Fluence rate dependence of red light-induced phosphorylation of plasma membrane H^+-ATPase in stomatal guard cells

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Stomatal opening is induced by red light as well as blue light. Recently, we established an immunohistochemical technique using whole leaves to study plasma membrane (PM) H^+-ATPase in guard cells, which is an important enzyme driving stomatal opening. Our technique revealed that red light illuminated to whole leaves induces photosynthesis-dependent phosphorylation of C-terminal penultimate residue of PM H^+-ATPase, threonine, in guard cells, which has been considered to be important for activation of PM H^+-ATPase, and we proposed that red light promotes stomatal opening via activation of PM H^+-ATPase in guard cells in whole leaves. Here, using our new immunohistochemical technique, we investigated fluence rate dependence of red light-induced phosphorylation of PM H^+-ATPase. We found that illumination of red light at 50 μmol m^{-2} s^{-1}, which was suggested to initiate photosynthesis, saturates phosphorylation of PM H^+-ATPase. Furthermore, we immunohistochemically confirmed decrease in amount of PM H^+-ATPase protein in a knock-out mutant of *AHA1*, an isogene encoding the major isoform of PM H^+-ATPase in guard cells, implying the importance of AHA1 as the major PM H^+-ATPase protein in guard cells for light-induced stomatal opening.

Keywords: *Arabidopsis thaliana*; red light; photosynthesis; guard cells; immunohistochemistry; phosphorylation; plasma membrane H^+-ATPase

The effect of red-light fluence rate on phosphorylation of plasma membrane H^+-ATPase in guard cells

Stomata consist of pairs of guard cells in the plant epidermis and control gas-exchange in response to various stimuli such as light, namely, red- and blue-light.[1,2] Blue light activates plasma membrane (PM) H^+-ATPase in guard cells via phosphorylation of its C-terminal penultimate residue, threonine, which provides driving force for stomatal opening.[2–7] Thus, disruption of the process of phosphorylation of PM H^+-ATPase results in suppression of stomatal opening.[8–12] Blue light-induced phosphorylation of PM H^+-ATPase is detectable by biochemical analyses using guard cell protoplasts (GCPs)[3,4,13] or

immunohistochemistry using isolated epidermis[14], indicating that it is a guard cell-autonomous reaction. Red light-induced stomatal opening is considered to depend on photosynthesis.[15–18]

Recently, we have established immunohistochemical technique for guard cells using whole leaves of Arabidopsis (*Arabidopsis thaliana*) instead of the isolated epidermis, and this technique allowed us to find red light-induced phosphorylation of the penultimate residue, threonine, of PM H^+-ATPase in guard cells. Our analyses revealed that red light-induced phosphorylation of PM H^+-ATPase correlates with stomatal opening in whole leaves and both are inhibited by photosynthetic electron transport inhibitor DCMU, suggesting that red light promotes stomatal opening in whole leaves via photosynthesis-dependent phosphorylation of PM H^+-ATPase in guard cells.[19]

Here, we applied our new immunohistochemical technique to investigating the effect of red-light fluence rate on phosphorylation of PM H^+-ATPase in guard cells. To this end, whole leaves were kept in the dark or illuminated with red light at 10, 50, 100, 300, and 600 μmol m^{-2} s^{-1} for 30 min and the phosphorylation levels were examined. As a positive control, leaves illuminated with red light (600 μmol m^{-2} s^{-1}) for 30 min and then blue light (5 μmol m^{-2} s^{-1}) superimposed on red light for 2.5 min were also examined. Consistent with our previous study,[19] illumination of red light at 600 μmol m^{-2} s^{-1} and the superimposition of blue light on red light successfully increased the phosphorylation level of PM H^+-ATPase in guard cells by 85% and 248%, respectively, compared to that of leaves kept in the dark (Fig. 1). We found that dim red light (10 μmol m^{-2} s^{-1}) was insufficient to induce phosphorylation of PM H^+-ATPase, and that red light-induced phosphorylation of PM H^+-ATPase was saturated even at 50 μmol m^{-2} s^{-1}, in which the phosphorylation level was comparable to those in leaves illuminated with red light at 100, 300, and 600 μmol m^{-2} s^{-1} (Fig. 1).

Given that almost similar fluence rate of red light appeared to induce photosynthesis and increase in stomatal conductance in the previous gas-exchange measurement,[18] it is suggested that signal(s) mediating red light-induced phosphorylation of PM H^+-ATPase in guard cells can be produced by initiation of photosynthesis in whole leaves. Previous our study suggested that neither sucrose, a primary photosynthetic product synthesized in guard cells and/or translocated from mesophyll to guard cells,[20] nor low CO_2 condition mimicking the reduction of intercellular CO_2 concentration (C_i) in whole leaves caused by photosynthesis induces phosphorylation of PM H^+-ATPase.[19] Therefore, the signal(s) might be other photosynthetic product(s). Alternatively, both reduced C_i and other signal(s) might be involved in red light-induced phosphorylation of PM H^+-ATPase, as CO_2 was indicated to have inhibitory effect on PM H^+-ATPase.[21] Further investigations would be required to verify these possibilities.

The major isoform of PM H^+-ATPase in guard cells

Arabidopsis have 11 isogenes coding PM H^+-ATPase, *AHA1–AHA11*, and the previous analysis using GCPs indicated that all isogenes are transcribed in guard cells.[13] A recent study revealed that *aha1* mutants show suppressed blue light-induced stomatal opening and delayed red light-dependent increase in stomatal conductance, and biochemical analysis using GCPs from *aha1* mutants suggested that AHA1 may be the major isoform of PM H^+-ATPase in guard cells.[6] Previous our study confirmed that red light-induced stomatal opening is delayed in *aha1-9*, a knock-out allele of *AHA1*, indicating that AHA1 is responsible for red light- as well as blue light-induced stomatal opening.[19] For further confirmation of the accumulation of AHA1 in guard cells, we immunohistochemically compared amount of PM H^+-ATPase between wild-type Col-0 and *aha1-9*. The results showed that the detection level of PM H^+-ATPase in guard cells of *aha1-9* decreased to

approximately 1/3 of that of Col-0 (Fig. 2), consistent with the previous study.[6] Taken together with our previous study,[19] these results suggest again that our technique appropriately works and reinforce the idea that AHA1 makes up the majority of PM H^+-ATPase in guard cells, which contributes to light-induced stomatal opening. A remaining question is how AHA1 becomes a major isoform of PM H^+-ATPase in guard cells. Posttranscriptional regulation is suggested to accumulate AHA1 protein in guard cells, as the transcript levels of other isogenes are suggested to be higher than that of *AHA1*.[6] The stability of mRNA of each isogene and/or lifetime of each isoform in guard cells would be future topics to elucidate the mechanism for AHA1 accumulation in guard cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Shimazaki K, Doi M, Assmann SM, Kinoshita T. Light regulation of stomatal movement. Annu Rev Plant Biol. 2007; 58:219–247. doi: 10.1146/annurev.arplant.57.032905.105434

2. Inoue S, Kinoshita T. Blue light regulation of stomatal opening and the plasma membrane H^+-ATPase. Plant Physiol. 2017; 174(2):531–538. doi: 10.1104/pp.17.00166

3. Kinoshita T, Shimazaki K. Blue light activates the plasma membrane H^+-ATPase by phosphorylation of the C-terminus in stomatal guard cells. EMBO J. 1999; 18(20): 5548–5558. doi: 10.1093/emboj/18.20.5548

4. Kinoshita T, Shimazaki K. Biochemical evidence for the requirement of 14-3-3 protein binding in activation of the guard-cell plasma membrane H^+-ATPase by blue light. Plant Cell Physiol. 2002; 43(11):1359–1365. doi: 10.1093/pcp/pcf167

5. Toda Y, Wang, Y, Takahashi A, Kawai Y, Tada Y, Yamaji N, Ma JF, Ashikari M, Kinoshita T. *Oryza sativa* H^+-ATPase (OSA) is involved in the regulation of dumbbell-shaped guard cells of rice. Plant Cell Physiol. 2016; 57(6):1220–1230. doi: 10.1093/pcp/pcw070

6. Yamauchi S, Takemiya A, Sakamoto T, Kurata T, Tsutsumi T, Kinoshita T, Shimazaki K. The plasma membrane H^+-ATPase AHA1 plays a major role in stomatal opening in response to blue light. Plant Physiol. 2016; 171(4):2731–2743. doi: 10.1104/pp.16.01581

7. Jezek M, Blatt MR. The membrane transport system of the guard cell and its integration for stomatal dynamics. Plant Physiol. 2017; 174(2):487–519. doi: 10.1104/pp.16.01949

8. Takemiya A, Kinoshita T, Asanuma M, Shimazaki K. Protein phosphatase 1 positively regulates stomatal opening in response to blue light in *Vicia faba*. Proc Natl Acad Sci U S A. 2006; 103(36):13549–12554. doi: 10.1073/pnas.0602503103

9. Takemiya A, Sugiyama N, Fujimoto H, Tsutsumi T, Yamauchi S, Hiyama A, Tada Y, Christie JM, Shimazaki K. Phosphorylation of BLUS1 kinase by phototropins is a primary step in stomatal opening. Nat Commun. 2013; 4:2094. doi: 10.1038/ncomms3094

10. Takemiya A, Yamauchi S, Yano T, Ariyoshi C, Shimazaki K. Identification of a regulatory subunit of protein phosphatase 1 which mediates blue light signaling for stomatal opening. Plant Cell Physiol. 2013; 54(1):24–35. doi: 10.1093/pcp/pcs073

11. Hayashi M, Inoue S, Ueno Y, Kinoshita T. A Raf-like protein kinase BHP mediates blue light-dependent stomatal opening. Sci Rep. 2017; 7:45586. doi: 10.1038/srep45586

12. Toh S, Inoue S, Toda Y, Yuki T, Suzuki K, Hamamoto S, Fukatsu K, Aoki S, Uchida M, Asai E, *et al.* Identification and characterization of compounds that affect stomatal movements. Plant Cell Physiol. 2018; 59(8):1568–1580. doi: 10.1093/pcp/pcy061

13. Ueno K, Kinoshita T, Inoue S, Emi T, Shimazaki K. Biochemical characterization of plasma membrane H^+-ATPase activation in guard cell protoplasts of *Arabidopsis thaliana* in response to blue light. Plant Cell Physiol. 2005; 46(6):955–963. doi: 10.1093/pcp/pci104

14. Hayashi M, Inoue S, Takahashi K, Kinoshita T. Immunohistochemical detection of blue light-induced phosphorylation of the plasma membrane H^+-ATPase in stomatal guard cell. Plant Cell Physiol. 2011; 52(7):1238–1248. doi: 10.1093/pcp/pcr072

15. Sharkey TD, Raschke K. Effect of light quality on stomatal opening in leaves of *Xanthium strumarium* L. Plant Physiol. 1981; 68(5):1170–1174. doi: 10.1104/pp.68.5.1170

16. Doi M, Shimazaki K. The stomata of the fern *Adiantum capillus-veneris* do not respond to CO_2 in the dark and open by photosynthesis in guard cells. Plant Physiol. 2008; 147(2):922–930. doi: 10.1104/pp.108.118950

17. Wang Y, Noguchi K, Terashima I. Photosynthesis-dependent and -independent responses of stomata to blue, red and green monochromatic light: differences between the normally oriented and inverted leaves of sunflower. Plant Cell Physiol. 2011; 52(3):479–489. doi: 10.1093/pcp/pcr005

18. Suetsugu N, Takami T, Ebisu Y, Watanabe H, Iiboshi C, Doi M, Shimazaki K. Guard cell chloroplasts are essential for blue light-dependent stomatal opening in Arabidopsis. PLoS One. 2014; 9(9):e108374. doi: 10.1371/journal.pone.0108374

19. Ando E, Kinoshita T. Red light-induced phosphorylation of plasma membrane H^+-ATPase in stomatal guard cells. Plant Physiol. 2018; In Press. doi: 10.1104/pp.18.00544

20. Daloso DM, Dos Anjos L, Fernie AR. Roles of sucrose in guard cell regulation. New Phytol. 2016; 211(3):809–818. doi: 10.1111/nph.13950

21. Edwards A, Bowling DJF. Evidence for a CO_2 inhibited proton extrusion pump in the stomatal cells of *Tradescantia virginiana*. J Exp Bot. 1985; 36(1):91–98. doi: 10.1093/jxb/36.1.91

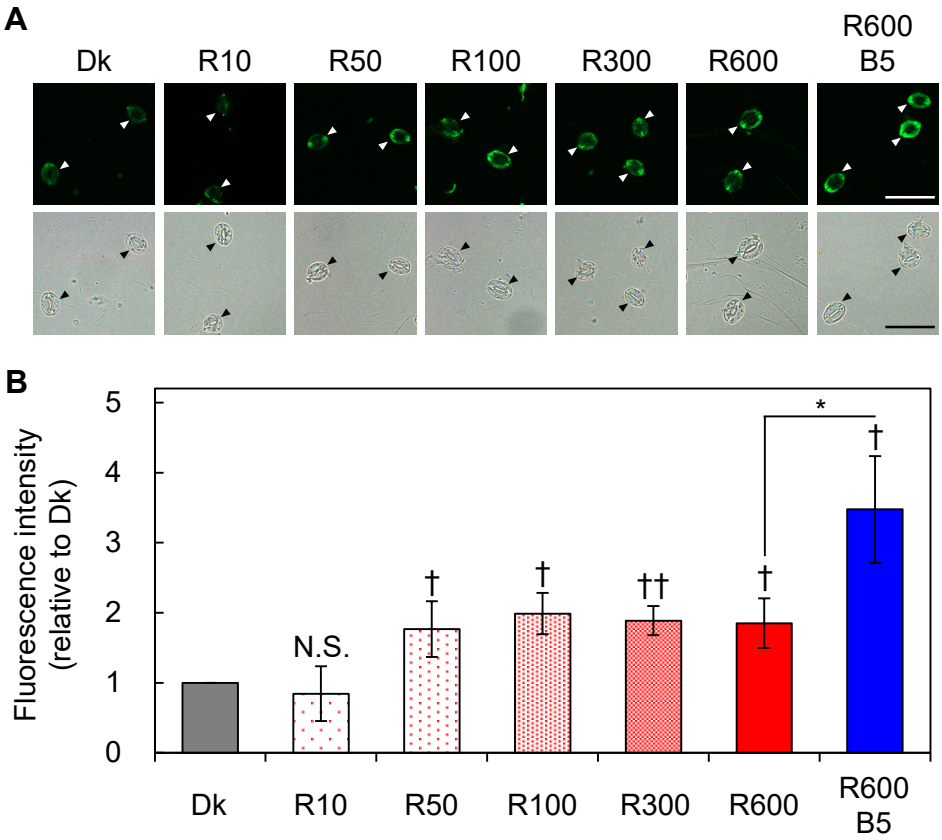

Figure 1. Fluence rate dependence of red light-induced phosphorylation of PM H^+-ATPase in guard cells. Mature leaves were harvested from dark-adapted plants and kept in the dark (Dk) or illuminated with red light at 10, 50, 100, 300, and 600 μmol m^{-2} s^{-1} (R10, R50, R100, R300, and R600, respectively) for 30 min. As a positive control, leaves illuminated with red light (600 μmol m^{-2} s^{-1}) for 30 min followed by blue light (5 μmol m^{-2} s^{-1}) superimposed on red light for 2.5 min (R600 B5) were examined. (A) Typical fluorescent (upper panel) and bright field (lower panel) images are shown. Arrowheads indicate guard cells. Scale bars represent 50 μm. (B) Quantification of the fluorescence intensities were calculated as previously described[19]. Data represent means of relative fluorescence intensities obtained from three biologically independent measurements with standard deviations (SDs). Daggers indicate that the mean is statistically significantly higher than Dk set to 1.0. N.S., not significant (one-tailed one-sample *t* test; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, N.S., $P = 0.72$). Asterisk indicates that blue light-induced increase in the phosphorylation level of PM H^+-ATPase is statistically significant (one-tailed Student's *t* test; $^{*}P < 0.05$).

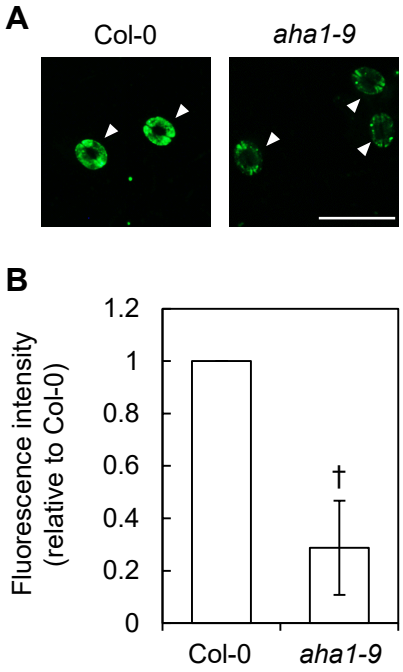

Figure 2. Immunohistochemical detection of guard-cell PM H^+-ATPase in wild-type Col-0 and *aha1-9*. (A) Typical fluorescent images are shown. Arrowheads indicate guard cells. Scale bar represents 50 μm. (B) Quantification of the fluorescence intensities were calculated as previously described.[19] Data represent a mean of relative fluorescence intensity obtained from three biologically independent measurements with SD. Dagger indicates that the mean is statistically significantly lower than Col-0 set to 1.0. (one-tailed one-sample *t* test; $^{\dagger}P < 0.05$).