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Phosphorylation, 14-3-3 protein and photoreceptor in blue light response of stomatal guard cells

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Blue light (BL) induces stomatal opening through activation of H^+ pump, which creates electrical gradient across the plasma membrane for K^+ uptake into guard cells. The pump is the plasma membrane H^+-ATPase and is activated via phosphorylation of the C-terminus with concomitant binding of the 14-3-3 protein. The opening is initiated by the perception of BL through phototropin (phot), which are recently identified as BL receptors in stomatal guard cells. In this study, we provide the biochemical evidence for phots as BL receptors in stomatal guard cells. vfphot was phosphorylated reversibly by BL, and phosphorylation levels of vfphot increased earlier than those of the plasma membrane H^+-ATPase. BL-dependent phosphorylations of vfphot and H^+-ATPase showed similar fluence dependency. Staurosporin, an inhibitor of serine/threonine protein kinase, and diphenyleneiodonium chloride (DPI), an inhibitor of flavoprotein, inhibited BL-dependent phosphorylations of vfphot and H^+-ATPase. These results indicate that vfphot acts as a BL-receptor mediating stomatal opening.

Key words: blue light, guard cell, phototropin, H^+-ATPase, phosporylation, 14-3-3 protein

INTRODUCTION

Blue light (BL) induces stomatal opening in higher plants, and the opening is initiated by the perception of BL through phototropins 1 (phot1) and 2 (phot2) [1], which are recently identified as BL receptors in stomatal guard cells [2]. The opening is mediated by an accumulation of K^+-salt in guard cells, and K^+ uptake is driven by an inside-negative, electrical potential across the plasma membrane [3, 4]. This electrical potential is created by BL-activated H^+ pump in the plasma membrane [5, 6]. Recent investigation has demonstrated that the H^+ pump is the plasma membrane H^+-ATPase, and that BL activates the H^+-ATPase through phosphorylation of serine and threonine residues in the C-terminus with concomitant binding of 14-3-3 protein to C-terminus [7]. An isoform vf14-3-3a specifically bound to the H^+-ATPase in *Vicia* guard cells *in vivo*, although guard cells expressed at least four isoforms of 14-3-3a, b, c, and d [8].

phot1 and phot2 in *Arabidopsis* mediate phototropism, chloroplast relocation, and stomatal opening as BL-receptors [1]. phot has two LOV domains in the N-terminus and a serine/threonine protein kinase domain in the C-terminus. A flavin mononucleotide (FMN) binds with the LOV domain as a chromophore. phot1 and phot2 in the etiolated seedling are autophosphorylated in response to BL *in vivo* and *in vitro*, suggesting that phosphorylation might be required for physiological responses. However, phosphorylation status of phot in the stomatal guard cells has not been investigated.

In the present study, we investigated *in vivo* phosphorylation status of phot in reponse to BL in *Vicia* guard cell protoplasts (GCPs). We concluded that phot acts as a BL receptor in stomatal guard cells.

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MATERIALS AND METHODS

Plant materials

Vicia faba (cv. Ryosai Issun) was cultured hydroponically in a green house as described previously [7]. GCPs were isolated enzymatically from the lower epidermis of 4- to 8-week-old leaves of *Vicia faba* according to the method described previously [7].

Determination of phosphorylation levels of vfphot and the plasma membrane H^+-ATPase

Phosphorylation levels of vfphot and the H^+-ATPase were determined by immunoprecipitation

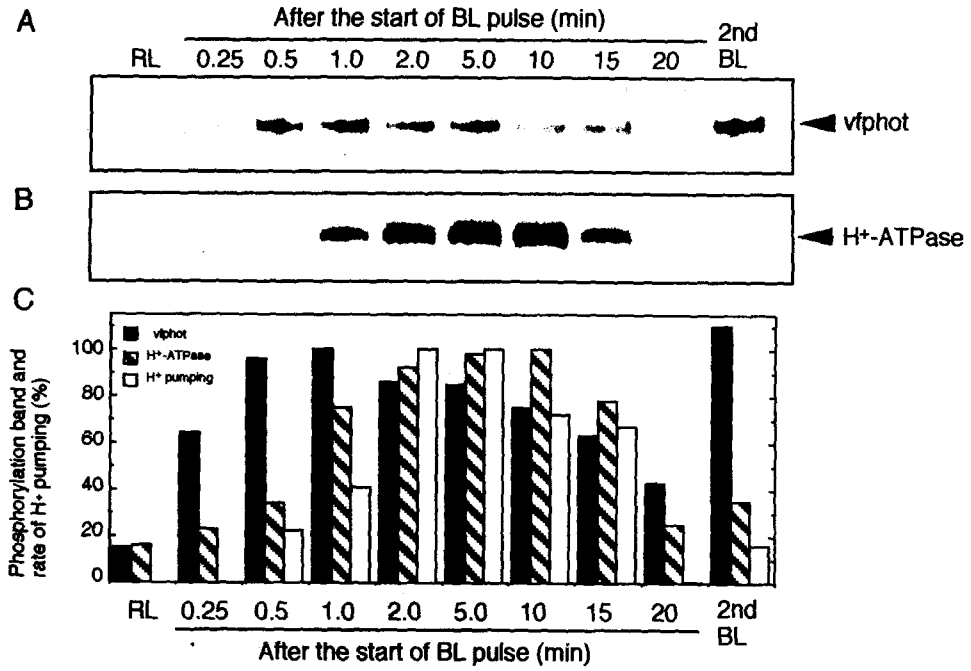

Figure 1 Changes in the phosphorylation levels of the vfphot and plasma membrane H^+-ATPase in GCPs in response to BL. (A) Autoradiogram of immunoprecipitated vfphot. (B) Autoradiogram of immunoprecipitated H^+-ATPase. (C) Rate of H^+ pumping and relative densities of phosphorylated vfphot and H^+-ATPase.

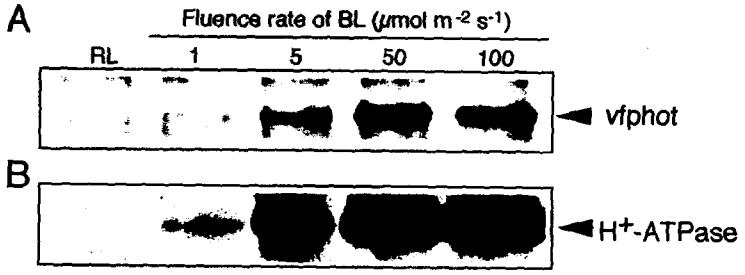

Figure 2 Fluence dependency of BL-dependent phosphorylations of vfphot and H^+-ATPase in GCPs (A) Autoradiogram of immunoprecipitated vfphot. (B) Autoradiogram of immunoprecipitated H^+-ATPase.

from ^{32}P-labeled GCPs as described previously [7].

Polyclonal antibodies

The antibodies raised against VHA1 were described previously [7]. The conserved region between LOV2 and kinase domain of *Arabidopsis* phot1 was amplified by PCR using cDNA from *Vicia* GCPs. The resulting amplified fragment was cloned into the *Bam*HI site of pGEX-2T and purified recombinant protein used as antigen for vfphot antibodies. vfphot antibodies recognized both vfphot1 and vfphot2, which are isoforms expressed in *Vicia* GCPs.

RESULTS AND DISCUSSION

To determine *in vivo* phosporylation levels of the vfphot and the plasma membrane H^+-ATPase, GCPs were incubated with ^{32}P-orthophosphate, and vfphot and H^+-ATPase were immunoprecipitated using specific antibodies. Autoradiograms reveal that

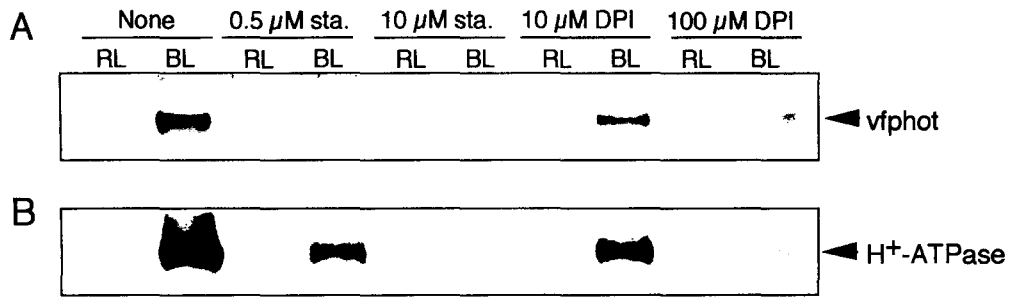

Figure 3 Effect of inhibitors of a protein kinase inhibitor, staurosporin, and flavoprotein, DPI, on BL-dependent phosphorylations of vfphot (A) and H^+-ATPase (B).

phosphorylation level of vfphot was increased by BL (Fig. 1A), reached a maximum 1.0 min after the BL pulse, and decreased gradually. When the second pulse of BL was applied 20 min after the first, vfphot was rephosphorylated. In contrast, phosphorylation level of the H^+-ATPase and rate of H^+ pumping was reached maximun around 2.0 min after the BL pulse (Fig. 1B, C). The results indicate that phosphorylation of vfphot is earlier than that of H^+-ATPase.

If phot acts as BL-receptor in stomatal guard cells, it is expected that phosphorylations of vfphot and H^+-ATPase show similar fluence dependency. To show this, we investigated phosphorylation levels of vfphot and H^+-ATPase as a function of BL fluence rate. BL-dependent phosphorylations of vfphot and H^+-ATPase showed similar fluence dependency (Fig. 2). Half-saturation of BL was 7.5 $\mu mol/m^2/sec$ when the pulse duration was 30 sec. BL-dependent H^+ pumping also showed similar fluence dependency (data not shown).

The phsophorylations of vfphot and H^+-ATPase were inhibited by staurosporin, an inhibitor of protein kinase, in a-concentration dependent manner (Fig. 3). DPI, an inhibitor of flavoprotein, also inhibited BL-dependent phosphorylations of vfphots and H^+-ATPase in a-concentration dependent manner. These inhibitors similarly inhibited BL-dependent H^+ pumiping (data not shown).

Phosphorylation of phot might be required for physiological responses [1]. In accord with this, our results showed that inhibition of phosphorylation of phot suppressed activation of the H^+-ATPase. From these results, we conclude that phot acts as a BL-receptor in the stomatal opening, and that phosphorylation of phot is required for activation of the H^+-ATPase..

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