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Blue light signaling in stomatal guard cells

Ken-ichiro Shimazaki[1*], Michio Doi[2] and Toshinori Kinoshita[1]
[1]Department of Biology, Faculty of Science, and [2]Research Center for Higher Education, Kyushu University, Ropponmatsu, 4-2-1 Fukuoka 810-8560, Japan

Blue light activates proton pump, and creates electrical gradient across the plasma membrane and drives K^+ uptake in stomatal guard cells. In this presentation, we provide evidence for regulatory mechanisms of the pump and the identification of blue light receptor. The pump is shown to be the plasma membrane H^+-ATPase and is activated through phosphorylation of the C-terminus. Phosphorylation occurred and 14-3-3 protein bound to the phosphorylation site. The binding of 14-3-3 protein was required for the H^+-ATPase activation. We also found that *phot1 phot2* double mutant does not respond to blue light but other mutants respond to blue light by stomatal opening. However, all these mutants are capable of stomatal opening in the presence of fusicoccin, an activator of the H^+-ATPase. These results suggest that both phot1 and phot2 act as blue light receptors in guard cells.

Key words: blue light, guard cells, H^+-ATPase, phot, phosphorylation, 14-3-3 protein

INTRODUCTION

Blue light (BL)-specific responses are ubiquitous in plant cells, and include phototropism, inhibition of stem elongation, and stomatal opening [1]. Stomatal pores surrounded by a pair of guard cells in the epidermis regulate gas exchange between leaves and the atmosphere, and thus allow CO_2 entry for photosynthesis and transpirational stream in higher plants [2]. The opening of stomata is mediated by an accumulation of K^+-salt in guard cells, and K^+ accumulation through the voltage-gated K^+ channel is driven by an inside-negative, electrical potential across the plasma membrane. This electrical potential is created by a BL-activated H^+ pump which has been suggested to be H^+-ATPase in the plasma membrane [2]. However, the mechanism by which the perception of BL is transduced into H^+ pump activation is largely unknown.

In this presentation, we provide evidence for the activation mechanisms of the plasma membrane H^+-ATPase and an identification of blue light receptor that mediate this response.

MATERIALS AND METHODS

Plant materials. *Vicia faba* (cv. Ryosai Issun) was cultured hydroponically in a green house. Guard cell protoplasts (GCPs) were isolated enzymatically form Vicia epidermis [3]. The Columbia ecotype Arabidopsis was used. Wild-type plants, the mutants of phot1-5, phot2-1 and npq1-2 and double mutant of phot1-5 phot2-1 were grown on soil.

Phosphorylation of the H^+-ATPase. Phosphorylation of the H^+-ATPase was determined after immunoprecipitation [3].

Stomatal aperture. Stomatal aperture were determined using "mature stomata" in epidermal strips from 3- to 5-week-old plants of Arabidopsis. Strips were illuminated with red light, or with blue light superimposed on the background red light, for 2 h. Stomatal aperture were measured from transmission images obtained using a laser scanning microscope.

RESULTS AND DISCUSSION

Activation mechanisms of the plasma membrane H^+-ATPase. We determined proton pumping in GCPs and ATP hydrolytic activity in the guard cell extract. As shown in Fig. 1A, proton pumping started 30s after the pulse of BL for 30s, and showed the maximum rate at 2.5 min after the pulse. In the same sample, ATP hydrolytic activity was determined. The rate of ATP hydrolysis paralleled to that of proton pumping and was sensitive to vanadate, indicating that proton pumping was mediated by the plasma membrane H^+-ATPase (Fig .1B).

Phosphorylation levels of the H^+-ATPase were determined after immunoprecipitation of the ATPase. Autoradiogram revealed that

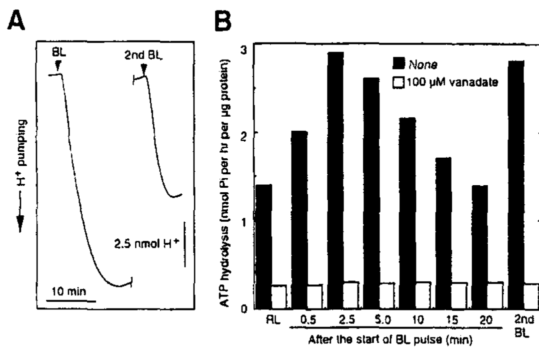

Figure 1. BL-dependent proton pumping in GCPs and BL-stimulated ATP hydrolysis in guard cell extracts from *Vicia*.

the levels coincided with the ATP hydrolytic activity

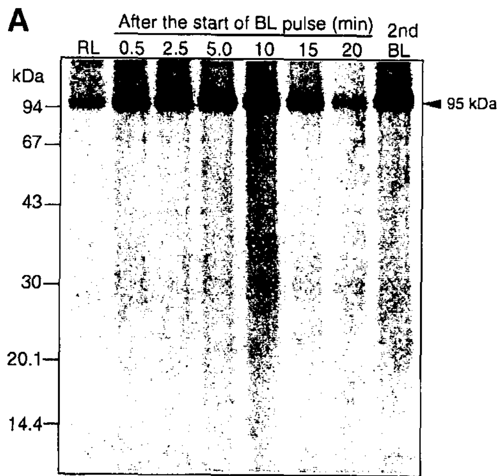

Figure 2. Changes in the phosphorylation levels of the plasma membrane H^+-ATPase in GCPs in response to BL.

(Fig. 2). In the presence of protein kinase inhibitor (K-252a), the phosphorylation in response to BL was inhibited (Fig.3B), and the proton pumping was also inhibited (Fig. 3A). The results suggest that protein phosphorylation of the plasma membrane H^+-ATPase is required for its activation.

Since the C-terminus is an autoinhibitory domain of the enzyme [4], it is likely that C-terminus is phosphorylated by BL. To determine this, we digested C-terminus using CNBr and phosphorylation levels were compared with that of the ATPase. As shown in Figure 4, all the radioactivity was recovered in the C-terminal fragments, suggesting that phosphorylation occurred exclusively on the C-terminus. The phosphorylated amino acids were determined to be serine and threonine residues. Further investigation indicate that the 14-3-3 protein binds to the phosphorylared threonine at the C-terminus, and 14-3-3 binding is required for the activation of the H^+-ATPase [3].

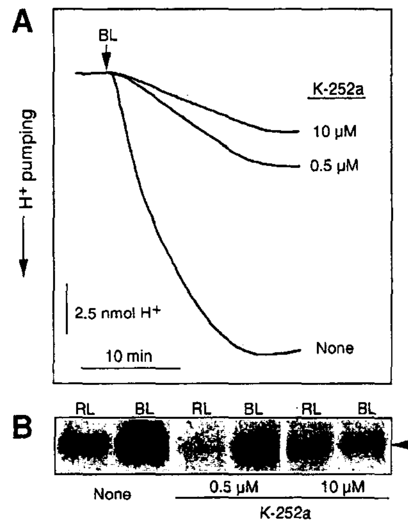

Figure 3. Inhibition of BL-dependent proton pumping (A) and phosphorylation of the H^+-ATPase by a protein kinase inhibitor (B).

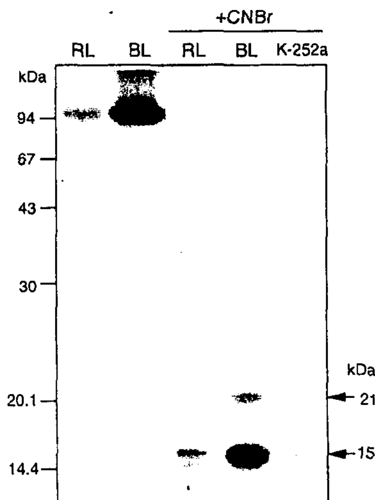

Figure 4. Exclusive phosphorylation of the C-terminus of the plasma membrane H^+-ATPase in guard cells by BL.

Identification of blue light receptor in guard cells

We then aim to determine the blue light receptor that mediates activation of the plasma membrane H^+-ATPase. We considered the phot1 protein and its homologue phot2 as candidate. The phot1 protein is a serine/threonine protein kinase, and absorption spectra of LOV domains of phot1 and

phot2 are closely resemble the action spectrum for stomatal opening [5].

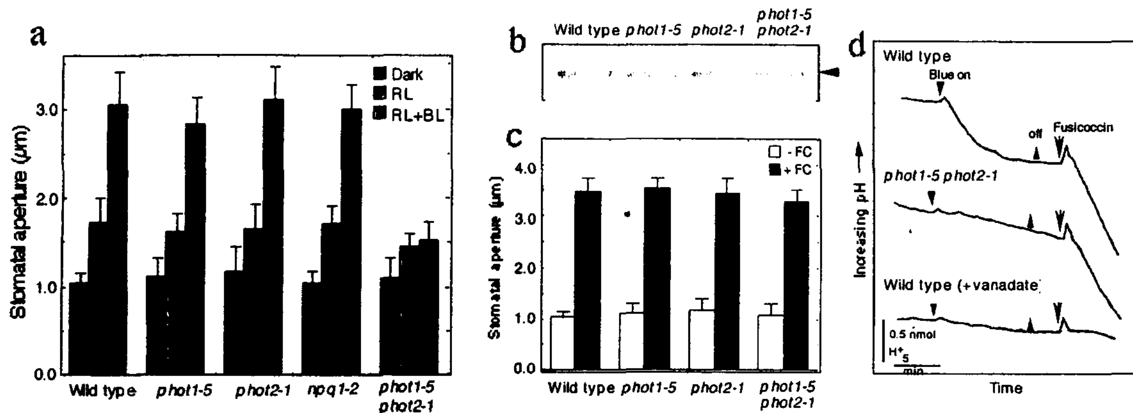

Figure 5. **a**: Stomatal opening in the epidermis under blue light. **b**: Amount of the plasma membrane H^+-ATPase determined by immunological method. **c**: Stomatal opening induced by fusicoccin in the dark.. **d**: Proton extrusion in the epidermal strips by BL and fusicoccin.

To investigate this question, we constructed a double mutant using a null allele of phot1 (*phot1-5*) and a chloroplast avoidance mutant *phot2-1* (*cav1-1*) in the Columbia background.Epidermal strips from wild type plants, single mutants of *phot1-5*, *phot2-1*, *npq1-2*, and a double mutant *phot1-5 phot2-1*, were used to investigate stomatal responses. The stomata closed in the dark but opened slightly under red light illumination of 50 mmol m^{-2} s^{-1} in strips from all the plants tested (Fig. 5a). The stomata opened much wider when a low fluence rate blue light (10 mmol m^{-2} s^{-1}) was added to the background of red light in wild type tissue, and in tissues from single mutants of *phot1-5*, *phot2-1*, and *npq1-2*. However, the stomata did not open in the *phot1-5 phot2-1* double mutant (Fig. 5a), suggesting that phot1 and phot2 are, in fact, the photoreceptors for stomatal opening.

However, it is still possible that the double mutation affected the activity of the plasma membrane H^+-ATPase, .which drives stomatal opening, and results in the prevention of stomatal opening. We are able to rule this possibility out because the amount of the plasma membrane H^+-ATPase did not differ in these plants when determined by immunological methods (Fig 5b). Moreover, the stomata opened widely in the double mutant as well as in other plants when the guard-cell plasma membrane H^+-ATPase was directly activated using the fungal toxin fusicoccin (Fig. 5c). We therefore conclude that the simultaneous impairment of *PHOT1* and *PHOT2* genes resulted in loss of blue light-mediated stomatal opening, probably through a lack of blue light perception.Essentially the same results as above were obtained using the intact *Arabidopsis* plants [6].

Stomatal opening is mediated by a plasma membrane H^+-ATPase, and epidermal strips extrude H^+ in response to blue light via this enzyme. We would therefore predict that epidermal strips taken from an *Arabidopsis phot1 phot 2* double mutant would be unable to respond to blue light in this manner. As expected, the epidermal strips from wild-type plants indeed actively extruded H^+ in response to blue light, while the strips from the double mutant did not (Fig. 5d). However, fusicoccin, an activator of the H^+-ATPase elicited H^+ extrusion in both plants, indicating that the lack of the H^+ extrusion was not due to a lesion in the H^+-ATPase in the double mutant, but because of a lack of blue light signal perception. The H^+ extrusions in response to blue light were similarly observed in single mutants of *phot1*, *phot2* and *npq1*. These H^+ extrusions were inhibited by vanadate, an inhibitor of the H^+-ATPase.

We then investigated the dependency of stomatal opening on blue light fluence, and found a different sensitivity between wild type and the single mutants of *phot1-5* and *phot2-1* (Fig. 6). When blue light of 1 mmol m^{-2} s^{-1} was added to the background red light, no effect of the blue light was observed in any of the plants tested. When the blue light fluence rate was increased to 5 mmol m^{-2} s^{-1}, the stomata opened fully in the wild type tissues, but opened only partially in

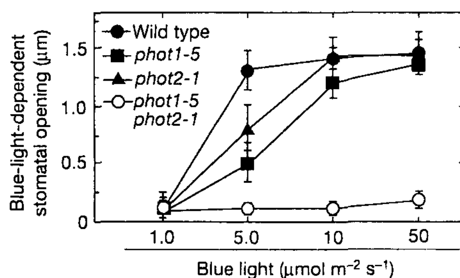

Figure 6. Fluence rate dependency of stomatal opening in response to blue light.

both of the single mutants. In most cases, the stomata in *phot2-1* opened wider than in *phot1-5* under these conditions. Interestingly, no difference in stomatal opening was observed among all plants tested, except in the double mutant under 10 mmol m^{-2} s^{-1} or higher fluence rates (Fig. 6). No blue light-dependent response was observed in the double mutant. These results indicate that the single mutation in the *PHOT1* and *PHOT2* genes decreased the sensitivity to blue light. Taken together, these results suggest that both phot1 and phot2 work simultaneously and redundantly as blue light receptors, at least under low-light intensity conditions (around 5 mmol m^{-2} s^{-1} or less).

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-*To whom correspondence should be addressed.
E-mail: kenrcb@mbox.nc.kyushu-u.ac.jp