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Blue-light-induced rapid chloroplast de-anchoring in *Vallisneria* epidermal cells

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Abstract In the outer periclinal cytoplasm of leaf epidermal cells of an aquatic angiosperm *Vallisneria*, blue light induces "chloroplast de-anchoring", a rapid decline in the resistance of chloroplasts against centrifugal force. Chloroplast de-anchoring is known induced within 1 min of irradiation with high-fluence-rate blue light specifically, preceding the commencement of chloroplasts migration toward the anticlinal cytoplasm. However, its regulatory mechanism has remained elusive, although pharmacological analysis suggested that a calcium release from intracellular calcium stores is necessary for the response. In search of the responsible photoreceptors, immunoblotting analysis using antibodies against phototropins demonstrated that cross-reactive polypeptides of 120-kDa exist in the plasma-membrane fraction prepared from the leaves. *In vitro* phosphorylation analysis revealed that 120-kDa polypeptides were phosphorylated by exposure to blue light in a fluence-dependent manner. The blue-light-induced phosphorylation activity was sensitive to a Ser/Thr kinase inhibitor, staurosporine, and unusually was retained at a high level for a long time in darkness. Furthermore, phototropin gene homologs (*Vallisneria PHOTOTROPIN1* and *PHOTOTROPIN2*) expressed in leaves were isolated. We propose that calcium-regulated chloroplast de-anchoring, possibly mediated by phototropins, is an initial process of the blue-light-induced avoidance response of chloroplasts in *Vallisneria*.

Keywords: Avoidance response of chloroplasts; blue-light response; chloroplast de-anchoring; cytoplasmic calcium; phototropin
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Research Article

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INTRODUCTION

In plant cells, organelles such as nuclei, mitochondria, chloroplasts, Golgi apparatus, and peroxisomes change their distribution patterns with the progression of cell cycle, cell growth and development, and also in response to a variety of environmental stimuli (Wada and Suetsugu 2004; Logan 2010; Takagi et al. 2011; Banaś et al. 2012). Regulation of distribution patterns of organelles is an important process for plants, which cannot move from their habitat, to adapt themselves to rapidly fluctuating environmental conditions. Chloroplasts are known to maintain advantageous distribution patterns under different conditions, and the redistribution of chloroplasts is accomplished through regulation of their motility (Wada et al. 2003; Takagi et al. 2009). Upon environmental stimuli, previously motionless chloroplasts become mobile, and relocate within the cell. Though the actin-based machinery drives chloroplast movement (Banaś et al. 2012; Higa and Wada 2015), many aspects in regulatory mechanism for chloroplast redistribution are still elusive.

In a monocot, *Lemna trisulca*, and in dicots, *Nicotiana tabacum*, and *Arabidopsis thaliana*, light-induced chloroplast relocation appears to be regulated by cytoplasmic calcium concentration (Tlalka and Fricker 1999; Anielska-Mazur et al. 2009; Aggarwal et al. 2013). On the other hand, in a fern, *Adiantum capillus-veneris*, a calcium influx through the plasma membrane seems necessary to induce chloroplast movement in response to mechanical stimulation, but not in response to light stimulation (Sato et al. 2001). More recently, Takamatsu and Takagi (2011) demonstrated that treatment with an actin-depolymerizing reagent or calcium over 1 μmol/L induced chloroplast detachment from plasma-membrane ghosts of spinach mesophyll cells, on which the cortical cytoplasm underlying the plasma membrane was exposed, and proposed that calcium-mediated "chloroplast de-anchoring" could be an initial process in environmentally induced chloroplast redistribution. Up to now, however, how the anchoring state of chloroplasts is modulated by environmental stimuli and whether calcium is involved in such modulation in living cells has been unclear.

Blue light is known to induce a transient increase in cytoplasmic calcium concentration (Harada and Shimazaki 2007). In *A. thaliana*, phototropins mediate a blue-light-induced intracellular calcium transient (Baum et al. 1999; Harada et al. 2003; Chen et al. 2008; Zhao et al. 2013). Phototropins are plant-specific UV-A and blue-light photoreceptors. *A. thaliana* harbors two isoforms of phototropin, phototropin1 and photortopin2, which mediate various blue-light responses, such as phototropism, stomatal opening, leaf flattening, and chloroplast relocation movement (Briggs and Christie 2002; Christie 2007). Among these responses, the avoidance response of chloroplasts is regulated specifically by phototropin2 (Kagawa et al. 2001; Sakai et al. 2001). It is also a characteristic feature of phototropins to mediate rapid

responses without transcriptional regulation, such as phosphorylation of the plasma-membrane H^+-ATPase in guard cells (Kinoshita et al. 2003), calcium influx through voltage-dependent calcium permeable channels on the plasma membrane of mesophyll protoplasts (Stoelzle et al. 2003), and induction of an intracellular calcium transient in mesophyll cells (Harada and Shimazaki 2009). Furthermore, Aggarwal et al. (2013) recently suggested the involvement of phosphoinositide-modulated calcium signaling in phototropin-regulated chloroplast relocation movement.

Phototropins absorb blue light at their two light, oxygen, or voltage domains (LOV domains) using flavin mononucleotides as the chromophore (Christie et al. 1999; Sakai et al. 2001; Swartz et al. 2001; Kasahara et al. 2002). Upon blue light absorption, phototropins are auto-phosphorylated by their own kinase activity raised from the C-terminal Ser/Thr kinase domain (Reymond et al. 1992; Inoue et al. 2008, 2010). The importance of phosphorylation of phototropins in blue-light signaling was first suggested by biochemical studies in *Zea mays* (Palmer et al. 1993a) and *Avena sativa* (Salomon et al. 1997) on coleoptile phototropism, and then proven by genetic studies in *A. thaliana* on the blue-light responses of phototropism, stomatal opening, leaf flattening, and chloroplast relocation movement (Inoue et al. 2008).

The leaf epidermal cells of an aquatic monocot, *Vallisneria* (Alismatales Hydrocharitaceae), are rectangular parallelepiped-shaped and aligned orderly in a monolayer, serving as an excellent experimental system for microscopic studies. Chloroplasts in the epidermal cells exhibit light-dependent redistribution, whereas the mode of movement of these chloroplasts appears to differ from that demonstrated in a moss (Yamashita et al. 2011), a fern (Tsuboi and Wada 2012), and in *A. thaliana* (Kadota et al. 2009). In those plants, chloroplasts exhibit phototactic movement driven by the conspicuous short actin filaments associated with their outer envelope (Kong and Wada 2014). On the contrary, as recognized for more than a century, chloroplasts in the Hydrocharitaceae, *Elodea*, and *Vallisneria*, seem to move only passively together with mobile cytoplasm (Senn 1908; Seitz 1964, 1967). In such cases, regulation of chloroplast anchoring should be more important to precisely determine the motility of chloroplasts at appropriate regions, than in the case of terrestrial plants, in which the motility of each chloroplast is regulated independently of cytoplasmic streaming.

In *Vallisneria* epidermal cells, the accumulation of chloroplasts into the outer periclinal cytoplasm is induced effectively by low-fluence-rate red light (Izutani et al. 1990; Dong et al. 1995). Under low-fluence-rate light, chloroplasts gain motility in a red/far-red light reversible manner, and subsequently become anchored depending on the operation of photosynthesis (Dong et al. 1996, 1998). On the other hand, the avoidance response is induced by high-fluence-rate blue light (Izutani et al. 1990) and is insensitive to the inhibitors of photosynthetic electron transport (Sakai and Takagi 2005). Nevertheless, in the redistribution process under high-fluence-rate blue light, chloroplasts, once they have migrated from the outer periclinal cytoplasm, are anchored in the anticlinal cytoplasm in a photosynthesis-dependent manner (Sakai and Takagi 2005). Consequently, in *Vallisneria* epidermal cells, multiple photosensory systems; photosynthesis, phytochromes, and unidentified blue-light receptors, work coordinately to regulate chloroplast distribution patterns under a range of light conditions.

Although several physiological studies have predicted that phototropin mediates the blue-light-induced chloroplast avoidance response (Izutani et al. 1990; Sakurai et al. 2005) and chloroplast de-anchoring (Sakai and Takagi 2005) in *Vallisneria*, molecular evidence for the presence of phototropins has been lacking. In this study, by applying a centrifugation method to living epidermal cells, we attempted to verify that chloroplast de-anchoring is the initial process of the chloroplast avoidance response. We further implicate the involvement of calcium released from intracellular stores in the process, and demonstrate the presence of *Vallisneira* phototropins.

RESULTS

Blue-light-induced chloroplast de-anchoring in *Vallisneria* epidermal cells

In dark-adapted epidermal cells of *Vallisneria*, the outer periclinal cytoplasm is occupied by a large number of chloroplasts that are apparently motionless. The number of chloroplasts located in the outer periclinal cytoplasm starts to decrease within several minutes of continuous irradiation with blue light over 10 μmol/m^2 per s (Izutani et al. 1990; Sakurai et al. 2005). Here, we evaluated the anchorage of chloroplasts based on resistance to centrifugation (Sakai and Takagi 2005). We divided the number of chloroplasts visible in the centripetal half of the cell (Np) by the total number of chloroplasts in the outer periclinal cytoplasm (Nt) and define this as the coverage ratio (Np/Nt). When chloroplasts are fully anchored, as they are in the dark, the ratio equals about 0.5, but it is much lower when the chloroplasts are de-anchored and hence pelleted by the centrifuge (Figure 1A). Although it has been demonstrated that chloroplast de-anchoring could be detected within one minute of blue-light irradiation using a centrifuge microscope of the stroboscopic type (Takagi et al. 1991), precise regulation of the total fluence and duration of actinic irradiation was difficult in that experimental system. Consequently, here we took pains to overcome these limitations to characterize this rapid response.

When dark-adapted cells were centrifuged for 5 min at various centrifugal forces, the coverage ratio was maintained at around 0.5 for forces below 680 *g*, but became slightly lower at 1,160 *g* (0.42 $\pm$ 0.01) (Figure 1B). On the other hand, even at a lower centrifugal force of 416 *g*, the coverage ratio markedly declined after irradiation with high-fluence-rate blue light, an effect that was clear after even 5 min of irradiation (Figure 1B). These results suggest that chloroplasts are anchored in the dark-adapted cells, whereas they become de-anchored within 5 min of blue-light irradiation. To know whether chloroplast de-anchoring precedes a decrease in the number of chloroplasts located in the outer periclinal cytoplasm, we examined the time courses of these two responses. First, we evaluated the coverage ratio at 680 *g* immediately after the termination of blue-light irradiation (70 μmol/m^2 per s; light-emitting diodes) for different durations. Blue-light irradiation for 30 s slightly decreased the coverage ratio, whereas blue-light irradiation for 60 s and up to 300 s induced larger and significant

decreases in the coverage ratio (Figure 1C). Next, we examined the kinetics of the chloroplast avoidance response, without centrifugation, by irradiating leaves directly on the stage of the microscope with blue light (80 μmol/m^2 per s). A decrease in the number of chloroplasts in the outer periclinal cytoplasm was only detectable 100–115 s after the start of blue-light irradiation (Figure 1E), which was substantially longer than needed for blue light to evoke de-anchoring. These data imply that chloroplast de-anchoring precedes the directed motility phase of the avoidance response.

We further evaluated the coverage ratio at 680 *g* after irradiation with blue light of various fluence rates. The obtained values were plotted against the total incident fluence in Figure 1D. After 1 min irradiation with blue light (470 nm)

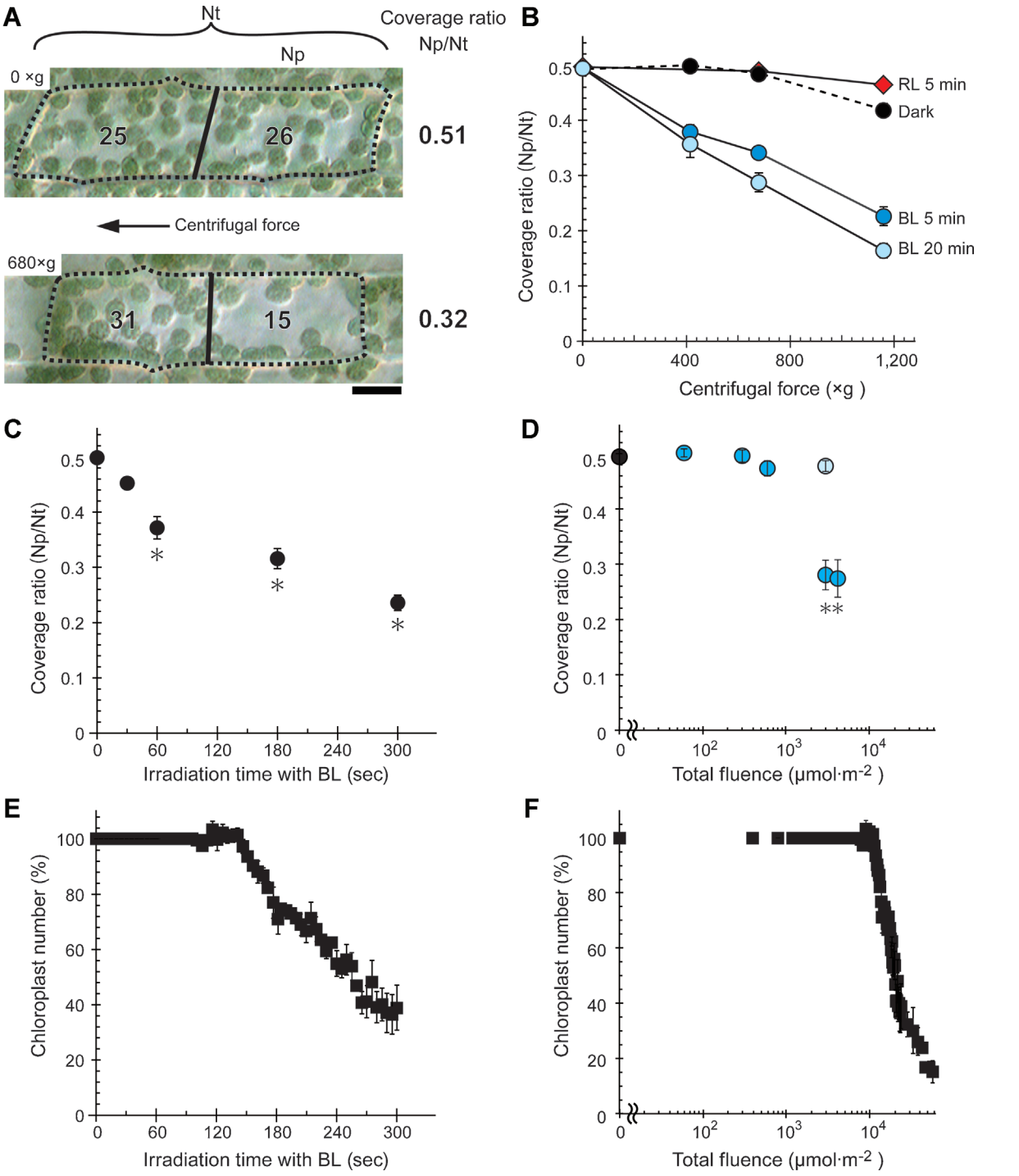

Figure 1. Continued.

below 5 μmol/m^2 per s, the coverage ratio did not change from 0.5. Similarly, longer irradiation with blue light of 5 μmol/m^2 per s for 10 min hardly decreased the coverage ratio. On the other hand, although the total incident fluence (3,000 μmol/m^2) was identical, 1 min irradiation with blue light of a higher fluence rate 50 μmol/m^2 per s remarkably decreased the coverage ratio (Figure 1D). In contrast, when we plotted the fluence dependence of the blue-light-induced decrease in the relative number of chloroplasts in the outer periclinal cytoplasm observed without centrifugation (Figure 1F), the inductive fluence range was about half an order of magnitude larger than in the centrifugation assay, again consistent with our proposal that chloroplast de-anchoring is a prerequisite for the chloroplast avoidance response.

Irradiation with red light of 70 μmol/m^2 per s for 5 min exerted little if any affect on the coverage ratio against the centrifugal force (Figure 1B). In addition, even when the cells were treated with an inhibitor of photosynthetic electron transport, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the fluence dependence of blue-light-induced chloroplast de-anchoring was indistinguishable from that in the untreated cells (Figure S1). These results strongly suggest that chloroplast de-anchoring in *Vallisneria* epidermal cells is a rapid response specifically regulated by blue-light receptors.

Effects of intracellular calcium-transient inhibitors on blue-light-induced chloroplast de-anchoring

Regulatory roles of calcium have been suggested for chloroplast photorelocation (Tlalka and Fricker 1999; Anielska-Mazur et al. 2009; Aggarwal et al. 2013). To elucidate the possible involvement of calcium in blue-light-induced chloroplast de-anchoring, we examined the effects of reagents used to inhibit calcium influx into the cytoplasm from different calcium stores. Lanthanum was used to block plasma membrane calcium channels (Takata et al. 1966; Takagi and Nagai 1985) and 3,4,5-trimethoxybenzoate 8-(*N*,*N*-diethyl amino) octylester (TMB-8) was used to block phospholipase C-mediated calcium transient (Poutrain et al. 2009). After irradiation with blue light of 70 μmol/m^2 per s for 1 min, the coverage ratio was evaluated at different centrifugal forces (Figure 2A). In the lanthanum-treated cells, the coverage ratio was not significantly different from the untreated cells at every centrifugal force. On the contrary, in the TMB-8-treated cells, the coverage ratio at centrifugal force over 680 *g* was significantly higher than that in the untreated cells. Consequently, blue-light-induced chloroplast de-anchoring is sensitive to the inhibitor for phospholipase C-mediated calcium release from intracellular stores, but not to the plasma-membrane calcium-channel blocker. As predicted, TMB-8 substantially delayed the start of blue-light-induced avoidance response of chloroplasts, while lanthanum did not (Figure 2B). Another inhibitor of phospholipase C, neomycin, also suppressed chloroplast avoidance (Figure S2).

120-kDa polypeptides on the plasma membrane cross-reacted with anti-phototropin antibodies

Since physiological characterization of blue-light-induced chloroplast de-anchoring suggested the involvement of blue light receptor phototropins, we attempted to demonstrate the presence of phototropin in *Vallisneria*. First, we carried out immunoblotting analysis using polyclonal antibodies against *A. thaliana* phototropin proteins. Although the antibodies were designed against 425 amino acids (Gln_{426}-Glu_{678}) in the central region of AtPHOT2, the antibodies cross-react with both phototropin1 and phototropoin2 in *A. thaliana*, which can be distinguished as two different bands separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Takemiya et al. 2005). Among the total proteins in a

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Figure 1. Blue-light-induced chloroplast de-anchoring in *Vallisneria* epidermal cells

(**A**) Chloroplast distribution in the outer periclinal cytoplasm of blue-light-irradiated cells before (upper image) and after (lower image) centrifugation. Immediately after the termination of irradiation with blue light (470 nm) of 80 μmol/m^2 per s for 5 min, cells were centrifuged at 680 *g* for 5 min in the direction indicated by a black arrow. The ratio of the number of chloroplasts in the centripetal half (Np; 26 for the upper cell and 15 for the lower cell) to the total number of chloroplasts (Nt; 51 for the upper cell and 46 for the lower cell) in each cell was calculated to be the coverage ratio (Np/Nt; 0.51 for the upper cell and 0.32 for the lower cell). For details, see Materials and Methods. Scale bar = 10 μm. (**B**) Effects of centrifugal force on the coverage ratio Np/Nt obtained in dark-adapted and blue-light- or red-light-irradiated cells. Dark-adapted cells (dark: black circles) were irradiated with blue light of 80 μmol/m^2 per s for 5 min (BL 5 min: blue circles) and 20 min (BL 20 min: light-blue circles) or with red light of 80 μmol/m^2 per s for 5 min (RL 5 min: red diamonds). After centrifugation for 5 min at different centrifugal forces, the coverage ratio (Np/Nt) was calculated and plotted against the centrifugal force. Bars indicate standard error. $n = 36–52$. (**C**) Time course of blue-light-induced chloroplast de-anchoring. Dark-adapted cells were irradiated with blue light (BL; 470 nm) of 70 μmol/m^2 per s for different periods of time. The coverage ratio obtained after centrifugation at 680*g* for 5 min was plotted against the irradiation time. Bars on the symbols indicate standard error. Asterisks denote significant differences from dark-adapted cells ($P < 0.01$ with Student's *t*-test). $n = 40–59$. (**D**) Fluence dependence of blue-light-induced chloroplast de-anchoring. Dark-adapted cells were irradiated with blue light of different fluence rates for 1 min (blue circles) or blue light of 5 μmol/m^2 per s for 10 min (light blue circle). The coverage ratio (Np/Nt) obtained after centrifugation at 680*g* for 5 min was plotted against the total incident fluence. Bars indicate standard error. Asterisks denote significant differences from dark-adapted cells ($P < 0.01$ with Student's *t*-test). $n = 36–54$. (**E**) Time course of blue-light-induced chloroplast avoidance response. Dark-adapted cells were continuously irradiated with blue light (BL; 488 nm) of 80 μmol/m^2 per s on the stage of an epi-fluorescence microscope. At different time points, the number of chloroplasts located in the outer-periclinal cytoplasm of each cell was counted and plotted as percentages of those immediately before the start of blue-light irradiation. Bars indicate standard error. $n = 3$. (**F**) Fluence dependence of blue-light-induced chloroplast avoidance response. From Figure 1E, the relative number of chloroplasts located in the outer-periclinal cytoplasm was replotted against the total incident fluence of blue light. Bars indicate standard errors.

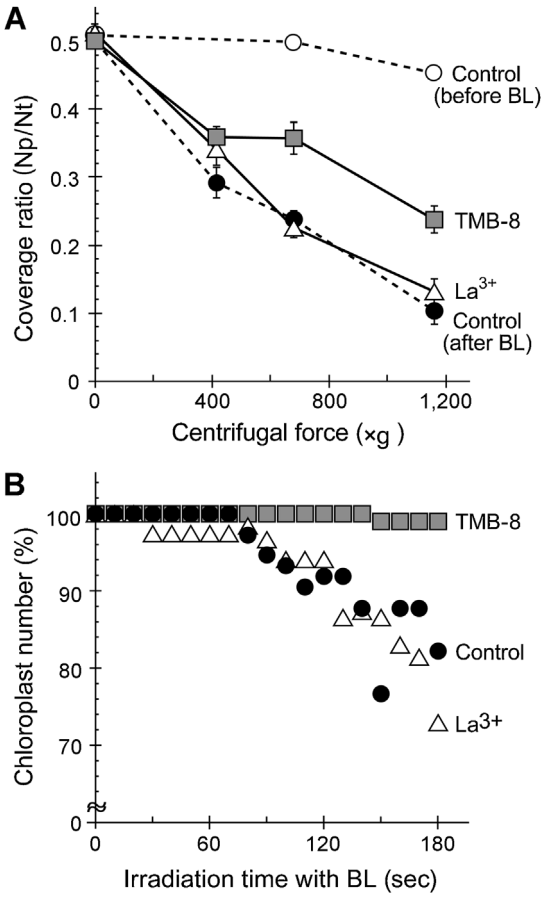

Figure 2. Effects of the inhibitors for cytoplasmic calcium transient on the blue-light-induced chloroplast de-anchoring and avoidance response in *Vallisneria* epidermal cells
(**A**) Dark-adapted cells treated with 100 μmol/L lanthanum (La^{3+}; open triangles) or 1 mmol/L TMB-8 (TMB-8; gray squares) were irradiated with blue light (470 nm) at 70 μmol/m^2 per s for 1 min. After centrifugation for 5 min at different centrifugal forces, the coverage ratio (Np/Nt) was calculated and plotted against the centrifugal force together with the values obtained in the untreated cells before (open circles) and after (closed circles) blue-light irradiation. Bars indicate standard errors. Asterisks denote significant differences from the blue-light-irradiated, untreated cells (closed circles) at the same centrifugal force ($P < 0.01$ with Student's *t*-test). $n = 39–51$. (**B**) Dark-adapted cells treated with 100 μmol/L lanthanum (La^{3+}; open triangles) or 200 μmol/L TMB-8 (TMB-8; gray squares) were continuously irradiated with blue light (488 nm) at 80 μmol/m^2 per s on the stage of an epi-fluorescence microscope. Optical images of the cells were sequentially captured with a time-lapse imaging system. The number of chloroplasts located in the outer periclinal cytoplasm was counted at 10-s intervals and plotted as the percentage against the irradiation time with blue light (BL). The experiments were repeated three times, and the results obtained in each representative cell are shown.

plasma-membrane-enriched fraction prepared from dark-adapted *Vallisneria* leaves, the antibodies detected a single band of 120-kDa polypeptides (Figure 3). The size of the polypeptides was similar to that of phototropin1 (120 kDa) rather than phototropin2 (110 kDa) of *A. thaliana* (Huala et al. 1997; Sakai et al. 2001). Even when larger amounts of total proteins were loaded, no other bands were detected. We suggest that the detected 120-kDa polypeptides are *Vallisneria* phototropins localized on the plasma membrane.

Blue-light-induced phosphorylation of 120-kDa polypeptides
We next asked whether the 120-kDa polypeptides are phosphorylated by blue light irradiation, by using a fiberscope as the light source. In both isolated plasma membrane fractions and microsomal fractions, the 120-kDa polypeptide was the only component phosphorylated in a blue-light-dependent manner (Figure 4). Phosphorylation of 55-kDa polypeptides was also detected, but it was independent of blue-light irradiation. To investigate a fluence-response relationship in the blue-light-induced phosphorylation of 120-kDa polypeptides, the microsomal fraction was irradiated for 30 s with blue light of various fluence rates. The response was detectable at 1 μmol/m^2 per s, suggesting the involvement of phototropin1-type proteins. The phosphorylation level of 120-kDa polypeptides increased with the blue-light fluence rate, with the response almost saturated at 50 μmol/m^2 per s (Figure 5A). Red light irradiation at 100 μmol/m^2 per s for 30 s did not induce detectable phosphorylation of the 120-kDa polypeptides (Figure 5A).

To elucidate whether high-fluence blue light can induce phosphorylation of other polypeptides, aiming to explore the

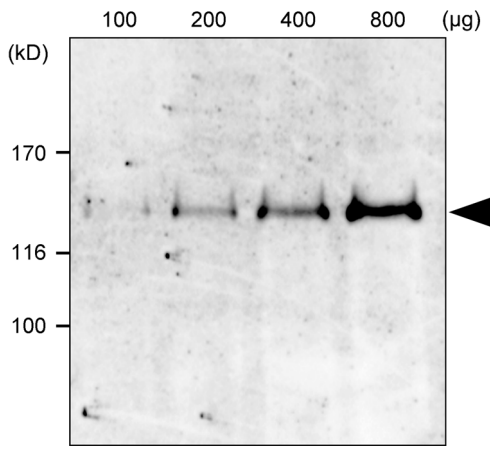

Figure 3. Immunoblotting of *Vallisneria* plasma-membrane fraction using the anti-phototropin antibodies
A plasma-membrane fraction prepared from the dark-adapted leaves was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using the polyclonal antibodies against *A. thaliana* phototropins. Each lane contains 100, 200, 400, and 800 μg of protein, respectively. An arrowhead indicates the position of 120-kDa polypeptides. The molecular masses of standard proteins are indicated on the left in kDa.

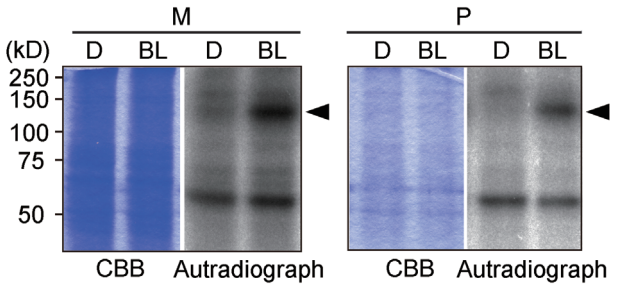

Figure 4. Blue-light-induced *in vitro* phosphorylation of membrane proteins of *Vallisneria*
Microsomal (M) and plasma-membrane (P) fractions prepared from the dark-adapted leaves were kept in darkness (D) or exposed to blue light (450 nm) of 100 μmol/m^2 per s for 30 s (BL). After incubation with radiolabeled adenosine triphosphate (ATP), each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie brilliant blue (CBB) or further subjected to autoradiography (Autoradiograph). Each lane contains 20 μg of protein. Arrowheads indicate the position of 120-kDa polypeptides. The molecular masses of standard proteins are indicated on the left in kDa.

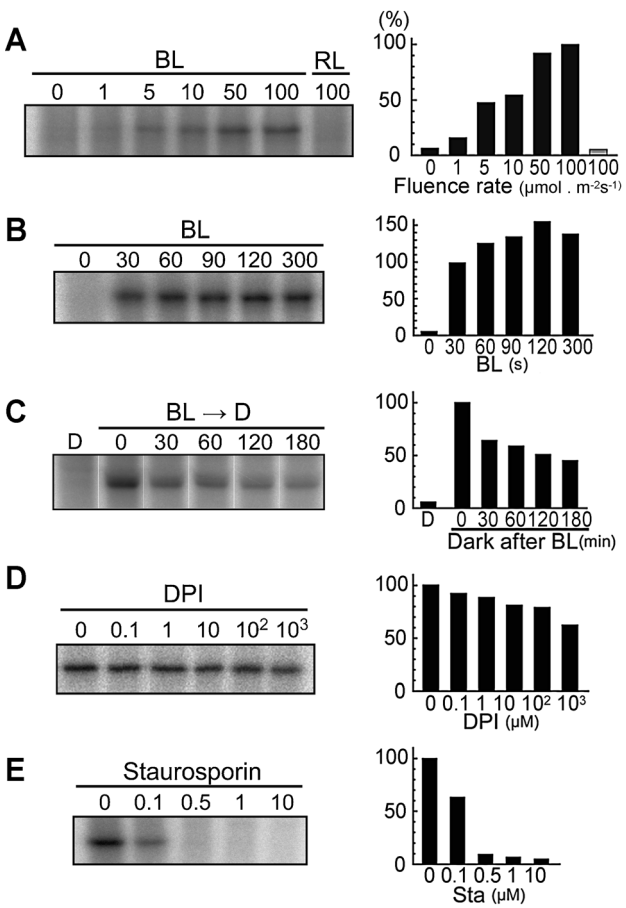

Figure 5. Continued.

presence of candidates for phototopin2-type proteins, we examined phosphorylated polypeptides in a microsomal fraction exposed to blue light at 100 μmol/m^2 per s for longer time. Longer irradiation resulted in higher level of phosphorylation of the 120-kDa polypeptides, and blue-light irradiation of 12,000 μmol/m^2 was sufficient for the maximal phosphorylation (Figure 5B). However, even after blue-light irradiation of 30,000 μmol/m^2, no additional positive band was detected.

We further investigated the retention of the blue-light-induced phosphorylation activity. The microsomal fraction was irradiated with blue light at 100 μmol/m^2 per s for 30 s, then incubated in darkness before the addition of radio-labeled ATP. When compared with the phosphorylation level of 120-kDa polypeptides immediately after the termination of the blue-light irradiation, phosphorylation levels were maintained at 60% after 30 min of dark incubation and at 45% even after 180 min of dark incubation (Figure 5C). In summary, blue light induces phosphorylation of the 120-kDa polypeptides in the membrane fraction in a fluence-dependent manner, and the activity is retained at high level for a long time in darkness.

We next examined the effect on phosphorylation of an inhibitor of flavoprotein function, diphenylene iodonium (DPI) (O'Donnell et al. 1993; Kinoshita et al. 2003), and a Ser/Thr

Figure 5. Characterization of blue-light-induced *in vitro* phosphorylation of 120-kDa polypeptides of *Vallisneria*
After actinic irradiation of a microsomal fraction prepared from the dark-adapted leaves, *in vitro* phosphorylation of 120-kDa polypeptides was examined as described in the legend to Figure 4, unless additional modifications were described, and demonstrated in the left panels. From the autoradiographs, the phosphorylation levels of 120-kDa polypeptides were semi-quantified and shown in the right panels, being normalized to the level obtained after exposure to blue light (450 nm) of 100 μmol/m^2 per s for 30 s. Each lane contains 20 μg of protein. (**A**) Effects of blue light of different fluence rates (0, 1, 5, 10, 50, and 100 μmol/m^2 per s) (BL) and red light of 100 μmol/m^2 per s for 30 s (RL) on the phosphorylation of 120-kDa polypeptides. (**B**) Effects of blue light of 100 μmol/m^2 per s for various periods of time (0, 30, 60, 90, 120, and 300 s) on the phosphorylation of 120-kDa polypeptides. (**C**) Effects of dark incubation on the blue-light-induced phosphorylation activity. After exposure to blue light of 100 μmol/m^2 per s for 30 s, the microsomal fraction was kept in darkness for different durations (0, 30, 60, 120, and 180 min) in the absence of adenosine triphosphate (ATP). After incubation with radio-labeled ATP, the fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then to autoradiography. For each test condition, the microsomal fraction was subjected to SDS-PAGE in duplicate lanes. The figure was edited from the one set of results. (**D**, **E**) Effects of diphenylene iodonium (DPI) (**D**) and staurosporine (**E**) on the blue-light-induced phosphorylation of 120-kDa polypeptides. A microsomal fraction was irradiated with blue light of 100 μmol/m^2 per s for 30 s in the presence of different concentrations of DPI (0, 01, 1, 10, 100, and 1,000 μmol/L) or staurosporine (0, 0.1, 0.5, 1, and 10 μmol/L). After incubation with radiolabeled ATP, the fractions were subjected to SDS-PAGE and then to autoradiography.

protein kinase inhibitor, staurosporine (Tamaoki et al. 1986). DPI suppressed phosphorylation slightly at 100 μmol/L (i.e., by 20%), and even at 1 mmol/L only by 40% (Figure 5D). On the other hand, staurosporine dramatically inhibited the phosphorylation of the 120-kDa polypeptides in a concentration-dependent manner, with complete inhibition at 0.5 μmol/L (Figure 5E). Staurosporine also inhibited phosphorylation of 55-kDa polypeptides, which occurred independent of blue-light irradiation (data not shown). This suggests that Ser/Thr-kinase activity is necessary for the blue-light-induced phosphorylation of 120-kDa polypeptides.

Phototropin genes of *Vallisneria*

Our results suggest that putative phototropin proteins exist and function as blue-light photoreceptors on the plasma membrane of *Vallisneria* leaves. To isolate phototropin genes, we conducted reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers constructed against the conserved sequences in homologs of *PHOTOTROPIN1* and *PHOTOTROPIN2* (Kinoshita et al. 2003; Kagawa et al. 2004). Two different PCR-amplified fragments were subcloned and their full-length cDNA sequences were determined by 5′- and 3′-rapid amplification of cDNA ends (RACE), and then designated *Vallisneria PHOTOTROPIN1* (*ValliPHOT1*, AB986569) and *PHOTOTROPIN2* (*ValliPHOT2*, AB986570). *ValliPHOT1* and *ValliPHOT2* contain putative open reading frames of 2,934 and 2,727 bp, respectively. The deduced proteins ValliPHOT1 and ValliPHOT2 consist of 975 and 909 amino acids with predicted molecular masses of 109 and 103 kDa, respectively (Figure 6A). ValliPHOT1 contains the highly conserved light-sensory LOV1 (amino acids 172–278) and LOV2 (454–460) domains and the C-terminal Ser/Thr kinase domain (641–933). ValliPHOT2 contains LOV1 (98–204), LOV2 (380–486), and Ser/Thr kinase (567–860) domains. The LOV domains possess conserved cysteine residues for FMN-adduct formation, Cys_{208} and Cys_{490} in ValliPHOT1 and Cys_{136} and Cys_{417} in ValliPHOT2 (Figure 6A, asterisks). In *Vallisneria* phototropins, several key residues are conserved in the kinase domain including serine residues, which are autophosphorylated under blue light, and a lysine residue essential for kinase activity (Figure 6A). Furthermore, ValliPHOT2 has a unique insertion of additional four residues, Ser_{593}-Glu_{596}, near the conserved Lys_{600} in the Ser/Thr kinase domain (Figure 6A, red characters).

Amino acid sequences of ValliPHOT1 and ValliPHOT2 were compared with those of previously identified phototropin homologs enumerated in Table 1. Phylogenetic analysis revealed that ValliPHOT1 belongs to the monocot PHOTOTROPIN1 clade and ValliPHOT2 belongs to the monocot PHOTOTROPIN2 clade (Figures 6B, S3, S4).

Semi-quantitative RT-PCR demonstrated that the expression levels of both *ValliPHOT1* and *ValliPHOT2* were higher in leaves than in roots (Figure 6C).

DISCUSSION

Chloroplast de-anchoring induced by high-fluence-rate blue light in *Vallisneria* epidermal cells

In living *Vallisneria* epidermal cells, it was previously observed that passive displacement of chloroplasts due to centrifugal force is induced approximately one minute after the start of blue-light irradiation (Takagi et al. 1991). Here, based on detailed, semi-quantitative characterization of blue-light-induced chloroplast de-anchoring, we conclude that de-anchoring is an initial step of the avoidance response of chloroplasts. The following findings support our conclusion. First, de-anchoring is rapidly induced within one minute of blue-light irradiation, preceding a decrease in the number of chloroplasts actually located in the outer periclinal cytoplasm (Figure 1C, E). It has been shown that previously motionless chloroplasts began to sway in random directions in 1–2 min after the start of continuous irradiation with blue light (60 $\mu mol/m^2$ per s) (Sakurai et al. 2005). It was assumed that, before the commencement of unidirectional migration toward the anticlinal cytoplasm, the anchored chloroplasts are de-anchored upon blue-light irradiation, and then transiently exhibit randomly directed, agitational movement. Second, both de-anchoring and avoidance response of chloroplasts are specifically induced by high-fluence-rate blue light (Figure 1D, Izutani et al. 1990), while avoidance is detectable only at a higher total fluence than is de-anchoring (Figure 1D, F). Finally, both de-anchoring and avoidance are sensitive to reagents used to inhibit phospholipase C-mediated calcium release from intracellular stores, but not to a reagent that blocks calcium entry through the plasma membrane (Figures 2A, B, S2).

While accumulation of chloroplasts into the outer periclinal cytoplasm is induced by low-fluence-rate light, this type of chloroplast movement is accelerated by red light and decelerated by far-red light (Dong et al. 1996). It was also shown that cytoplasmic motility in *Vallisneria* epidermal cells is accelerated within a few seconds of light irradiation in a red/far-red light reversible manner (Takagi et al. 2003). However, red light never induced chloroplast de-anchoring (Figure 1B), indicating that phytochrome-mediated acceleration of the cytoplasmic motility could not solely induce chloroplast de-anchoring. On the other hand, in epidermal cells exposed to low-fluence-rate light, chloroplasts become anchored depending on photosynthesis (Dong et al. 1998). Such mechanisms might interfere with the de-anchoring processes of chloroplasts, especially under low-fluence-rate blue light. Our result that DCMU never affected the blue-light-induced chloroplast de-anchoring (Figure S1) may rule out the involvement of photosynthesis in the response.

A possible involvement of phototropins in the blue-light-induced chloroplast de-anchoring

Phototropins generally mediate rapid responses such as phototropism, stomatal opening, and chloroplast photorelocation movement, while cryptochromes mediate slower developmental responses through transcriptional regulation (Christie 2007). It is also established that phototropins are involved in more rapid intracellular processes with a time constant of a minute or less, such as membrane depolarization (Folta and Spalding 2001), phosphorylation of plasma-membrane H^+-ATPase (Kinoshita et al. 2003), and induction of transient increase in the cytoplasmic calcium concentration (Harada et al. 2003). Chloroplast de-anchoring could also be categorized into the blue-light-induced rapid intracellular processes regulated by phototropins (Figure 1C, Takagi et al. 1991). Furthermore, chloroplast de-anchoring is induced only by blue light fluence rates higher than 50 $\mu mol/m^2$ per s, and only at total incident blue light fluence larger than

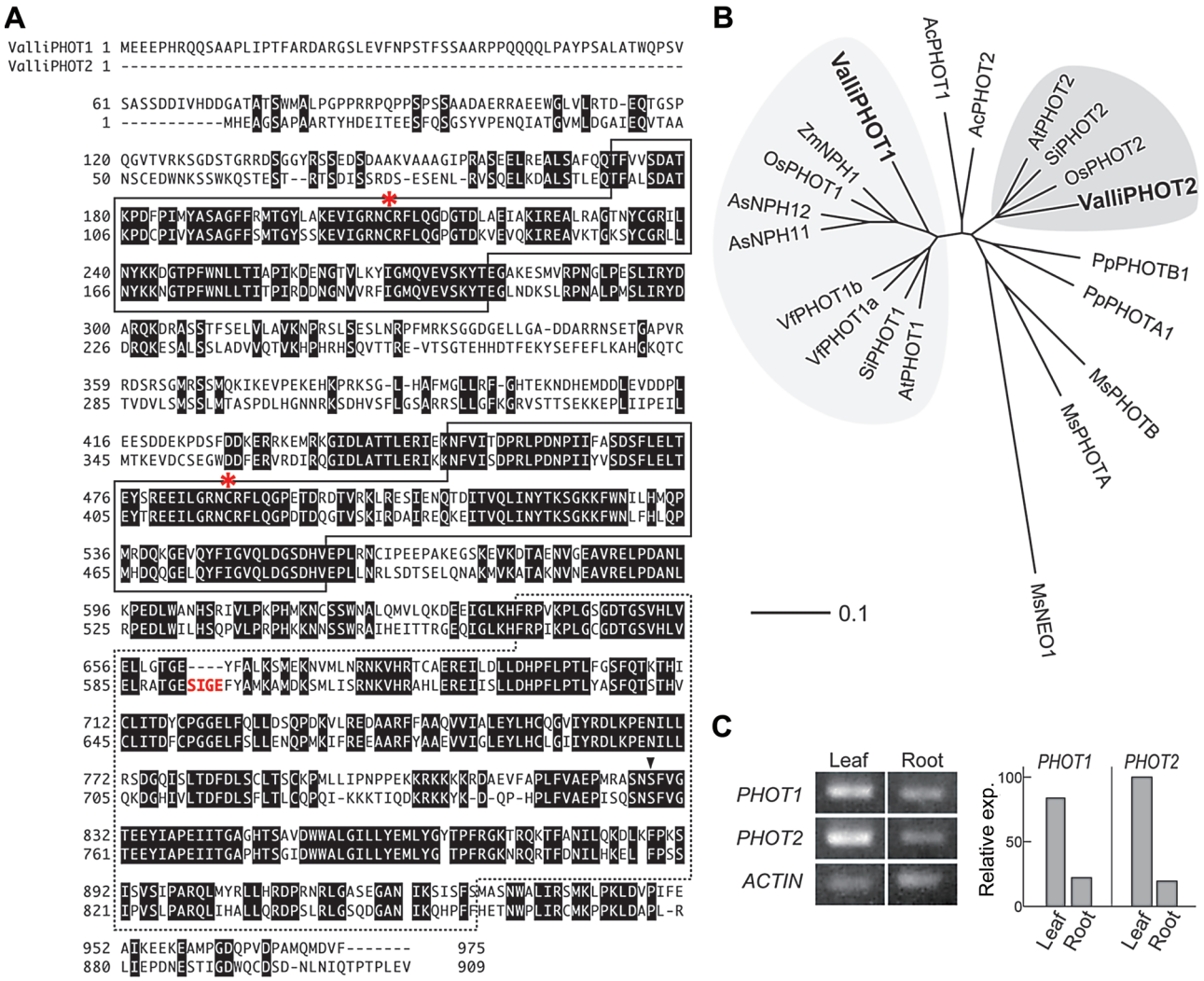

Figure 6. Phototropins of *Vallisneria*

(**A**) Alignment of deduced amino acid sequences of ValliPHOT1 and ValliPHOT2 by Multiple-Alignment program of GENETIX-MAC. Dashes; gaps introduced to allow for optimal alignment of sequences, frames with solid line; LOV domains, frame with dotted line; Ser/Thr kinase domain, red asterisks; conserved Cys residues in LOV domains, arrowheads; conserved Ser residues in Ser/Thr kinase domain. Red characters indicate ValliPHOT2-specific additional residues. (**B**) Phylogenetic relationship of phototropins including ValliPHOT1 and ValliPHOT2 deduced from a comparison of amino acid sequences using the ClustalW program. The scale represents 0.1 substitutions per site. For details, see the text and Table 1. (**C**) Expression levels of *ValliPHOT1* and *ValliPHOT2* examined by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed using 5 ng of total RNA prepared from leaves and roots of *Vallisneria*. The gel image (right panel) is a representative set of triplicate among the three individual PCR. From the gel images, expression level of each gene was evaluated with Image J program, and relative expression levels of *ValliPHOT1* and *ValliPHOT2* were normalized with *ACTIN* as reference gene (left panel) ($n = 3$).

3,000 μmol/m^2 (Figure 1D). These characteristics are similar to those reported for the phototropin2-mediated avoidance response of chloroplasts in *A. thaliana* (Sakai et al. 2001), raising the possibility that chloroplast de-anchoring is a process under the control of phototropin2. According to Figure 5B, 3,000 μmol/m^2 blue light induced phosphorylation of 120-kDa polypeptides at approximately 70% of the maximal phosphorylation level. We can postulate that this level of phosphorylation of putative *Vallisneria* phototropins is required to induce chloroplast de-anchoring.

In *Vallisneria* epidermal cells, the flavoprotein inhibitor, DPI, delayed a change in the mode of chloroplast movement from random sway to unidirectional migration, but not the induction of random sway itself (Sakurai et al. 2005). This may be consistent with our present results that DPI only slightly inhibited the blue-light-induced phosphorylation of 120-kDa polypeptides (Figure 5D). Since chloroplast de-anchoring is thought to be a prerequisite for the induction of randomly directed chloroplast movement, both responses are mediated by phototropins and thus insensitive to DPI. On the other hand,

Table 1. Phototropin homologs

Name	ID	Organism	
AtPHOT1	AF030864	*Arabidopsis thaliana*	Huala et al. 1997
AtPHOT2	AF053941		Jarillo et al. 1998
VfPHOT1a	AB095909	*Vicia faba*	Kinoshita et al. 2003
VfPHOT1b	AB095910		
SlPHOT1	EF06335	*Solanum lycopersicum*	
SlPHOT2	EU021291		
OsPHOT1	AJ252142	*Oryza sativa*	Kanegae et al. 2000
OsPHOT2	AB018443		
AsNPH1-1	AF033096	*Avena sativa*	Huala et al. 1997
AsNPH1-2	AF033097		
ZmNPH1	AF033263	*Zea mays*	Zacherl et al. 1998
AcNPH1	AB037188	*Adiantum capillus-veneris*	Nozue et al. 2000
AcPHOT2	AB115546		
PpPHOTA1	XM_001774204	*Physcomitrella patens*	
PpPHOTA2	XM_001774562		
PpPHOTB1	XM_001766357		
PpPHOTB2	XM_001785674		
MsPHOTA	AB206968	*Mougeotia scalaris*	Suetsugu et al. 2005
MsPHOTB	AB206964		
MsNEO	AB206961		

staurosporine dramatically inhibited the blue-light-induced phosphorylation of 120-kDa polypeptides (Figure 5E), agreeing with a well-established nature of phototropins. The initial step of phototropin-mediated blue-light signal transduction is auto-phosphorylation depending on the intrinsic Ser/Thr kinase activity of phototropins (Christie 2007). Although it was confirmed that blue-light-induced avoidance response of chloroplasts in *Vallisneria* epidermal cells is substantially suppressed in the presence of staurosporine at 10 μmol/L, staurosporine occasionally induced cytoplasmic streaming in mesophyll cells (N Sakurai-Ozato, pers. comm., 2007). Since non-specific effects of staurosporine on cytoplasmic motility could not be ruled out, we did not examine its effects on chloroplast de-anchoring further. As another inhibitor of Ser/Thr kinase K252-a killed cells, whether Ser/Thr kinase activity is necessary for blue-light-induced chloroplast de-anchoring remains to be elucidated.

A possible involvement of calcium release from intracellular calcium stores in the blue-light-induced chloroplast de-anchoring

The blue-light-induced chloroplast de-anchoring and avoidance responses of chloroplasts were sensitive to the inhibitors of a phospholipase C-mediated calcium transient but not to the plasma membrane calcium-channel blocker (Figures 2A, B, S2). These results are consistent with reports emphasizing an important role of calcium transported from internal calcium stores in the induction of chloroplast photo-relocation movement (Sato et al. 2001; Banaś et al. 2012), and further suggest that a target process of calcium in the response is chloroplast de-anchoring. Calcium may exert its effects through reorganization of actin cytoskeleton associated with immobilized chloroplasts (Kadota et al. 2009; Takamatsu and Takagi 2011).

Blue light induces rapid cytoplasmic calcium transient in several plant species (Harada and Shimazaki 2007). In *A. thaliana*, phototropin1 and phototropin2 mediate a calcium transient, which occurs within a few tens of seconds upon blue-light irradiation (Harada et al. 2003). Harada et al. (2003) further clarified that phototropin1 and phototropin2 share partly redundant but distinct roles in the blue-light-induced calcium transient. Phototropin1 mainly functions under low fluence rate blue light (0.1–50 μmol/m^2 per s), while phototropin2 acts under higher fluence rate blue light (1–250 μmol/m^2 per s). In addition, both phototropin1 and phototropin2 appear to induce a calcium transient through activation of plasma membrane calcium permeable channels, whereas only phototropin2 could trigger phospholipase C-mediated calcium release from intracellular calcium stores (Harada et al. 2003; Stoelzle et al. 2003; Zhao et al. 2013). Taken together, though we have not yet succeeded in detecting a blue-light-induced calcium transient in *Vallisneria* epidermal cells, a rapid induction of chloroplast de-anchoring within 60 s of blue-light irradiation (Figure 1C, Takagi et al. 1991) and its sensitivity to TMB-8 (Figure 2A) support our proposal that the response is mediated by a phototropin2-type blue-light receptor through calcium release from intracellular calcium stores.

In *Vallisneria* mesophyll cells, cytoplasmic streaming is controlled by calcium; lower calcium is favorable for cytoplasmic streaming while higher calcium is inhibitory (Takagi and Nagai 1985, 1986). Red light most effectively induces the cytoplasmic streaming, while far-red light inhibits it

(Takagi and Nagai 1985). In this case, red and far-red light appear to modulate calcium fluxes across the plasma membrane to regulate the cytoplasmic concentration of calcium (Takagi and Nagai 1988). Although we do not know at present whether a rapid increase in cytoplasmic calcium depending on intracellular stores is necessary for the induction of cytoplasmic streaming in mesophyll cells, we suppose this is unlikely since a simple treatment of dark-adapted mesophyll cells with EGTA could induce the cytoplasmic streaming in darkness (Takagi and Nagai 1986). Consequently, though the cytoplasmic streaming itself is under the control of calcium concentration, the mode of regulation of chloroplast motility in *Vallisneria* epidermal and mesophyll cells might be different.

Phototropins of *Vallisneria*

We succeeded in isolating two phototropin gene homologs, expressed at similar levels in leaves of *Vallisneria*, *ValliPHOT1* and *ValliPHOT2* (Figure 6). This is apparently the first information on aquatic angiosperm phototropins, providing full-length sequences of open reading frames. *Vallisneria*, together with *Elodea* and *Lemna*, are classified into the family Hydrocharitaceae in the order Alismatales. The alismatales lineage is evidently the second oldest blanch in the monocot phylogeny (APG III 2009). In accordance with this, the amino-acid identity of ValliPHOT1 to AtPHOT1 (64.9%) does not differ much from that to OsPHOT1 (65.9%). This is also the case of ValliPHOT2.

On the other hand, we detected only a single band of 120 kDa in immunoblotting analysis and *in vitro* protein-phosphorylation assay (Figures 3–5). According to the deduced amino-acid sequences of ValliPHOT1 and ValliPHOT2, their molecular masses are estimated to be 109 and 103 kDa, respectively. Considering posttranslational protein modification such as phosphorylation, there is a possibility that ValliPHOT1 and ValliPHOT2 are not separable by SDS-PAGE. In *A. thaliana*, photosensitivity of kinase activity of phototropin1 appears to be much higher than that of phototropin2, and the kinase activation could be detected after blue-light irradiation of only less than 1,000 μmol/m^2 (Sakai et al. 2001; Okajima et al. 2012). *In vitro* phosphorylation of the 120-kDa polypeptides by blue-light irradiation of 150/μmol per m^2 (Figure 5A) may suggest that at least phototropin1-type proteins are present among the 120-kDa polypeptides of *Vallisneria*.

In maize, an *in vitro* protein-phosphorylation assay demonstrated that the phosphorylation level of membrane-associated 114-kDa polypeptides substantially declined in the absence of ATP during dark incubation after the end of blue-light irradiation for 30 s, and the blue-light-induced phosphorylation activity almost completely disappeared after 20 min of dark incubation (Palmer et al. 1993b). A similar phenomenon has also been reported in oats (Salomon et al. 1996). Contrary to these results, the phosphorylation level of 120-kDa polypeptides of *Vallisneria* remained at 45% even after a 3-h dark incubation (Figure 5C). Phototropins of the aquatic angiosperm *Vallisneria* might have photochemical properties that differ from those of terrestrial plants, which would be advantageous to amplify the blue-light signal. Characterization of immunoreactivity and blue-light-induced kinase activity using recombinant proteins should provide more valuable information on the presence and molecular properties of *Vallisneria* phototropins.

MATERIALS AND METHODS

Plant materials

Young plants of giant vallisneria (*Vallisneria* sp.) were purchased at a tropical-fish store. This plant material is closely related to *Vallisneria gigantea* Graebner, which was used for many previous studies. Although it is recently reported that the *V. gigantea* is most likely a synonym of *V. nana* (Les et al. 2008), we have not yet identified the specific epithet of our *Vallisneria* isolate. Plants were grown under a 12-h light (50 μmol/m^2 per s) and 12-h dark regime at 20–25 °C. The light source was a bank of 20-W fluorescent lamps (FL20S-PG; National, Kadoma, Japan).

Observation of chloroplast avoidance response

Preparation of specimens for light microscopic observation of leaf epidermal cells was performed according to Sakurai et al. (2005) with several modifications. After 12–16 h of dark adaptation, the specimen was irradiated with blue light (488 nm, 80 μmol/m^2 per s) on the stage of a microscope (BX50; Olympus, Tokyo, Japan) from above through the objective lens, using the excitation system with a dichroic mirror and a mercury lamp. The fluence rate of blue light was adjusted with neutral-density filters (Fuji Photo Film, Tokyo, Japan) and measured with a quantum sensor and data logger (LI-1400; LI-COR, Nebraska, USA). Before and during the actinic blue-light irradiation, the optical images were captured digitally with a charge-coupled device camera (RETIGA 2000RV; Roper Industries, Florida, USA) at 5-s intervals using dim green light (550 nm, 10 μmol/m^2 per s) from below produced by combination of an interference filter (KL-55; Toshiba, Tokyo, Japan) and a cut-off filter (O-54; Toshiba) as described in Izutani et al. (1990). The number of chloroplasts located in the outer-periclinal cytoplasm of the cell was counted on each image at appropriate time points. When the effects of 100 μmol/L $LaCl_3$ or 10 μmol/L neomycin were examined, specimens were treated with each chemical for 2 h in darkness before the start of observation.

Centrifugation of specimens

Preparation of the specimens and centrifugation experiments were performed according to Sakai and Takagi (2005) with several modifications. Before centrifugation, specimens were irradiated from above the outer periclinal sides of the cells for a defined period of time either with blue light (470 nm) or red light (660 nm) by using light-emitting diodes (MIL-B18 or MIL-R18; SANYO, Moriguchi, Japan). Fluence rates of blue light and red light were measured with a quantum sensor and data logger (LI-1400). Immediately after centrifugation, the specimen was fixed either with 1.5% formaldehyde in a buffered solution or with boiling water. When the effects of inhibitors for calcium transient were examined, specimens were treated for 2 h in darkness with 100 μmol/L $LaCl_3$ or 1 mmol/L TMB-8. All of experiments were carried out between12 and 20 h after the beginning of the dark treatment.

Index of chloroplast anchoring

From the bright-field images, the ratio of the number of chloroplasts in the centripetal half (Np) to the total number of

chloroplasts (Nt) in each cell was calculated (Figure 1A). The coverage ratio (Np/Nt) was used as the index of chloroplast anchoring.

Preparation of crude microsomal and plasma-membrane-enriched fractions

A microsomal fraction and a plasma-membrane-enriched fraction were prepared from dark-adapted leaves of *Vallisneria* according to Harada et al. (2002b), with minor modifications. The final microsomal and plasma-membrane fractions were suspended in a buffer solution containing 250 mmol/L sucrose, 0.1 mmol/L DTT, and 10 mmol/L Mops-KOH at pH 7.6. All the procedures were carried out at 0–4 °C under dim red light (0.1 μmol/m^2 per s). The total protein content of each fraction was determined by the method of Bradford (1976).

Immunoblotting

The total proteins in the plasma-membrane fraction were concentrated by a TCA/acetone precipitation method and separated by SDS-PAGE in a 9.0% (w/v) polyacrylamide gel. Immunoblotting was performed according to Kinoshita et al. (2003) using the polyclonal antibodies against *Arabidopsis thaliana* PHOTOTROPIN2 (Takemiya et al. 2005).

In vitro phosphorylation assay

An *in vitro* phosphorylation assay on the microsomal and plasma-membrane fractions, each containing 1 mg/mL protein, was performed using radiolabeled ATP according to Kinoshita et al. (2003) with several modifications. Blue light (450 nm) was applied using a fiberscope (FI-150T; Sugiura Lab, Tokyo, Japan) equipped with a halogen lamp (JCR; Ushio) and a combination of an interference filter (BP-45; Kenko, Tokyo, Japan) and a cut-off filter (Y-44; Kenko). Red light (660 nm) was applied by using light-emitting diodes (MIL-R18; SANYO). After actinic irradiation of the membrane fraction, 20 μg of total proteins were separated by SDS-PAGE on a 10.0% (w/v) gel. Autoradiography was carried out by exposing the de-stained and dried gels to an imaging plate, followed by visualization with an imaging analyzer (BAS-2500; Fujifilm). The total intensity of pixels in the 120-kDa band was quantified with Image J program, and was normalized to the value obtained after exposure to blue light of 100 μmol/m^2 per s for 30 s. When the effects of inhibitors were examined, DPI of an appropriate concentration was added to the reaction mixture 30 min before the start of irradiation with blue light, and staurosporine of an appropriate concentration was added 5 min before. Each assay was repeated at least twice to confirm reproducible results throughout the experiments.

Cloning of *Vallisneria* phototropins

Total RNA was isolated from fresh green leaves of *Vallisneria* according to cetyltrimethyllammonium bromide methods as described in Harada et al. (2002a) with slight modifications. The first-strand cDNAs were prepared from total RNA using SuperScript II reverse transcriptase (Invitrogen) with oligo(-dT)12–18 primer. The following PCR was carried out with two sets of degenerate primers: 5′-TAYTCYTCNGTNCCNACRAA-3′ and 5′-CCNGAYAAYYCCNATHATHTTYGC-3′ for *PHOTOTROPIN1*, 5′-GGIATHGAYYTIGCIACIACIYTIGARMG-3′ and 5′-GCDATRTAYTCYTCIGTICCIACRAAISWRTT-3′ for *PHOTOTROPIN2* (Kinoshita et al. 2003, Kagawa et al. 2004). Gene fragments were amplified in 30 PCR cycles; 95 °C (2 min); 95 °C (30 s), 45–50 °C (30 s), 72 °C (1 min) – 30 cycles; 72 °C (10 min). The resulting PCR products were sub-cloned into pCR4-TOPO vector (Invitrogen) and sequenced. Using SMART RACE Kit (Clontech), 5′- and 3′-RACE were carried out with total RNA according to the manufacturer's instructions. To clone full lengths of cDNA sequences, RT-PCR was performed using gene specific primers conjugated with additional sequences containing appropriate restriction sites at the 5′ ends: 5′-TATAGAAAAATGGAAGAGGAGCCCCA-3′ and 5′-GACGGCTCAGAACACGTCCATTTGCA-3′ for *ValliPHOT1*, 5′-AATTGCGGGATGCATGAGGCAGGATC-3′ and 5′-AGCATTTCAGAAAGCACAACGCACAC-3′ for *ValliPHOT2*.

Semi-quantitative RT-PCR analysis

To assess *ValliPHOT*s transcription levels, total RNA was isolated from fresh leaves and roots of *Vallisneria* at the end of the light period of growth. Reverse transcription was performed from 5 ng total RNA using the Prime Script Reverse Transcriptase (Takara Bio) and oligo-dT primer according to the manufacturer's instructions. The primers used were as follows: 5′-AGAATCATTAAACAGGCCATTCATGA-3′ and 5′-CTATTTCATCATCTTTTTGTAAGACC-3′ for *ValliPHOT1*, 5′-TCAAGTTACAACTAGAGAGGTTACCT-3′ and 5′-CCACGTGTAGTAATCTCATGTATTG-3′ for *ValliPHOT2*, and 5′-GGTCGACCACGGCACACCGGTG-3′ and 5′-GTACGGCCACTGGCATACAGAG-3′ for *ACTIN* (AF237626) as an endogenous control. All genes were amplified in 30 PCR cycles; 98 °C (30 s); 98 °C (10 s), 55 °C (30 s), 72 °C (1 min) – 5 cycles; 98 °C (10 s), 50 °C (30 s), 72 °C (1 min) – 25 cycles; 72 °C (5 min). The PCR products were resolved on a 0.8% agarose gel and visualized by ethidium bromide staining. The total intensity of pixels in each band was quantified with Image J program, and was normalized to the value obtained for *ACTIN* as reference gene.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site

Figure S1. Effects of DCMU on the blue-light-induced chloroplast de-anchoring in *Vallisneria* epidermal cells

Figure S2. Effects of neomycin on the blue-light-induced chloroplast avoidance response

Figure S3. Alignment of amino-acid sequences of PHOTOTROPIN1

Figure S4. Alignment of amino-acid sequences of PHOTOTROPIN2