Phototropins (phot1 and phot2) are autophosphorylating blue-light receptor kinases that mediate blue-light responses such as phototropism, chloroplast accumulation, and stomatal opening in Arabidopsis (Arabidopsis thaliana). Only phot2 induces the chloroplast avoidance response under strong blue light. The serine (Ser) residues of the kinase activation loop in phot1 are autophosphorylated by blue light, and autophosphorylation is essential for the phot1-mediated responses. However, the role of autophosphorylation in phot2 remains to be determined. In this study, we substituted the conserved residues of Ser-761 and Ser-763 with alanine (S761A S763A) in the phot2 activation loop and analyzed their function by investigating the phot2-mediated responses after the transformation of phot1 phot2 double mutant with this mutant phot2 gene. Transgenic plants expressing the mutant phot2 protein exhibited impaired responses in chloroplast movement, stomatal opening, phototropic bending, leaf flattening, and plant growth; and those expressing phot2 with S761D S763D mutations showed the normal responses. Substitution of both Ser-761 and Ser-763 with alanine in phot2 did not significantly affect the kinase activity in planta. From these results, we conclude that phosphorylation of Ser-761 and Ser-763 in the activation loop may be a common primary step for phot2-mediated responses.
changes are thought to trigger the C-terminal kinase domain activation. In fact, the LOV2 domain, not the LOV1 domain, binds to the kinase domain and inhibits kinase activity in the dark; the LOV2 domain dissociates from the kinase domain and the kinase phosphorylates casein as a substrate upon irradiation (Matsuoka and Tokutomi, 2005; Kong et al., 2007; Tokutomi et al., 2008; Kaiserli et al., 2009).

The blue-light-activated phototropin kinase exhibits phosphorylation of phototropin itself, called auto-phosphorylation (Christie et al., 1998, 2002; Sakai et al., 2001). Recent studies showed that autophosphorylation occurred in the N-terminal region upstream from the LOV1 domain, and Hinge1 region between LOV1 and LOV2 domains in Avena phot1a and Vicia phot1s (Kinoshita et al., 2003; Salomon et al., 2003). More recent studies using mass spectrometric analyses directly identified that the autophosphorylation sites of Arabidopsis phot1 were Ser-58, Ser-170, and Ser-185 in the N terminus; Ser-350, Ser-376, and Ser-410 in the Hinge1 region; Ser-851 in the kinase activation loop; and Thr-993 in the C terminus (Inoue et al., 2008a; Sullivan et al., 2008). Ser-849 is thought to be also a phosphorylation site by functional analysis with point mutation (Inoue et al., 2008a). Among these sites, the autophosphorylation of Ser-849 and Ser-851 in the activation loop was essential to phot1-mediated responses; other sites were not required (Inoue et al., 2008a). Recently, it was demonstrated that Ser-851 is a primary autophosphorylation site required for the internalization and endocytic recruitment of phot1 protein in response to blue light (Kaiserli et al., 2009). In phot2, two Ser residues in the activation loop of the kinase domain are highly conserved as Ser-761 and Ser-763 (Matsuoka et al., 2007; Inoue et al., 2008a; Fig. 2A). However, the functional role of these two Ser residues in phot2-mediated responses has not been determined and the autophosphorylation sites of phot2 have not been identified.

In this study, we investigated the roles of Ser-761 and Ser-763 in the kinase activation loop by complementation experiments using mutated phot2 constructs in which each of these Ser residues had been substituted with Ala or Asp. We suggested that phosphorylation of both Ser residues is required for phot2-mediated responses. We also provided information on in vivo autophosphorylation sites in phot2.

RESULTS

Blue-Light-Induced Autophosphorylation of Arabidopsis Phototropin2

Etiolated seedlings of transgenic Arabidopsis (WT-11 and D720N-1 lines: see Table I) were irradiated with blue light for 1 min, and microsomal membranes were immediately prepared from the seedlings. Phot2 proteins were isolated by immunoprecipitation from the membranes. Phot2 showed a low phosphorylation level in the dark, and this increased significantly by the blue light (Fig. 1A), with a simultaneous upward mobility shift on SDS-PAGE (Fig. 1B). This phosphorylation is blue-light-induced autophosphorylation in vivo because the phot2 protein in the kinase-dead D720N-1 line showed an increase in neither the phosphorylation level nor the mobility shift by blue light. We confirmed the expression of phot2 proteins in WT-11 and D720N-1 lines (Fig. 1B).

Generation of Transgenic Arabidopsis Plants Expressing Various Phot2 Mutant Proteins

We previously demonstrated that the blue-light-induced autophosphorylation of Ser-849 and Ser-851 in the kinase activation loop was essential for phot1-mediated responses (Inoue et al., 2008a), and found that the corresponding Ser residues in phot2 were conserved Ser-761 and Ser-763 (Fig. 2A). To investigate whether Ser-761 and Ser-763 in phot2 kinase have important roles in the phot2-mediated responses, we constructed single- and multiple-site-mutated phot2 cDNAs by site-directed mutagenesis via substitutions of these Ser residues, and transformed the phot1 phot2 double mutant (glabra1 [gl1] phot1-5 phot2-1) with these constructs, which included constructs of an inactive kinase (D720N; Kong et al., 2007) and an activation loop substituted (S761A, S763A, and S761A/D S763A/D; Table I). We expressed the mutant phot2 proteins under the control of native PHOT1 promoter and expected high expression of the proteins in the etiolated seedlings. A reason is described in the “Construction of Transformation Vector” section in “Materials and Methods.” We first selected the transgenic plants that expressed phot2 proteins with levels similar to those of the controls (gl1) in rosette leaves (Fig. 2B, top section). As expected, the phot2 protein was highly expressed in etiolated seedlings of these transgenic plants, while no detectable amount of phot2 was expressed in gl1 (Fig. 1B, bottom section). This is probably because the activation of PHOT2 promoter requires light and because PHOT2 mRNA is not sufficiently expressed in etiolated seedlings in the dark in the Arabidopsis plant (Jarillo et al., 2001; Kagawa et al., 2001). In etiolated seedlings of gl1, the amount of phot2 protein increased with light irradiation, and phot2 substantially accumulated at 14 h after the start of irradiation (Fig. 2C, top section). In contrast, etiolated seedlings of the transgenic WT-11 line expressed phot2 proteins in the dark, and the amounts of phot2 proteins gradually decreased by light as reported in native phot1 protein (Fig. 2C, bottom section; Sakamoto and Briggs, 2002).

Functional Analyses of Ser-761 and Ser-763 in Phot2-Mediated Responses

Chloroplast Movement

We assayed the blue-light-induced chloroplast movement by a slit band assay (Kagawa et al., 2001;
Table 1. List of transgenic plants with various phot2 constructs

| Construct | Description |
|-----------|-------------|
| WT        | No mutation |
| D720N (Kong et al., 2007) | Kinase dead: binding site of Mg²⁺-ATP in phot2 kinase is mutated |
| S761A     | Substitution of the Ser-761 in the activation loop with Ala |
| S763A     | Substitution of the Ser-763 in the activation loop with Ala |
| S761A S763A | Simultaneous substitutions of the Ser-761 and Ser-763 in the activation loop with Ala |
| S761D S763D | Simultaneous substitutions of the Ser-849 and Ser-851 in the activation loop with Ser |

Suetsugu et al., 2005). Phot2 induces chloroplast accumulation and avoidance response under the low and high intensities of blue light, respectively (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001). The blue-light intensity in which the accumulation response was turned to the avoidance response was between 16 and 32 μmol m⁻² s⁻¹ (Jarillo et al., 2001; Suetsugu et al., 2005). Phot2 induces chloroplast accumulation from 0.1 to 5 μmol m⁻² s⁻¹ of blue light (Fig. 3, A and C; Supplemental Fig. S1A) and did the avoidance above 30 μmol m⁻² s⁻¹ (Fig. 3, B and C; Supplemental Fig. S1B). The phot1 mutant (g11 phot1-5) leaves showed a less-sensitive accumulation response than g11, with light sensitivity from 1 to 5 μmol m⁻² s⁻¹, but the mutant exhibited an avoidance response similar to that in g11. The phot2 mutant (g11 phot2-1) leaves showed only the accumulation response under the low to high intensities of blue light, and the leaves of phot1 phot2 double mutant did not show any response. The results confirmed that the phot1 protein induces only the chloroplast accumulation response irrespective of light intensity, and that the phot2 protein induces both chloroplast accumulation and avoidance responses and the change from the accumulation to avoidance occurs between 5 and 30 μmol m⁻² s⁻¹ of blue light (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001). The results are in accord with recent findings on the red-light transmittance of leaves (de Carbonnel et al., 2010), indicating the reliability of our results.

The leaves of WT-11 showed a response curve of chloroplast movement similar to that found in the leaves of the phot1 mutant (Fig. 3, C and D), but those of kinase-dead D720N-1 did not show any response. These results suggest that only the phot2 protein is functional in WT-11 and that the chloroplast movement depends on the kinase activity of introduced phot2. S761A-3 leaves responded similarly to WT-11, but the S763A-11 leaves showed the normal accumulation response with a small magnitude of avoidance response (Fig. 3, A, B, and D). Sensitivity to blue light in the avoidance response did not change. Furthermore, the leaves of S761A S763A-3 showed neither accumulation at 5 μmol m⁻² s⁻¹ nor avoidance at 30 μmol m⁻² s⁻¹. Interestingly, the avoidance response emerged over 90 μmol m⁻² s⁻¹ and the magnitude increased with the intensity of blue light (Fig. 3D). These results indicate that Ser-761 or Ser-763 is sufficient to induce chloroplast accumulation, and that Ser-763 is essential for the avoidance response but Ser-761 is not. Furthermore, the simultaneous disruption of both Ser-761 and Ser-763 brings about greater impairment in any of the responses than does the disruption of either residue, and one or more unidentified sites other than the two Ser residues also seem to work in the avoidance response. The leaves of the S761D S763D-3 line exhibited similar responses to those of the WT-11 line. The result indicates that the two Asp residues mimic the phosphorylation of the Ser-761 and Ser-763.

Figure 1. Blue-light-dependent in vivo autophosphorylation of phot2 in etiolated seedlings. Etiolated seedlings of the transgenic lines (see Table I) were kept in the dark (Dk) or irradiated with blue light at 500 μmol m⁻² s⁻¹ for 1 min (BL). The phot2 protein was isolated by immunoprecipitation from the microsomes (300 μg) of the seedlings and subjected to SDS-PAGE. A, Phosphorylation of the immunopurified phot2 proteins. Phosphorylated proteins were detected by phos-tag, the phosphorylation-specific indicator. B, Immunoblot of the immunopurified phot2 proteins. Dashed line indicates the highest mobility edges of the bands. Experiments repeated on three occasions gave similar results.

Table I. List of transgenic plants with various phot2 constructs

| Construct | Description |
|-----------|-------------|
| WT        | No mutation |
| D720N (Kong et al., 2007) | Kinase dead: binding site of Mg²⁺-ATP in phot2 kinase is mutated |
| S761A     | Substitution of the Ser-761 in the activation loop with Ala |
| S763A     | Substitution of the Ser-763 in the activation loop with Ala |
| S761A S763A | Simultaneous substitutions of the Ser-761 and Ser-763 in the activation loop with Ala |
| S761D S763D | Simultaneous substitutions of the Ser-849 and Ser-851 in the activation loop with Ser |

Role of Activation Loop of Phototropin2 in Arabidopsis
We further investigated the location of chloroplasts in mesophyll cells of transgenic leaves in response to weak (5 μmol m⁻² s⁻¹) and strong (90 μmol m⁻² s⁻¹) blue light, and under the dark conditions. Chloroplasts of gl1, WT-11, and the S761D S763D-3 leaves gathered at the periclinal cell surfaces in response to weak blue light and moved to the anticalin cell surfaces by strong blue light (Fig. 4, low and high). Chloroplasts of D720N-1 leaves exhibited random localization in response to blue light. Chloroplasts of S761A S763A-3 leaves did not accumulate at the periclinal cell surfaces in response to weak blue light. A small proportion of the chloroplasts moved to the anticalin cell surfaces but a few chloroplasts of S761A S763A-3 leaves accumulated at the periclinal cell surfaces by strong blue light (Supplemental Fig. S2). These observations again indicate that the two Ser residues are important for the phot2-mediated chloroplast movement.

Under the dark, most chloroplasts in gl1, WT-11, and S761D S763D-3 lines accumulated in the bottom of mesophyll cells as reported previously (Fig. 4, dark; Suetsugu et al., 2005; Kong et al., 2007; Aihara et al., 2008). In contrast, chloroplasts of D720N-1 and S761A S763A-3 lines did not accumulate in the bottom and were localized randomly as in phot1 phot2 double mutant (Suetsugu et al., 2005). The results indicate that the dark positioning requires both the kinase activity and phosphorylation of the Ser residues in phot2.

Stomatal Opening, Phototropism, and Leaf Flattening

We measured other phot2-mediated responses in the transgenic lines. Stomata in the isolated epidermis from WT-11 leaves opened, and those in Col and gl1 showed a similar response to blue light, but stomata in the kinase-dead D720N-1 line did not open as well as those of the phot1 phot2 double mutant (Fig. 5A; Supplemental Fig. S1C). The blue-light-specific stomatal opening was partially impaired in both S761A-3 and S763A-11 leaves and largely impaired in S761A S763A-3 leaves.

We then determined phototropic curvature. The hypocotyls of the WT-11 line showed normal bending in response to unilateral blue light as well as those of Col and gl1, but those of the D720N-1 line did not (Fig. 5B; Supplemental Fig. S1D). The hypocotyl bending was impaired partially in the S761A-3 line and largely in the S763A-11 and S761A S763A-3 lines.

We inspected the leaf shape of the transgenic plants grown under white light. Leaves of Col, gl1, phot1 mutant, phot2 mutant, the WT-11 line, the S761A-3 line, and the S763A-11 line were flat, but those of the phot1 phot2 double mutant and the D720N-1 line were severely curled downward (Fig. 5, C and D; Supplemental Fig. S1E). The leaves of S761A S763A-3 showed partial curling and grew larger than those of the phot1 phot2 double mutant and the D720N-1 line.

The S761D S763D-3 line exhibited almost full responses of stomatal opening, phototropism, and leaf flattening (Fig. 5). The results suggest that the substitution of Ser-761 and Ser-763 with Asp mimicked the phosphorylation of Ser, and that the phosphorylation of these Ser residues was required for all phot2-mediated responses tested.

Phot2 Kinase Activities in Transgenic Plants

We determined blue-light-dependent autophosphorylation of mutant phot2 proteins in vivo using transgenic plants by the mobility shift of the proteins. The mobility shift was clearly observed in phot2 protein from the WT-11 line by blue light (Fig. 6). This shift was dependent on the autophosphorylation of phot2 protein, because the shift was not found in the kinase-dead D720N-1 line. We also found shifts in mutant phot2 proteins in both S761A S763A-3 and S761D S763D-3 lines. These results indicate that the amino acid substitutions in the activation loop of the kinase domain did not affect significantly the autophosphorylation activity of phot2 in vivo. The same
situation was reported for phot1 protein (Inoue et al., 2008a).

**Identification of in Vivo Autophosphorylation Sites of Phot2**

In phot1, a 14-3-3 protein is bound to autophosphorylated phot1 protein in a blue-light-dependent manner (Kinoshita et al., 2003; Inoue et al., 2008a; Supplemental Fig. S3A, top section). However, a 14-3-3 protein was not bound to autophosphorylated phot2 protein after the irradiation of blue light even at high intensities (Supplemental Fig. S3B, top section). This confirmed the recent results (Sullivan et al., 2009). A 14-3-3 protein bound to the binding motifs in the Hinge1 region of phot1, which include Set-350, Ser-376, and Ser-410 (Inoue et al., 2008a; Sullivan et al., 2009), and these motifs were not found in the Hinge1 region of phot2 (Supplemental Fig. S3C). The homologies of the N-terminal and Hinge1 regions between phot1 and phot2 were low (Supplemental Fig. S3, C and D). The autophosphorylation sites in phot2 may differ from those in phot1, and no autophosphorylation sites in phot2 have been identified so far.

We thus determined in vivo the autophosphorylation sites of phot2 using etiolated seedlings of WT-11 lines. These seedlings were irradiated with blue light for 2 min. We collected phosphorylated phot2 proteins by immunoprecipitation as described above (Fig. 1) and confirmed the phosphorylation by both phos-tag blotting and the mobility shift on SDS-PAGE (Fig. 7, A and B). The isolated phot2 proteins on SDS-PAGE were stained by Coomassie Brilliant Blue (Fig. 7C). The protein was excised from the gel and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the phosphorylation sites (Inoue et al., 2008a). Two independent experiments consistently revealed that 25 sites were phosphorylated in phot2. The sites included Ser-9, Ser-22, Ser-30, Thr-34, Ser-37, Thr-38, Ser-39, Ser-53,
Ser-54, Thr-67, Ser-88, Ser-105, Ser-111, Ser-114, and Ser-121 in the N terminus; and Ser-284, Ser-289, Ser-291, Thr-294, Ser-300, Ser-301, Thr-302, Thr-303, and Thr-305 in the Hinge1 region (Fig. 7D). The results indicate that both the N terminus and the Hinge1 region were densely phosphorylated in phot2. Unfortunately, we could not find the phosphorylation of Ser-761 or Ser-763 in the kinase activation loop because of a lack of informative fragment ions.

**DISCUSSION**

**Functional Analyses of Deduced Phosphorylation Sites in the Activation Loop of Phot2**

Both phot1 and phot2 induce phototropism, chloroplast accumulation, stomatal opening, leaf flattening, and leaf positioning (Briggs and Christie, 2002; Christie, 2007; Inoue et al., 2008b), whereas only phot2 induces chloroplast avoidance under relatively strong light (Kagawa et al., 2001; Sakai et al., 2001) and chloroplast gathering in the bottom of mesophylls in the dark (Suetsugu et al., 2005; Kong et al., 2007; Aihara et al., 2008). Phot2 does not contribute to the growth inhibition of hypocotyls (Folta and Spalding, 2001). In phot1, we previously demonstrated that the autophosphorylation of the Ser-849 and Ser-851 in the kinase activation loop is required for the phot1-mediated responses mentioned above. Ser-851 undergoes reversible phosphorylation and dephosphorylation in response to blue light in planta (Inoue et al., 2008a). We found that these two Ser residues were conserved as Ser-761 and Ser-763 in the activation loop of phot2 (Fig. 2A), and we suspected that phototropin-mediated responses such as phototropism, chloroplast accumulation, stomatal opening, and leaf flattening may require the phosphorylation of the two Ser residues but the responses of phot2-specific chloroplast avoidance and dark positioning may not. To investigate this, we expressed phot2 mutant proteins of S761A, S763A, and S761A S763A in the phot1 phot2 double mutant and inspected these phenotypes. As we expected, phototropism and stomatal opening were partially impaired in both the S761A-3 and S763A-11 lines (Fig. 5, A and B), and all phototropin-mediated responses tested were largely impaired in the S761A S763A-3 line (Figs. 3–5). The chloroplast avoidance response was also impaired partially in the S763A-11 line and largely in the S761A S763A-3 line (Figs. 3, B and D, and 4). The chloroplast dark positioning was impaired in the S761A S763A-3 line (Fig. 4). These results indicate that the Ser residues in the activation loop are important for all phot2-mediated responses tested in Arabidopsis. However, there were differences in the contributions of the phosphorylation of the two Ser residues to phot2-mediated responses. For example, the S761A-3 line was partially impaired in stomatal opening and phototropism but was not impaired in the responses of chloroplast accumulation, avoidance, and leaf flattening (Figs. 3 and 5). The S763A-11 line was not affected in leaf flattening but was partially impaired in other responses. No difference in phototropism was found between the S763A-11 and the S761A S763A-3 line. There were different degrees of impairment in the phot2-mediated responses in the same mutant line as described above, but the
reasons for these differences cannot be explained at present. We should note, however, that in all of the phot2-mediated responses, the double mutations in the two Ser residues consistently resulted in greater or similar impairment than that of one mutation.

The S761D S763D-3 line showed almost complete responses in our phenotypic analyses (Figs. 3–5). The mutations mimicked constitutive autophosphorylation of two Ser residues, but the physiological responses absolutely required blue light. For example, the S761D S763D-3 line showed a light response curve similar to that of the WT-11 line in chloroplast movement (Fig. 3D), and did not show any light response in the absence of light (Figs. 4 and 5A; Supplemental Fig. S4). These results indicate that the phosphorylation of Ser residues in the activation loop is not sufficient for downstream signaling, and that both the phosphorylation in the loop and the conformational change in LOV domains by blue light are required for the responses. Very recently, it was demonstrated that a protein phosphatase 2A (PP2A) dephosphorylated phot2 that had been autophosphorylated by blue-light irradiation. When the PP2A activity was reduced by the mutation in a regulatory subunit of PP2A in planta, phot2-mediated phototropism and stomatal opening showed higher sensitivities to blue light in the mutant than in the wild type (Tseng and Briggs, 2010). The autophosphorylation levels of phot2 were sustained for a longer time in the mutant than in the wild type. These results suggested that autophosphorylation is required for phot2 signaling and also affects the photosensitivity of phot2-mediated responses. However, since our present results indicated that a mimic of phosphorylation in the activation loop of phot2 did not affect photosensitivity, we thought that the autophosphorylation of Ser-761 and Ser-763 residues in the
loop might not be responsible for the photosensitivity and that other autophosphorylation sites may be involved in photosensitivity.

In S761A S763A-3 leaves, most of the chloroplasts moved to the anticlinal cell surfaces over 90 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for 1 min (BL). Microsomal membranes from the seedlings were prepared and the microsomal proteins (40 \( \mu \text{g} \)) were subjected to immuno blot analysis with antiphot2 antibodies. Dashed line indicates the highest mobility edges of the bands. Experiments repeated on two occasions gave similar results.

Figure 6. Autophosphorylation kinase activity in the transgenic plants. Autophosphorylation activity of phot2 kinase in vivo was determined by a mobility shift of phot2 protein on the SDS-PAGE gel. Etiolated seedlings were kept in the dark (Dk) or irradiated with a blue-light pulse at 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) for 1 min (BL). Microsomal membranes from the seedlings were prepared and the microsomal proteins (40 \( \mu \text{g} \)) were subjected to immuno blot analysis with antiphot2 antibodies. Dashed line indicates the highest mobility edges of the bands. Experiments repeated on two occasions gave similar results.

Generation and Utilization of PHOT2-Expressing Transgenic Plants under the Control of the PHOT1 Promoter

In this study, we used the PHOT1 promoter to express PHOT2 genes in the complementation experiments (Fig. 2B) because the PHOT1 promoter strongly expresses phot1 protein in etiolated seedlings (Sakamoto and Briggs, 2002) but PHOT2 promoter does not express this protein in etiolated seedlings in the dark (Aihara et al., 2008; Fig. 2C). This procedure was important to obtain a sufficient amount of phosphorylated phot2 protein from etiolated seedlings for analysis by LC-MS/MS. In etiolated seedlings, the expression level of phot2 protein in the WT-11 line was much higher than in gll, but the levels of phot2 proteins showed no difference in these rosette leaves (Fig. 2B). The high expression level of phot2 protein in the seedlings of WT-11 enabled us to identify the phosphorylation sites of phot2 (Fig. 7, C and D).

Hypocotyls of the WT-11 line bent 80° in response to unilateral blue light at 0.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), while those of the phot1 mutant bent less than 10° at the same light intensity (Fig. 5B). Previous reports have demonstrated that the robust phototropic bending of the phot1 mutant required more than 10 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) of blue light (Sakai et al., 2001; Inada et al., 2004; Cho...
et al., 2007). The high sensitivity to blue light in WT-11 is probably due to the high expression of phot2 protein. In etiolated seedlings, the levels of phot2 protein in gll1 were low, while those in the WT-11 line were high in the dark (Fig. 2C). The efficiency of phototropic bending was at least 20-times higher in WT-11 than in phot1-5 (Supplemental Fig. S5). These results indicate that the sensitivity of phototropic curvature is largely affected by the amount of photoreceptor protein.

Identification of Phosphorylation Sites of Phot2 in Arabidopsis

In this study, we identified 25 in vivo phosphorylation sites by LC-MS/MS (Fig. 7D) in the N terminus and Hinge1 regions, but we could not find the phosphorylation sites in the C terminus that include the kinase domain. There were 4 times as many phosphorylation sites than there were in the same regions of phot1 (Inoue et al., 2008a; Sullivan et al., 2008). The presence of multiple phosphorylation sites by blue light would result in a large mobility shift of phot2 protein on SDS-PAGE (Figs. 1B and 7B). However, the phot2 fragment lacking the N terminus and Hinge1 regions induces chloroplast avoidance response in Adiantum (Kagawa et al., 2004), and the kinase domain of phot2 constitutively causes chloroplast avoidance and stomatal opening in Arabidopsis (Kong et al., 2007). Therefore, we thought that the 25 phosphorylation sites might not be important for phot2 signaling and therefore did not perform functional analyses of these sites in this study.

Although our functional analyses revealed the importance of the phosphorylation of Ser-761 and Ser-763, we could not detect the phosphorylation of Ser-761 and Ser-763 in vivo by mass spectrometric analysis (Fig. 7D). This is because the peptide fragments including the activation loop were not obtained as appropriate short peptides by trypsin digestion. In accord with this, there are no Arg and Lys residues around the two Ser residues. We generated antibodies against a synthetic peptide STQSNpSFVGEYY1, including phosphorylated Ser-763. Unfortunately, the antibodies recognized phot2 in both phosphorylated and unphosphorylated states and could not determine in vivo phosphorylation of phot2. The functional phenotypic analyses showed that the S761A S763A construct only slightly restored the phot2-mediated responses, but the S761D S763D construct fully restored all of the responses (Figs. 3–5). These results suggest that Asp mimicked the phosphorylation of Ser-761 and Ser-763 and that these two Ser residues may be phosphorylated in vivo by blue light.

Function of Autophosphorylation and Other Potential Phosphorylation Sites in the Activation Loop

The S761A S763A-3 line showed the blue-light-induced mobility shift of phot2 protein (Fig. 6). This shift indicates that the autophosphorylation activity of phot2 was not significantly affected by the substitutions in planta. However, all of the phot2-mediated responses in the same line were inhibited (Figs. 3–5). These results suggest that autophosphorylation of the two Ser residues is likely required for signaling rather than conferring catalytic activity.

Phototropins mediate divergent physiological responses, including phototropism, chloroplast movement, nuclear positioning, stomatal opening, rapid inhibition of hypocotyl growth, leaf flattening, and leaf positioning (Folta and Spalding, 2001; Briggs and Christie, 2002; Christie, 2007; Iwabuchi et al., 2007; Inoue et al., 2008b, 2010). Among these, four responses—phototropism, chloroplast movement, stomatal opening, and leaf flattening—all required phosphorylation of the Ser residues in the activation loop (Figs. 3–5; Inoue et al., 2008a), suggesting that autophosphorylation may be a common step for downstream signaling for all of the phot-mediated responses. Each signaling likely diverges from the interacting partners of phototropins. In fact, some phototropin-interacting proteins, such as NONPHOTOTROPIC HYPOCOTYL3 (NPH3), ROOT PHOTOTROPISM2 (RPT2), and PHYTOCHROME KINASE SUBSTRATE1 (PKS1), PKS2, and PKS4 were found in Arabidopsis (Motchoulski and Liscum, 1999; Sakai et al., 2000; Inada et al., 2004; Lariguet et al., 2006), and they function as signal transducers in the distinct responses (Demarsy and Fankhauser, 2009). NPH3 acts in leaf flattening, leaf positioning, and phototropism, and contributes to growth enhancement in response to blue light, but does not affect the rapid inhibition of hypocotyl growth, chloroplast movement, and stomatal opening (Folta and Spalding, 2001; Inada et al., 2004; Inoue et al., 2008b; de Carbonnel et al., 2010). RPT2 only serves in phototropism and stomatal opening (Inada et al., 2004). PKSs regulate phototropism, leaf positioning, and leaf flattening and are not responsible for chloroplast movement and stomatal opening (Lariguet et al., 2006; Boccalandro et al., 2008; Schepens et al., 2008; de Carbonnel et al., 2010). PKS1 was reported to be phosphorylated by blue light (Lariguet et al., 2006) and PKSs may be substrates of phototropin kinases, although the phosphorylation of other PKSs has yet to be determined. The divergence mechanism of blue-light signaling via phosphorylation is an important subject in the study of phototropin responses (Inoue et al., 2010).

We should note that the phot2-mediated responses in the S761A S763A-3 line were strongly, but not completely, impaired (Figs. 3–5). In contrast, the impairment of phot1-mediated responses in the S849A S851A-6 line was almost complete (Inoue et al., 2008a). Phot2 seems to have additional phosphorylation sites in the activation loop that are required for signaling. Human PAS kinase, which has a high structural homology to phototropins in the functional domains, undergoes autophosphorylation of Thr-1161 and Thr-1165 in its activation loop (Rutter et al., 2001; Tokutomi et al., 2008). Thr-1161 and Thr-1165 correspond to Ser-
763 and Thr-767 in phot2 in the sequences, respectively (Supplemental Fig. S6A), and thus autophosphorylation of Thr-767 may be important for phot2-mediated responses. Therefore, we transformed the phot1 phot2 double mutant with the phot2 mutant construct of T767A or T767D and performed functional analyses. The T767A-2 line exhibited impairment in phot2-mediated responses (Supplemental Fig. S6B), but this is probably due to the loss of kinase activity (Supplemental Fig. S6C). The transgenic lines expressing the phot2 T767D construct could not be obtained (Supplemental Fig. S6D).

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

Plants of Arabidopsis (Arabidopsis thaliana) gl1 (Col background), gl1 phot1-5, gl1 phot2-1, and gl1 phot1-5 phot2-1 mutant and all of the transgenic plants were grown on soil for 3 to 5 weeks under a 14-h fluorescent light (50 μmol m⁻² s⁻¹)/10-h dark cycle. The plants were grown at 24°C under relative humidity of 55% to 75%. These plants were used for functional analyses, including chloroplast movement, stomatal opening, leaf flattening, and growth. Etiolated seedlings were kept in the dark or illuminated with blue light at 500 μmol m⁻² s⁻¹/C176.

**Identification of Phosphorylation Sites**

Isolation of Phot2 by Immunoprecipitation for Etiolated Seedings

Detection of Phot2 Phosphorylation Levels in Etiolated Seedlings

**Generation of Polyclonal Antibodies**

The N-terminal region of PHOT2 (PHOT2N) was expressed in Escherichia coli cells, and the recombinant protein was purified and used as an antigen. A 357-bp cDNA fragment encoding Met-1 to Pro-118 of PHOT2 was amplified by PCR. The amplified DNA fragment was cloned into the pET30a vector (Novagen), and the plasmids were transformed into the E. coli BL21 strain. The polypeptide was expressed as a fusion protein with His-tag and was purified using the Profinity IMAC Ni-charged resin (Promega). The His-tagged PHOT2N was obtained by elution with imidazole and was used to immunize the rabbits (AGC TechnoGlass).

**Immunoblotting and Protein Blotting**

Immunoblotting of phot1 and phot2, and protein blotting of a 14-3-3 protein (GF-14a) to phot1 and phot2 (Supplemental Fig. S3), were performed according to previous methods (Kinoshiba and Shimazaki, 1999; Kinoshiba et al., 2003).

**Detection of Phot2 Phosphorylation Levels in Etiolated Seedlings**

One-thousand etiolated seedlings (WT-11 or D720N-1 line; see Table I) were kept in the dark or illuminated with blue light at 500 μmol m⁻² s⁻¹ for 1 min. Microsomal membranes were prepared, and 300 μg microsomal proteins was used for immunoprecipitation as described previously (Inoue et al., 2008a). Immunoprecipitation was performed using specific antibodies against PHOT2N. Phosphorylation of the immunopurified phot2 was determined by blotting with phos-tag-biotin (NARD Institute Ltd.) according to a previous method (Kinoshiba et al., 2006).

**Isolation of Phot2 by Immunoprecipitation for Identification of Phosphorylation Sites**

Six-thousand etiolated seedlings of the WT-11 line were illuminated with blue light at 500 μmol m⁻² s⁻¹ for 2 min. Microsomal membranes (2.5 mg) were immediately prepared from the seedlings of the WT-11 line and were used for immunoprecipitation. The immunopurified phot2 proteins were subjected to SDS-PAGE and used for mass spectrometric analyses, Coomassie Brilliant Blue staining, phos-tag blotting, and immunoblotting.

Determination of in Vivo Phosphorylation Sites by Mass Spectrometric Analysis

The phosphorylation sites of phot2 proteins were identified by LC-MS/MS analysis according to a previous method (Inoue et al., 2008a).

**Construction of Transformation Vector**

We aimed to purify phot2 protein from etiolated seedlings to determine the phosphorylation sites by mass spectrometric analysis (Fig. 7). Since native PHOT2 promoter seems to have low activity in the dark (Jarillo et al., 2001; Kagawa et al., 2001; Aihara et al., 2008), we expressed the mutant phot2 proteins under the control of native PHOT1 promoter. We changed a previously generated vector bearing the genomic phot1 gene (Inoue et al., 2008a) and constructed a gene transfer vector bearing the PHOT2 cDNA under the control of the native PHOT1 promoter, as follows. The inverse PCR was performed using the oligonucleotide primers 5'-CCA-TGGTTTTGAGATTTGGACTAAACGTTTATCG-3' and 5'-CATCCCATGTTATCAGAGGCGCAGCGTACG3'. The phosphorylated linear DNAs were self ligated. The full-length PHOT2 cDNA was amplified by reverse transcription-PCR using the total RNA from wild-type seedlings with oligonucleotide primers 5'-TACCCATGTTATGAGCAGCGCAGCGTACG3' and 5'-CACCCATGTTATCAGAGGCGCAGCGTACG3'. The cDNA was cloned into the vector with the Ncol site. The resulting vector was verified by DNA sequencing.

**Site-Directed Mutagenesis of Gene Transfer Vector**

Single and multiple amino acid substitutions were done using the QuickChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Nucleotide substitutions were introduced into the PHOT2 gene in the gene transfer vector as templates for PCR reactions. The PCR reactions were conducted using oligonucleotide primers: for D720N, 5'-GCTGACATAAGCTGCTTTTCTGCTGCTTGTTAGC3' and 5'-CTGTGATGTTGAATCGCTGTGTACG3'; for S761A, 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'; for T762A, 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'; for T762D, 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'; for T762D/S761A 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'; for T762D/S763, 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'; for T762D/S763/S761A 5'-CCATCTCGAGGGCATGCGCAGGCGGACG3' and 5'-GGTCTCTGAGGGCATGCGCAGGCGGACG3'. All constructs were sequenced to verify specific mutations.

**Transformation of Arabidopsis**

Arabidopsis plants were transformed by an Agrobacterium tumefaciens-mediated method (Clough and Bent, 1998). We selected the transgenic plants according to their resistance against hygromycin on the agar plates.

**Phenotypic Analyses**

Measurements of chloroplast accumulation and avoidance responses, stomatal aperture, phototropic curvature, and leaf flattening were performed according to previous methods (Inoue et al., 2008a).

**Preparation of Mesophyll Cell Protoplasts**

Mesophyll cell protoplasts were prepared enzymatically from 4- to 5-week-old Arabidopsis plants as reported previously (Ueno et al., 2005) and kept in the dark until use. The protoplasts were used for the experiments of blue-light fluence rate dependencies of autophosphorylation in phot1 and phot2 (Supplemental Fig. S3).
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF030864 and AF053941.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison of phot-mediated responses of Col with those of gi1.

Supplemental Figure S2. Chloroplast positioning of mesophyll cells in the WT-11 and the S761A/S763A-3 leaves under the strong blue light.

Supplemental Figure S3. Differences in autophosphorylation between phot1 and phot2.

Supplemental Figure S4. Chloroplast positioning in WT-11 and S761D S763D-3 lines.

Supplemental Figure S5. Fluence rate-dependent curves of hypocotyl phototropism in phot1 mutant and the WT-11 line.

Supplemental Figure S6. Functional analysis of potential autophosphorylation sites in the activation loop of phot2.

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