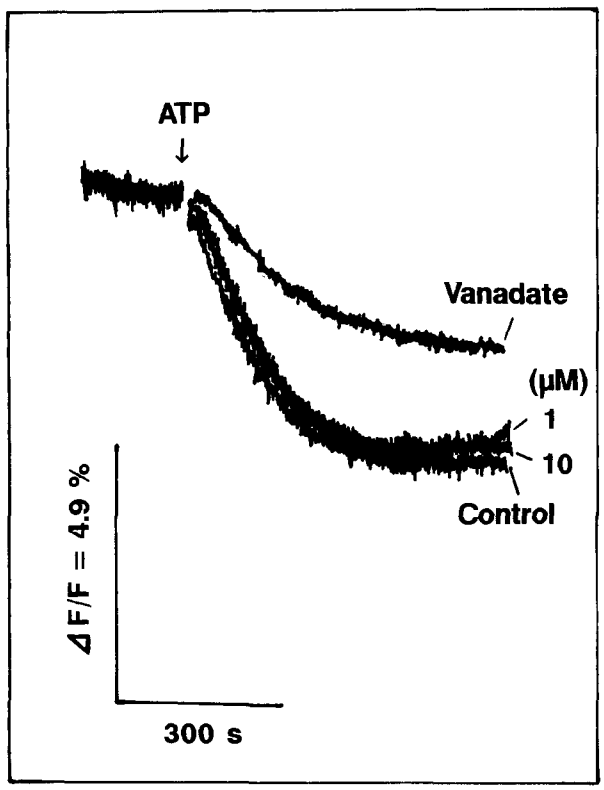


**Figure 7.** Effect of ABA on $H^+$ pumping in microsome vesicles from GCPs of *Vicia*. The basal reaction mixture (250 μL) contained membrane vesicles (10 μg of protein), 10 mM Mops-KOH (pH 7.0), 0.25 M mannitol, 5 mM MgC $l_2$, 1 mM EGTA, 50 mM $KNO_3$, 5 μg/mL oligomycin, and 1 μM quinacrine. Vanadate at 100 μM and ABA at 1 or 10 μM were added to the basal reaction mixture. The concentration of free $Ca^{2+}$ in the reaction mixture was $10^{-8}$ M. ΔF/F, Change in fluorescence divided by the initial fluorescence.

examined further the effect of $ABA^-$ on the $H^+$ pumping (Fig. 6B). When the $ABA^-$ concentration was increased from 8 to 500 nM, while the ABAH concentration was kept at 2 nM, there was no increase in inhibition, suggesting that $ABA^-$ was not responsible for the inhibition of BL-dependent $H^+$ pumping. Since ABAH can enter guard cells much more easily than $ABA^-$ (Kondo et al., 1980; Schwartz et al., 1994), a possible explanation for the effectiveness of ABAH is that the phytohormone acts inside the cells. Consistent with this interpretation is the fact that the time requirement for full inhibition of BL-dependent $H^+$ pumping after the addition of ABA (Fig. 3) suggests that the accumulation of ABA in the cells may be needed to inhibit the pump.

Whether the primary ABA receptors are located in the external or the internal sides of the plasma membrane of guard cells, or in both, remains the subject of vigorous debate (Assmann, 1994). Strong evidence for the presence of intracellular ABA receptors has been reported by Schwartz et al. (1994), who demonstrated that microinjection of ABA into the cytosol of guard cells elicits stomatal closure in *Commelina* and inhibits inward $K^+$ current in *Vicia*, and by Allan et al. (1994), who showed that the microinjected caged ABA induces an increase in cytosolic $Ca^{2+}$ and stomatal closure in *Commelina*. Other evidence and our data indicating that ABA acts more effectively under acidic than alkaline conditions in guard cells (Ogunkanmi et al., 1973; Kondo et al., 1980; Paterson et al., 1988; Anderson et al., 1994) are in accord with the intracellular location for ABA receptors. In contrast, Hartung (1983) showed that $ABA^-$ was as effective as ABAH in causing stomatal closure of *Valerianella locusta*. Recently, Anderson et al. (1994) indicated that external application to *Commelina* epidermis of a final concentration of 10 μM ABA at pH 8.0, in which $ABA^-$ predominated, inhibited stomatal opening by about 60% and suggested the contribution of an extracellular receptor for ABA in guard cells. More recently, MacRobbie (1995) showed that 10 μM ABA at pH 8.0 induced $^{86}Rb^+$ efflux from *Commelina*; this suggested the carrier-mediated uptake of $ABA^-$ in guard cells. However, the concentration of ABAH is calculated to be 6 nM in the external medium of 10 μM ABA at pH 8.0 according to the Henderson-Hasselbalch equation. ABAH at 2 to 6 nM in the incubation medium has a strong inhibitory effect on BL-dependent $H^+$ pumping in various pH ranges (Figs. 5 and 6). Moreover, the ABAH concentration required for 50% of the maximum inhibition in stomatal opening of *Commelina* epidermis can be estimated to be 0.4 nM (McAinsh et al., 1990). Thus, it may be possible that ABAH enters guard cells by diffusion even at high medium pH, accumulates, and acts inside the cells when the epidermis and protoplasts are incubated in the medium for a sufficiently long time (e.g. 2 h).

It is clear that ABA does not inhibit the pump in isolated microsomal membranes of guard cells (Fig. 7). ABA seems to inhibit the pump through unidentified mechanisms in vivo. Recent investigations have indicated that ABA induces an increase in $Ca^{2+}$ concentration up to 1 μM (McAinsh et al., 1990; Schroeder and Hagiwara, 1990; Gilroy et al., 1991; Allan et al., 1994) and pH by up to 0.4 unit in the cytosol (Irving et al., 1992; Blatt and Armstrong, 1993) of guard cells. More recently, we showed that the $H^+$ pump in the plasma membrane of guard cells was inhibited by submicromolar concentrations of $Ca^{2+}$ (Kinoshita et al., 1995). Moreover, it is well known that the $H^+$ pump has an optimum pH of approximately 6.8 (Becker et al., 1993), and its activity will decrease with an increase in cytosolic pH. Therefore, the increased concentration of $Ca^{2+}$ and/or the pH increase in the cytosol of guard cells caused by ABA may be responsible for the inhibition of BL-dependent $H^+$ pumping. We note that ABA may also inhibit the signaling pathway by which the perception of BL is transduced into the activation of the $H^+$ pump.

Our results show that ABA does not cause complete inhibition of BL-dependent $H^+$ pumping in the maximum response (Fig. 2). Such partial inhibition has been reported previously, with a maximum of 40% inhibition by 50 μM ABA (Shimazaki et al., 1986). The difference in the sensitivity of BL-dependent $H^+$ pumping to ABA may be due to cultivar or cultural and experimental conditions. However, the extent of inhibition was partial in both cases. Such partial inhibition of BL-dependent $H^+$ pumping by the phytohormone seems to be important to maintain inside-negative membrane potential and

prevent the damage to guard cells under drought stress. Complete inhibition of the pump may depolarize the membrane strongly and cause excessive turgor loss and deterioration of the cells.

Received October 16, 1995; accepted March 10, 1996.


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