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EFFECTS OF CAFFEINE AND 2,5-DI-(*tert*-BUTYL)-1,4-BENZOHYDROQUINONE ON BLUE LIGHT-DEPENDENT H^+ PUMPING IN GUARD CELL PROTOPLASTS FROM *Vicia faba* L.

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Abstract – The sensory transduction processes of blue light in guard cells have been suggested the involvement of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) or MLCK-like proteins. The source of Ca^{2+} required for the signal transduction process was investigated in guard cell protoplasts (GCPs). The GCPs showed the typical H^+ pumping activity by blue light (200 μmol m^{-2} s^{-1}) and fusicoccin (10 μM) under background red light (600 μmol m^{-2} s^{-1}). The blue light-dependent H^+ pumping was not significantly affected by the externally changed Ca^{2+} concentrations. The addition of 1 mM Ca^{2+} in the bathing medium ratherly inhibited the H^+ pumping. In contrast, the blue light-dependent H^+ pumping was inhibited by caffeine and 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ), inhibitor of Ca^{2+}-ATPase in endoplasmic reticulum (ER) without inhibiting the H^+ pump. The inhibition by caffeine and BHQ was fully reversible. The extent of inhibition by caffeine and BHQ was larger when they were added together than when added separately. The results suggest that Ca^{2+} required for the blue light-dependent H^+ pumping may be released from the intracellular Ca^{2+} stores, probably ER in guard cells.

INTRODUCTION

Stomatal pores in the leaf epidermes control gas exchange between leaves and the atmosphere including CO_2 uptake, necessary for photosynthesis, and H_2O loss by transpiration. To date, it has been largely known that there are many types of signals as factors affecting such stomatal function. Light is a primary environmental signal for opening stomata. Especially, blue light (BL)† plays a dominant role in stomatal opening to light. BL is shown to activate the plasma membrane H^+ pump in guard cells.[1-3] The pump activation creates an electrical potential across the plasma membrane and drives the K^+ uptake through the voltage-gated K^+ channels,[4] leading to stomatal opening. However, our current understand-ing of the mechanisms on the sensory transduction of BL is still very poor.

BL-activated responses in plant cells have been known to employ Ca^{2+} as a signaling agent.[5] However, it has so far not been characterized in plant cells that Ca^{2+} may play a key role in triggering light-stimulated responses.

Recent investigations using pharmacological tools have showed that Ca^{2+}/calmodulin (CaM)-dependent MLCK or MLCK-like proteins might be involved in the signal transduction process of BL-dependent H^+ pumping in guard cells.[6,7] This reflects an increase of $[Ca^{2+}]_{cyt}$ in guard cells responding to BL because it is necessary for the activation of CaM. It may be accomplished by an influx of free Ca^{2+} from the bathing medium across the plasma membrane and/or a release of Ca^{2+} from intracellular stores into cytosol. Available data have provided for Ca^{2+} entry through the plasma membrane into cytosol in guard cells; non-selective Ca^{2+}-permeable channels,[8,9] and stretch-activated Ca^{2+} channels[10] are identified in the plasma membrane of guard cells. Non-selective Ca^{2+}-permeable channels open by abscisic acid, and stretch-activated Ca^{2+} channels open in response to hypotonic treatment. On the other hand, three discrete Ca^{2+} channels were identified in vacuole of plants as a pathway for Ca^{2+} release from the intracellular stores. Those Ca^{2+} channels were inositol trisphosphate (IP_3)-, cyclic adenosine 3',5'-diphosphoribose (cADPR)-, and voltage-sensitive, respectively.[11,12] IP_3- and voltage-sensitive Ca^{2+} channels in the vacuole [13-15] seem to be responsible for the stomatal closure through

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†*Abbreviations:* BHQ, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone; BL, blue light; $[Ca^{2+}]_{cyt}$, free Ca^{2+} concentration in the cytosol; CaM, calmodulin; cADPR, cyclic adenosine 3',5'-diphosphoribose; CPA, cyclopiazonic acid; DMSO, dimethylsulfoxide; FC, fusicoccin; GCPs, guard cell protoplasts; IP_3, inositol trisphosphate; ER, endoplasmic reticulum; MLCK, myosin light chain kinase.

an inactivation of anion channels and/or an inactivation of inward-rectifying K^+ channels by Ca^{2+}.[8,16] Functional role of cADPR-sensitive Ca^{2+} channels in the vacuole was not yet clarified. In animal cells, influxes of Ca^{2+} into cytosol from the intracellular stores occur through at least two discrete Ca^{2+} channels. One of them is activated by IP_3 and the other is by Ca^{2+}, both of them are located in the endoplasmic reticulum (ER). In plant cells, the ER accumulates Ca^{2+} and may act as Ca^{2+} stores, although there are no such channels identified in the ER.[17]

In this study the effects of externally changed Ca^{2+} concentrations, and caffeine and BHQ, inhibitor of the ER Ca^{2+}-ATPase in animal cells on the BL-dependent H^+ pumping in *Vicia* guard cell protoplasts were investigated. These experiments suggest that Ca^{2+} from the ER stores may be used to mediate H^+ pumping by BL in GCPs.

MATERIALS AND METHODS

Plant material. Seeds of *Vicia faba L.*(Ryosai Issun) were germinated on vermiculite in a controlled environmental growth chamber (25°C, 12 h of light/12 h of dark) for 1 week, and the seedlings were grown hydroponically in a greenhouse (20 ± 2°C) under sunlight as previously described.[6]

Isolation of guard cell protoplasts. Guard cell protoplasts (GCPs) were isolated enzymatically from the epidermes according to the method of Goh *et al.* (1995).[18] The epidermal strips were digested in a medium (pH 5.4) containing 1 m*M* $CaCl_2$ throughout the isolation procedures. The released protoplasts in medium were washed twice with a chilled solution of 0.4 *M* mannitol and 1 m*M* $CaCl_2$ and collected by centrifugation. The isolated protoplasts were suspended and stored in the dark in a solution of 0.4 *M* mannitol and 1 m*M* $CaCl_2$ unless otherwise stated.

In order to investigate the effects of external Ca^{2+} concentration on the activity of BL-dependent H^+ pumping, the released protoplasts in medium were washed twice with a chilled solution of 0.4 *M* mannitol and collected by centrifugation. The isolated protoplasts were suspended and stored in the dark in a solution of 0.4 *M* mannitol and kept on ice until use.

Measurement of H^+ pumping. BL-dependent H^+ pumping and pH change in the GCPs suspension were determined with glass pH electrode connected to a pH meter.[6] BL (200 μmol m^{-2} s^{-1}) was applied as a short pulse (30 s) 1 h after the onset of background red light (600 μmol m^{-2} s^{-1}) irradiation. Red light was obtained from the lamp by filtering the light through a glass cutoff (Corning 2-61, Corning, NY) and Cinemoid 5A filters. BL was from a tungsten lamp through a glass filter (Corning 5-60). The basal reaction mixture (1.0 mL) consisted of 0.125 m*M* Mes-NaOH (pH 6.2), 0.4 *M* mannitol, and 10 m*M* KCl with and without 1 m*M* $CaCl_2$. Fusicoccin (FC)-induced H^+ pumping was measured in the same reaction mixture under irradiation by red light. FC was added at 10 μ*M*. The amounts of protoplasts used for all experiments were 20 μg of protein/mL. The added protoplasts were preincubated for 10 min in darkness before the irradiation of red light.

Determination of external Ca^{2+} concentration. The isolated protoplasts were washed twice with a chilled solution of 0.4 *M* mannitol without $CaCl_2$, and then centrifuged at 110 g for 7 min. In order to determine the external Ca^{2+} concentration during the H^+ pumping by BL took places in GCPs, the GCPs suspension was collected and centrifuged when the magnitude of H^+ pumping was maximal. The supernant was collected for the direct determination of the amount of Ca^{2+}. Concentration of Ca^{2+} was determined by an atomic absorption spectrometry (model Z-9000, Hitachi, Tokyo, Japan).

Protein determination. Protein was determined by the method of Bradford (1976)[19] with BSA as a standard.

RESULTS AND DISCUSSION

Proton pumping by blue light and fusicoccin

The H^+ pumping by blue light (BL) in GCPs has been postulated to be activated by plasma membrane H^+-ATPase as a terminal target,[3,20,21] although the participation of plasma membrane redox chain[22,23] cannot be excluded. The properties of H^+ pumping activated by BL in GCPs have been well characterized elsewhere.[3,18,24,25,37] Figure 1A shows the time course of BL-dependent H^+ pumping in GCPs. The H^+ Pumping was induced by a pulse of BL (200 μmol mm^{-2} s^{-1}, 30s) superimposed on background red light (600 μmol m^{-2} s^{-1}). Protons were extruded continuously for 12 to 16 min after the cessation of the pulse. The maximum magnitude of H^+ pumping was 0.346 nmol H^+ μg $protein^{-1}$ $pulse^{-1}$. Fusicoccin (FC) at 10 μ*M*, an activator of H^+ pump also induced the H^+ pump (Fig. 1B). The maximum rates of H^+ pumping occurred around 5 min and showed 2.53 nmol H^+ μg $protein^{-1}$ h^{-1}. The action of FC on higher plants is usually attributed to activation of the H^+-ATPase of plasma membrane.[26] The results showed that the isolated GCPs had typical properties of H^+ pumping by BL and FC in GCPs, respectively. Calcium ions may act as a second messenger in light-response coupling in plant cells. Thus, the BL photoreceptor cells will provide a model from where Ca^{2+} required for the light response may originate.

Effects of external Ca^{2+} concentration in BL-dependent H^+ pumping

The magnitude of H^+ pumping by BL in GCPs was not significantly affected by the changes of Ca^{2+} concen-trations ranging from 0.1 to 10 m*M* in the bathing medium (Table 1). They also did not cause a considerable change in the time course of the BL-dependent H^+ pumping in GCPs (data not shown). When GCPs preparation was washed with a solution of 0.4 *M*

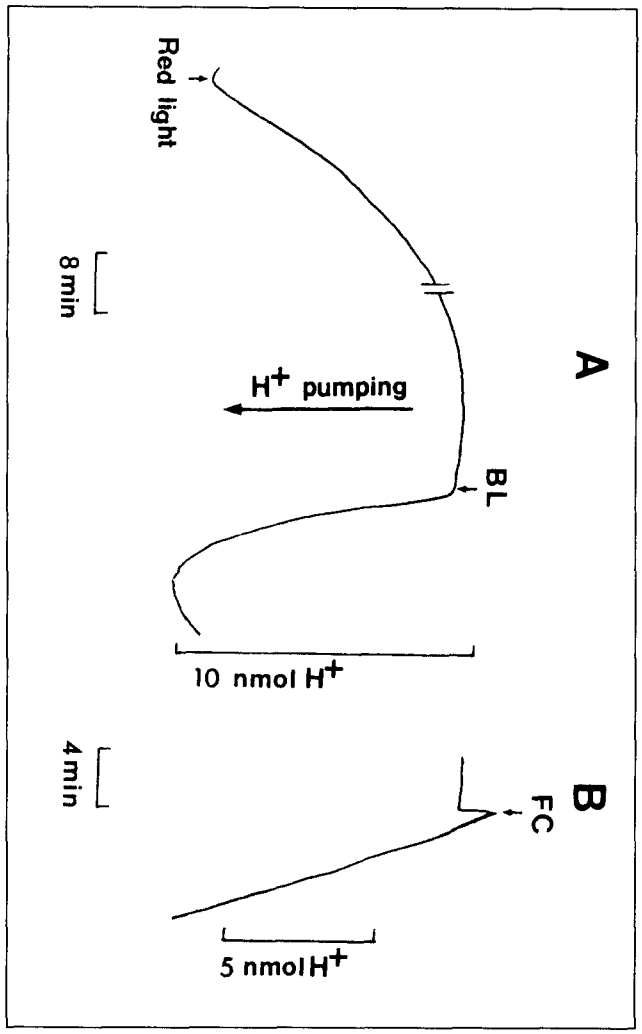

Figure 1. H^+ pumping by blue light (A) and fusicoccin (B) in *Vicia* guard cell protoplasts under background red light (600 μmol m^{-2} s^{-1}). A) A pulse of blue light (200 μmol m^{-2} s^{-1}, 30s) was given at the time indicated by arrow. The amount of acid equivalents was determined by addition of 10 nmol of H^+ at the end of each experiment. B) Fusicoccin was added to GCPs suspension at a final concentration of 10 μM in 0.5% DMSO at the time indicated by arrow. The amount of acid equivalents was determined by addition of 10 nmol of H^+ 10 min before the addition of fusicoccin. The reaction mixture (1.0 mL) contained 0.125 m*M* Mes-NaOH (pH 6.2), 0.4 *M* mannitol, 10 m*M* KCl, and 1 m*M* $CaCl_2$. The amounts of GCPs added in the reaction mixture were 20 μg of protein/mL. All measurements were carried out at 24°C.

mannitol by centrifugation, the magnitude of H^+ pumping by BL was decreased by 35% (Table 2A). However, the removal of external Ca^{2+} by washing the GCPs preparation in this system was not an efficient technique. The concentration of external Ca^{2+} in GCPs preparation was estimated to be 1.57 ± 0.45 μM, not to be zero by using atomic absorption spectrometry. In addition, the

Table 1. Effects of external Ca^{2+} concentration on blue light-dependent H^+ pumping in *Vicia* guard cell protoplasts.

$CaCl_2$	Magnitude of BL-dependent H^+ pumping (nmol H^+ μg protein^{-1} pulse^{-1})
10[a]	0.281 ± 0.024
1.0	0.248 ± 0.010
0.1[b]	0.278 ± 0.026

[a, b] The final concentration of Ca^{2+} in the bathing medium was controlled by the addition of the reaction solution (0.4 *M* mannitol, 10 m*M* KCl, 0.125 m*M* Mes-NaOH, pH 6.2) with 11.11 μM and 0.11 μM $CaCl_2$, respectively. Other experimental conditions were the same as in Fig. 1. All measurements are significant at the 0.1 level by Student's *t* test.

Table 2. Effects of external Ca^{2+} removal from the bathing medium on blue light-dependent H^+ pumping in *Vicia* guard cell protoplasts.

	$CaCl_2$	Magnitude of BL-dependent H^+ pumping (nmol H^+ μg protein^{-1} pulse^{-1})
A)	1	0.386 ± 0.049
	0[a]	0.252 ± 0.008
B)	0	0.229 ± 0.010
	1[b]	0.160 ± 0.019

A) GCPs preparation was suspended in 0.4 *M* mannitol and 1 m*M* $CaCl_2$. [a]GCPs preparation was washed twice with a solution of 0.4 *M* mannitol. Values represent the means $\pm$ SE of three replicates. B) GCPs preparation was suspended in 0.4 *M* mannitol. [b]The final concentration of 1 m*M* Ca^{2+} in the suspending medium was added to GCPs suspension 21 min before the application of BL. Values represent the means $\pm$ SE of six replicates. Other experimental conditions were the same as in Fig. 1. All measurements are significant at the 0.1 level by Student's *t* test.

concentration of external Ca^{2+} was 9.57 ± 0.01 μM during the induction of H^+ pumping by BL took place in GCPs. Any sudden increase of Ca^{2+} concentration in the bathing medium may be derived from the broken GCPs during the measurement of H^+ pumping. In the other experiments, we failed to measure the H^+ pumping by BL in GCPs when the external Ca^{2+} was depleted by Ca^{2+} chelator, ethylenediaminete-tracetic acid (data not shown). We further tested a possibility of Ca^{2+} entry from the external space for the activation of BL-dependent H^+ pumping in GCPs. To lower the external Ca^{2+} concentration, GCPs preparation was also washed twice and centrifuged with a solution of 0.4 *M* mannitol. The GCPs preparation had the magnitude of H^+ pumping showing 0.229, but it was decreased to 0.160 nmol H^+ μg protein^{-1} pulse^{-1} when 1 m*M* $CaCl_2$ was

Table 3. Inhibition of blue light-dependent H^+ pumping in *Vicia* guard cell protoplasts by caffeine, 2,5-di-(*tert*-butyl)-1,4-benzohydroquinone (BHQ), and thapsigargin.

Inhibitors	Concentrations	Magnitude of BL-dependent H^+ pumping (nmol H^+ μg protein^{-1} pulse^{-1})	Rate of FC-induced H^+ pumping (nmol H^+ μg protein^{-1} h^{-1})
Caffeine	0	0.495	2.13
(mM)	5	0.118	N.D.
	10	0.060	2.25
BHQ	0	0.415	2.42
(μM)	1	0.322	2.32
	10	0.100	2.45
Thapsigargin	0	0.341	N.D.
(μM)	20	0.342	N.D.

The inhibitors were added to GCPs suspension 21 min before the application of BL. Caffeine was dissolved in the reaction solution, and BHQ and thapsigargin were dissolved in DMSO, respectively. The final concentration of DMSO was 0.5%. Other experimental conditions were as shown in Fig. 1. At least three separate experiments were done for individual inhibitors, and the typical values were given. N.D., Not determined.

supplied to the bathing medium 21 min before the application of BL (Table 2B). These indicate that cytosolic free Ca^{2+} required for the activation of H^+ pumping by BL in GCPs may be not originated from the external space.

Effects of caffeine and BHQ in BL-dependent H^+ pumping.

We examined a possible involvement of Ca^{2+} that was stored in intracellular organelles, especially in the ER, in the BL-dependent H^+ pumping of GCPs. The H^+ pumping was inhibited by caffeine about 76 % at 5 m*M* and 89 % at 10 m*M* when GCPs were incubated with caffeine for 21 min prior to the application of BL (Table 3). An addition of FC (10 μ*M*) to GCPs suspen-sion induced the H^+ pumping similar to those of the controls; it did not inhibit the activity of the H^+ pump itself. These results reflect that caffeine affects the signal transduction process between the photoreceptor and the H^+ pump. Caffeine may deplete the free Ca^{2+} of intracellular stores by activating the efflux of Ca^{2+} outside of cells through the plasma membrane, probably due to releasing of the Ca^{2+} from the stores such as ER. We, therefore, expect that emptying of intracellular Ca^{2+} stores in guard cells suppresses the BL-dependent H^+ pumping. The ER Ca^{2+}-ATPase inhibitor, BHQ is shown to empty Ca^{2+} pools in smooth muscle cells by inhibiting Ca^{2+} uptake into ER.[27] As shown in Table 3, BHQ at 10 μ*M* strongly inhibited the BL-dependent H^+ pumping in a concentration dependent manner. The pump activity itself was also not

Table 4. Restoration of the blue light-dependent H^+ pumping activity in caffeine- and BHQ-treated guard cell protoplasts.

Magnitude of BL-dependent H^+ pumping (nmol H^+ μg protein^{-1} pulse^{-1})			
Controls	Caffeine-treated	Controls	BHQ-treated
0.275	0.231 (+84%)	0.315	0.338 (+107%)
0.357	0.216 (+61%)	0.374	0.356 (+95%)
0.336	0.346 (+103%)	0.318	0.308 (+96%)

Caffeine (10 m*M*) and BHQ (10 μ*M*) were pretreated for 20 min at room temperature in darkness, and then GCPs preparation was washed twice with a solution of 0.4 *M* mannitol and 1 m*M* $CaCl_2$. At least three separate experiments were done for individual inhibitors, and the typical values were given. Other experimental conditions were the same as shown in Fig. 1.

Table 5. Effects of 1 m*M* caffeine and 1 μ*M* BHQ on blue light-dependent H^+ pumping in *Vicia* guard cell protoplasts.

Inhibitors	Magnitude of BL-dependent H^+ pumping (nmol H^+ mg protein^{-1} pulse^{-1})
Caffeine BHQ	0.444 ± 0.037
+ Caffeine BHQ	0.406 ± 0.037
Caffeine + BHQ	0.379 ± 0.031
+ Caffeine + BHQ	0.278 ± 0.026

The inhibitors were added to GCPs preparation 21 min before the application of BL. Values represent the means ± SE of six replicates. Other experimental conditions were as in Fig. 1. All measurements are significant at the 0.1 level by Student's *t* test.

inhibited by BHQ because the addition of FC induced the H^+ pumping in a similar rate to those of controls. We further examined the effect of thapsigargin, the most selective inhibitor of ER Ca^{2+}-ATPase in a variety of animal cell types, such as lym-phocytes, smooth muscle cells, and neuronal cells,[28] on the BL-dependent H^+ pumping. However, thapsigargin had no inhibitory effect on the pumping (Table 3). The other experiments with cyclopiazonic acid (CPA), a specific inhibitor of the Ca^{2+}-ATPase in the sarcoplasmic reticulum of animal cells[29,30] and in the ER of plant cells,[31] showed that the treatment at 10 μ*M* 21 min before the application of BL inhibited the BL-dependent H^+ pumping by 60 %.[32] Such inhibitory effects strongly suggest that Ca^{2+} release from the intracellular stores may be responsible for transducing the BL signal into the activation of the pump, although thapsigargin does not inhibit the BL-dependent H^+ pumping.

On the other hand, caffeine-treated and BHQ-treated GCPs responded almost completely to BL upon the removal of caffeine and BHQ by washing. When the GCPs that had been preincubated with 10 m*M* caffeine

or 10 μM BHQ for 20 min were washed twice with a solution of 0.4 M mannitol and 1 mM $CaCl_2$, GCPs restored the activity of the BL-dependent H^+ pump up to 100% (Table 4). Caffeine and BHQ seem to empty the intracellular Ca^{2+} by activating the Ca^{2+}-induced Ca^{2+} release through Ca^{2+} channels on ER. If caffeine and BHQ affect the same intracellular store, we can expect the combined inhibitory effects of caffeine and BHQ on the BL-dependent H^+ pumping when they were added to GCPs suspension at the same time. We tested this possibility using low concentrations of these reagents. The H^+ pumping was inhibited by 9% at 1 mM caffeine, and 15% at 1 μM BHQ, respectively and showed about 40% inhibition by the combination of 1 mM caffeine and 1 μM BHQ (Table 5). This synergistic effect in the inhibition of BL-dependent H^+ pumping by two inhibitors in GCPs indicated the same action site.

CONCLUSION

BL-activated responses in guard cells have been suggested to employ Ca^{2+} as the signaling agent.[6,7] It is our working hypothesis that the transduction processes of BL signal in guard cells become involved in an increase in $[Ca^{2+}]_{cyt}$. The increase of $[Ca^{2+}]_{cyt}$ can be achieved by the influx of Ca^{2+} from external space across the plasma membrane and/or release from the intracellular store(s). The increase of $[Ca^{2+}]_{cyt}$ required for the activation of H^+ pumping by BL in GCPs seems to be not originated from the external space (Table 1 and 2). In plant cells, the ER accumulates Ca^{2+} through the Ca^{2+}-ATPase, and several evidence demonstrated the presence of the ER Ca^{2+}-ATPase that is sensitive to CPA.[17,31,33,34] The inhibitory effects by caffeine, BHQ, and CPA strongly suggest that Ca^{2+} release from at least a part of intracellular stores, probably from the ER may be responsible for transducing the BL signal into the activation of the pump in guard cells. Since the inhibition of ER Ca^{2+}-ATPase caused an immediate drop in the Ca^{2+} concentration in Hella cells,[35] our results with the inhibitors may make it impossible to increase the $[Ca^{2+}]_{cyt}$ in BL responses of guard cells and thus it will diminish the activation signal for the H^+ pump. Therefore, it is most likely that the increase of $[Ca^{2+}]_{cyt}$ required for BL-dependent H^+ pumping is originated from the ER, although the exact mechanisms of caffeine and BHQ on plant cells have not been clarified.

There are several distinct intracellular Ca^{2+} stores that can release Ca^{2+} into cytosol of guard cells. Vacuole, ER, chloroplasts, and mitochondria are the candidate.[36] Vacuole is the largest Ca^{2+} stores in plant cells, and has Ca^{2+}-dependent, and cADPR-dependent Ca^{2+} channels on tonoplast.[11,12,15] However, no CPA and BHQ-sensitive component was identified on the tonoplasts of plant cells. In this context, further cytophysiological investigations are needed elsewhere. This will be helpful to our understanding of the mechanisms on the sensory transduction of BL in guard cells.

REFERENCES

1. Assmann, S. M. (1993) Signal transduction in guard cells. *Annu. Rev. Cell Biol.* **9**, 345-375.
2. Iino, M., T. Ogawa and E. Zeiger (1985) Kinetic properties of the blue-light response of stomata. *Proc. Natl. Acad. Sci. USA.* **82**, 8019-8023.
3. Shimazaki, K., M. Iino and E. Zeiger (1986) Blue light-dependent proton extrusion of guard cell protoplasts of *Vicia faba. Nature* **319**, 324-326.
4. Schroeder, J. I., K. Raschke and E. Neher (1987) Voltage dependence of K^+ channels in guard cell protoplasts. *Proc. Natl. Acad. Sci. USA.* **84**, 4108-4112.
5. Hepler, P. K. and R. O. Wayne (1985) Calcium and development. *Ann. Rev. Plant Physiol.* **36**, 397-439.
6. Shimazaki, K., T. Kinoshita and M. Nishimura (1992) Involvement of calmodulin and calmodulin-dependent myosin light chain kinase in blue light-dependent H^+ pumping by guard cell protoplasts from *Vicia faba* L. *Plant Physiol.* **99**, 1416-1421.
7. Shimazaki, K., K. Omasa, T. Kinoshita and M. Nishimura (1993) Properties of the signal transduction pathway in the blue light response of stomatal guard cells of *Vicia faba* and *Commelina benghalenis. Plant Cell Physiol.* **34**, 1321-1327.
8. Schroeder, J. I. and S. Hagiwara (1990) Repetitive increases in cytosolic Ca^{2+} of guard cells by abscisic acid activation of non-selective Ca^{2+} permeable channels. *Proc. Natl. Acad. Sci. USA.* **87**, 9305-9309.
9. Fairley-Grenot, K. A. and S. M. Assmann (1991) Evidence for G-protein regulation of inward K^+ current in guard cells of *Fava bean. Plant Cell* **3**, 1037-1044.
10. Cosgrove, D. J. and R. Hedrich (1991) Stretch-activated chloride, potassium, and calcium channels coexisting in plasma membrane of guard cells of *Vicia faba* L. *Planta* **186**, 143-153.
11. Hedrich, R. and E. Neher (1987) Cytosolic calcium regulates voltage-dependent ion channels in plant vacuoles. *Nature* **329**, 833-837.
12. Allen, G. J., S. R. Muir and D. Sanders (1995) Release of Ca^{2+} from individual plant vacuoles by both InsP3 and cyclic ADP-ribose. *Science* **268**, 735-737.
13. Gilroy, S., N. D. Read and A. J. Trewavas (1990) Elevation of cytoplasmic calcium by caged calcium or caged inositol trisphosphate initiates stomatal closure. *Nature* **346**, 769-771.
14. Blatt, M. R., G. Thiel and D. R. Trentham (1990) Reversible inactivation of K^+ channels of *Vicia* stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. *Nature* **346**, 766-769.
15. Ward J. M. and J. I. Schroeder (1994) Calcium-activated

K^+ channels and calcium induced calcium release by slow vacuolar ion channels in guard cell vacuoles implicated in the control of stomatal closure. *Plant Cell* **6**, 669-683.

16. Lemtiri-Chlieh, F. and E. A. C. MacRobbie (1994) Role of calcium in the modulation of *Vicia* guard cell potassium channels by abscisic acid: a patch-clamp study. *J. Membr. Biol.* **137**, 99-107.
17. Buckout, T. J. (1984) Characterization of Ca^{2+} transport in purified endoplasmic reticulum membrane vesicles from *Lepidium sativum* L. roots. *Plant Physiol.* **76**, 962-967.
18. Goh, C-H., O. Tatsuo and K. Shimazaki (1995) Properties of proton pumping in response to blue light and fusicoccin in guard cell protoplasts isolated from adaxial epidermis of *Vicia* leaves. *Plant Physiol.* **109**, 187-194.
19. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
20. Assmann, M. M., L. Simoncini and J. I. Schroeder (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. *Nature* **318**, 285-287.
21. Amodeo, G., A. Srivastava and E. Zeiger (1992) Vanadate inhibits blue light-stimulated swelling of *Vicia* guard cell protoplasts. *Plant Physiol.* **100**, 1567-1570.
22. Gautier, H., A. Vavasseur, G. Lasceve and A. M. Boudet (1992) Redox processes in the blue light response of guard cell protoplasts of *Commelina communis* L. *Plant Physiol.* **98**, 34-38.
23. Vani, T. and A. S. Raghavendra (1992) Plasma membrane redox system in guard cell protoplasts of pea (*Pisum sativum L.*). *J. Exp. Bot.* **43**, 291-297.
24. Zeiger, E. (1987) Cellular and functional properties of the stomatal response to blue light. *In* H. Senger, ed, Blue light response: Phenomena and occurrence in plants and microorganisms. Vol II, CRC Press, Boca Raton, FL, pp 110-120.
25. Mawson, B. J. and E. Zeiger (1991) Blue light modulation of chlorophyll a fluorescence transients in guard cell chloroplasts. *Plant Physiol.* **96**, 753-763.
26. Briskin, D. P. and M. C. Gawienowski (1996) Role of the plasma membrane H^+-ATPase in K^+ transport. *Plant Physiol.* **111**, 1199-1207.
27. Short, A. D., J. Bian, K. Ghosh, R. T. Waldron, S. L. Rybak and D. L. Gill (1993) Intracellular Ca^{2+} pool content in linked to control of cell growth. *Proc. Natl. Acad. Sci. USA* **90**, 4986-4990.
28. Takemura, H., A. R. Hughes, O. Thastrup and Jr. J. W. Putney (1989) Activation of calcium entry by the tumor promotor thapsigargin in parotid acinar cells. *J. Biol. Chem.* **264**, 12266-12271.
29. Goeger, D. E., R. T. Riley, J. W. Dorner and R. J. Cole (1988) Cyclopiazonic acid inhibition of the Ca^{2+}-transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. *Biochem. Pharmacol.* **37**, 978-981.
30. Seidler, N. W., L. Jona, M. Vegh and A. Martonosi (1989) Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J. Biol. Chem.* **264**, 17816-17823.
31. Hsieh, W-L., W.S. Pierce and H. Sze (1991) Calcium-pumping ATPase in vesicles from carrot cells. *Plant Physiol.* **97**, 1535-1544.
32. Shimazaki, K., C-H. Goh and T. Oku (1996) Inhibition of blue light-dependent proton pumping on guard cell protoplasts by caffeine and inhibitors of endoplasmic reticulum Ca^{2+}-ATPase. *Plant Cell Physiol.* abstract 411 (3aA12).
33. Giannini, J. L., J. Ruiz-Cristin, D. P. Briskin (1987) Calcium transport in sealed vesicles from red beet (*Beta vulgaris* L.) storage tissue: characterization of a Ca^{2+}-pumping ATPase associated with endoplasmic reticulum. *Plant Physiol.* **85**, 1129-1136.
34. Bush, D. S. and T. Wang (1995) Diversity of calcium-effulx transporters in wheat aleurone cells. *Planta* **197**, 19-30.
35. Monteo, M., M. Brini, R. Marsault, J. Alvalez, R. Sitia, T. Pozzan and R. Rizzuto (1995) Monitoring dynamic changes in free Ca^{2+} concentration in the endoplasmic reticulum of intact cells. *EMBO J.* **14**, 5467-5475.
36. Bush, D. S. (1993) Regulation of cytosolic calcium in plants. *Plant Physiol.* **103**, 7-13.
37. Goh, C-H., O. Tatsuo and K. Shimazaki (1996) Inhibition of blue light-dependent proton pumping by abscisic acid in guard cell protoplasts from *Vicia faba* L. *Plant Physiol.* **111**, 433-440.