A Dominant Mutation in the Light-Oxygen and Voltage2 Domain Vicinity Impairs Phototropin1 Signaling in Tomato1[C][W][OPEN]

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In higher plants, blue light (BL) phototropism is primarily controlled by the phototropins, which are also involved in stomatal movement and chloroplast relocation. These photoresponses are mediated by two phototropins, phot1 and phot2. Phot1 mediates responses with higher sensitivity than phot2, and phot2 specifically mediates chloroplast avoidance and dark positioning responses. Here, we report the isolation and characterization of a Nonphototropic seedling1 (Nps1) mutant of tomato (Solanum lycopersicum). The mutant is impaired in low-fluence BL responses, including chloroplast accumulation and stomatal opening. Genetic analyses show that the mutant locus is dominant negative in nature. In dark-grown seedlings of the Nps1 mutant, phot1 protein accumulates at a highly reduced level relative to the wild type and lacks BL-induced autophosphorylation. The mutant harbors a single glycine-1484-to-alanine transition in the Hinge1 region of a phot1 homolog, resulting in an arginine-to-histidine substitution (R495H) in a highly conserved Aα helix proximal to the light-oxygen and voltage2 domain of the translated gene product. Significantly, the R495H substitution occurring in the Hinge1 region of PHOT1 abolishes its regulatory activity in Nps1 seedlings, thereby highlighting the functional significance of the Aα helix region in phototropic signaling of tomato.

Being sessile in nature, plants have developed diverse sets of sensory mechanisms, integrating external cues such as light, water, and temperature to adapt their growth and development to the ambient environment. Plants have evolved a cohort of photoreceptors such as red/far-red light-sensing phytochromes (Chen and Chory, 2011), UV-A/blue light (BL)-sensing phototropins (Christie, 2007; Holland et al., 2009; Suetsugu and Wada, 2013), cryptochromes (Yu et al., 2010; Liu et al., 2011), Zeusipte (ZTL)/Flavin-binding, Kelch repeat, F-box protein1/light-oxygen and voltage (LOV)-kelch protein2 members of the ZTL/ADAGIO putative family of photoreceptors (Suetsugu and Wada, 2013), and UV-B light-sensing UV-B resistance8 (Heijde and Ulm, 2012), enabling them to sense nearly the full range of the solar spectrum. One of the most visually obvious photoresponses of flowering plants involves the growth and orientation of organs toward or away from light, particularly during the early stages of growth and the establishment of seedlings (Iino, 1990) and during gap-filling situations in dense canopy conditions (Ballaré, 1999) for optimizing photosynthesis and interspecies/intraspecies competition. Several studies involving the relative effectiveness of different wavelengths of the solar spectrum as well as monitoring of lateral differences in light intensity revealed that the directional growth of plants is specifically mediated by the UV-A/blue region of the visible spectrum. Molecular genetic analysis of Arabidopsis (Arabidopsis thaliana) mutants inhibited in hypocotyl curvature toward BL revealed that, among the UV-A light/BL-specific photoreceptors, the phototropins perceive ambient light as a cue for directional growth (Liscum and Briggs, 1995; Kagawa et al., 2001).
Phototropins have been identified in several plant species, ranging from the green alga *Chlamydomonas reinhardtii* to higher plants (Briggs et al., 2001). To date, two members of the phototropins have been reported from higher plants, phot1 and phot2, which share sequence homology (Sakai et al., 2001). Physiological analyses with Arabidopsis mutants lacking phot1 and phot2 have revealed that, in addition to regulating the hypocotyl curvature of seedlings toward BL (Huala et al., 1997; Christie et al., 1998), phototropins also regulate a diverse range of responses in flowering plants (Christie and Murphy, 2013; Hohm et al., 2013). These responses include chloroplast movements (Sakai et al., 2001), nuclear positioning (Iwabuchi et al., 2007), stomatal opening (Kinoshita et al., 2001), sun tracking (Inoue et al., 2008b), leaf flattening (Sakamoto and Briggs, 2002), and the rapid inhibition of the growth of etiolated hypocotyls (Folta and Spalding, 2001).

While both phot1 and phot2 overlap in function in regulating phototropism, chloroplast accumulation, leaf expansion, and stomatal opening, they also exhibit differential photosensitivity to BL, where phot1 is more sensitive to low-fluence BL than phot2. Both phot1 and phot2 redundantly regulate the chloroplast accumulation toward low-fluence BL, and phot2 exclusively regulates the chloroplast avoidance from high-fluence BL (Jarillo et al., 2001; Kagawa et al., 2001), while phot1 solely mediates the rapid inhibition of the elongation of etiolated hypocotyls (Folta and Spalding, 2001). Analysis of mutants downstream of blue light perception by phototropins revealed that the phototropin signaling branches out at an early step, and phot1 and phot2 trigger distinct photoresponses recruiting multiple signaling partners (Christie and Murphy, 2013; Hohm et al., 2013).

Molecular characterizations have shown that phototropins are plasma membrane-associated Ser/Thr kinases containing a photosensory domain (Briggs and Christie, 2002) in the N-terminal region composed of two LOV domains (LOV1 and LOV2) and the kinase domain at the C-terminal end. The LOV1 and LOV2 domains bind the FMN as chromophore and are responsible for BL sensing by phototropin. Although phototropins characteristically possess two LOV domains, the photoregulation of phototropin activity is predominantly mediated by LOV2 (Christie, 2007). The exposure to BL also causes adduct formation between the FMN and the Cys residue in LOV domains and leads to the phosphorylation of phototropin, which is believed to be the primary step in the transmission of phototropic signals (Christie et al., 1998; Sakai et al., 2000). To decipher the functions of different domains of phototropins, many different substitution mutants of phototropins have been generated, which have enabled the elucidation of the functional significance of the different domains (Matsuoka and Tokutomi, 2005; Jones et al., 2007; Kong et al., 2007; Inoue et al., 2008a). Inoue et al. (2008a) showed that the BL-induced autophosphorylation of Ser-851 in the C-terminal kinase domain of phototropin is the primary step for initiating stomatal opening, phototropism, chloroplast accumulation, and leaf flattening. Mutational studies also revealed that the photosensory N-terminal domain of phototropin acts as a kinase inhibitor, where the LOV2 domain inhibits the activity of kinase domain by binding to it, and BL exposure is required for the dissociation of the LOV2 domain, enabling phosphorylation of the kinase domain (Matsuoka and Tokutomi, 2005; Jones et al., 2007).

While our current understanding of phototropism has been greatly facilitated by the isolation of phototropins and their signaling mutants, the phot mutants identified to date are loss-of-function alleles. The lack of dominant-negative alleles or alleles with increased sensitivity to phototropic stimulus has hindered exploration into the roles of different domains of phot proteins in regulating phototropic signaling. In addition, the dearth of mutants defective in phototropin or phototropin-mediated responses has been a major bottleneck in furthering our understanding of the function of phototropins in crop species. Although phototropin homologs have been identified from a variety of crop species, including oat (*Avena sativa*; Zacherl et al., 1998), rice (*Oryza sativa*; Kanegae et al., 2000), and tomato (*Solanum lycopersicum*; Sharma et al., 2007; Sharma and Sharma, 2007), only the coleoptile *phototropin1* mutant of rice has been isolated, which is defective in BL phototropism (Haga et al., 2005).

Here, we report that in a mutant screen for non-phototropic seedlings under continuous BL, we recovered a strong dominant-negative mutation of *phot1*. The dominant-negative mutations are useful to elucidate redundant functions, as mutant protein in addition to suppressing its own functions can also suppress the function of its partners. The characterization of this new *phot1* mutant revealed that the dominant activity is caused by the substitution of an Arg residue located in the Aα helix in the Hinge1 region between the LOV1 and LOV2 domains. Our study shows the functional importance of the Aα helix (Halavaty and Moffat, 2007) in regulating phot1-mediated signaling in tomato.

**RESULTS**

**The Nonphototropic seedling1 Mutant Lacks Low-Fluence Phototropic Curvature**

The *Nonphototropic seedling1* (*Nps1*) mutant was isolated in a screen for nonphototropic tomato seedlings exposed to prolonged (12-h) unilateral low-fluence BL (Fig. 1A). The phototropic response of seedlings of the F2 population obtained by reciprocal crossing of the *Nps1* mutant with the wild type showed segregation of the *Nps1* and wild-type phenotypes at a ratio close to 3:1 (434 of 605 seedlings showed segregation of the *Nps1* and wild-type phenotypes at a ratio close to 3:1 (434 of 605 seedlings showed segregation of the *Nps1* and wild-type phenotypes). This result indicated that the *Nps1* phenotype is caused by a dominant-negative mutation at a single locus. *Nps1* seedlings showed a total loss of...
hypocotyl phototropism toward low-fluence BL (0.1–1 μmol m⁻² s⁻¹), indicating that the mutation at the Nps1 locus may have disrupted the function of phot1 (Fig. 1, B and C), since low-fluence BL phototropism is principally mediated by phot1. On exposing Nps1 seedlings to high-fluence BL (100 μmol m⁻² s⁻¹), the seedlings displayed curvature (approximately 20°) similar to that of the wild type (Fig. 1D), whereas the Nps1 seedlings exhibited a partial response to low-fluence BL (10 μmol m⁻² s⁻¹) similar to that of the phot1 mutant in Arabidopsis (Sakai et al., 2001). Surprisingly, the increasing BL fluence led to diminished curvature in wild-type seedlings similar to that observed for the glabrous1 mutant of Arabidopsis (Sullivan et al., 2008).

Figure 1. Isolation and phenotypic characterization of the Nps1 mutant. A, Isolation of a nonphototropic mutant of tomato (red arrow) under continuous directional BL (indicated by the blue arrow). B, Nps1 seedlings show no phototropic response to 0.1 or 1 μmol m⁻² s⁻¹ 3-h BL exposure. The white arrow indicates the direction of light. C and D, Phototropic curvature after low-fluence (C) and high-fluence (D) BL exposure. The response in D was measured after 6 h. E and F, Gravitropic curvature of etiolated (E) and deetiolated (F) seedlings. G and H, Hypocotyl (G) and root (H) lengths of 3-d-old seedlings. 1 and J, Representative leaflets from the 10th node of a 1-month-old plant: three terminal leaflets (I) and leaflets from the midrachis (J). WT, Wild type. [See online article for color version of this figure.]
Since the display of organ curvature is mediated by auxin, we also ascertained whether the observed lack of phototropic curvature was due to a reduction in auxin sensitivity of mutant hypocotyls. However, horizontally oriented mutant and wild-type seedlings showed a nearly similar time course of geotropic curvature, eliminating the above possibility (Fig. 1, E and F). Apart from the apparent disruption of the phototropic responses, Nps1 mutation also affected vegetative growth. Hypocotyls of etiolated seedlings of Nps1 were slightly shorter than those of the wild type (Fig. 1G). In the case of roots, both etiolated and deetiolated Nps1 seedlings showed shorter roots compared with the wild type (Fig. 1H). Under greenhouse conditions, Nps1 plants were dwarf compared with the wild type. An interesting feature of the mutant was altered morphology of the leaflets, with an increase in size, reduced serration of the leaf margin, and an increase in the size of leaflet lobes (Fig. 1, I and J).

The Nps1 Mutant Is Defective in the PHOT1 Homolog of Tomato

The loss of phototropism in Arabidopsis mutants grown under low-fluence BL is mostly caused by a defect or complete absence of phot1 (Liscum and Briggs, 1995; Christie et al., 1998). Therefore, the Nps1 mutant could possibly have either an insufficient amount or nonfunctional homolog of phot1. Western analysis of wild-type extract probed with Arabidopsis anti-PHOT1 antibodies (Doi et al., 2004) detected a 124-kD band, which is close to the molecular mass reported for tomato phot1 (Knieb et al., 2004). While a band of the same size was also detected in the mutant, its intensity was less than 10% of the intensity in the wild type (Fig. 2A). In contrast, the level of phot2 protein in the mutant and the wild type was nearly similar (Fig. 2B). Surprisingly, in F1 seedlings, western analysis showed a near absence of the phot1 band, indicating that mutated phot1 protein affected the stability of wild-type protein (Supplemental Fig. S1). Consistent with the above reduction, F1 seedlings showed highly reduced (80% inhibition) phototropic curvature (less than 5%).

The possibility that the protein detected by Arabidopsis anti-PHOT1 was indeed a receptor for BL was examined by studying BL-mediated phosphorylation of proteins in homogenates of etiolated wild-type and mutant seedlings. In vitro phosphorylation studies (Salomon et al., 2003) revealed BL-induced phosphorylation of a protein of approximately 124 kD in wild-type extracts, whereas there was an absence of it in the mutant extracts (Fig. 2C). Interestingly, the far-red insensitive (fri) mutant of tomato, which lacks low-fluence BL phototropism, retains BL-induced phosphorylation of a protein of similar molecular mass as in the wild type (Srinivas et al., 2004).

We pulled out two full-length complementary DNAs (cDNAs) encoding tomato PHOT1 (EF063359) and PHOT2 (EU021291) by cDNA library screening and subsequent RACE. Both Slphot1 and Slphot2 contain LOV1, LOV2, and Ser/Thr kinase domains, which are typically conserved in other reported phototropins from plants. The deduced protein sequences of Slphot1 consisted of 1,018 amino acids and shared 70% and 59% identity with Atphot1 and Atphot2, respectively, while Slphot2 consisted of 952 amino acids and had 68% and 61% identity with Atphot2 and Atphot1, respectively (data not shown).

Since physiological and biochemical evidence indicates that the Nps1 mutant is defective in phototropin-mediated responses, we next investigated the expression of SIPHOTs by reverse transcription (RT)-PCR analysis in etiolated and BL-treated wild-type and Nps1 seedlings. Interestingly, there was no detectable difference in the expression of PHOT1 and PHOT2 transcripts in both the wild type and the mutant. While the expression of PHOT1 transcripts was higher in etiolated seedlings for both the wild type and the mutant, the expression of PHOT2 transcripts was enhanced in seedlings irradiated with 25 μmol m⁻² s⁻¹ BL (Fig. 3A). This result agrees with the previous observations that light induces the expression of the PHOT2 gene (Kanegae et al., 2000; Jarillo et al., 2001; Kagawa et al., 2001).

The cloning and sequencing of PHOT1 and PHOT2 cDNA from the Nps1 mutant revealed a Gly-1484-to-Ala
transition in the mutant PHOT1, resulting in the replacement of Arg-495 with His (R495H) in the translated mutant protein in the Hinge1 region (Fig. 3B). The mutation is located in a highly conserved Aα helix in the N-terminal helix-turn motif (GIDLATTLERIEK) prior to the LOV2 domain in all available phot1 and phot2 sequences from the green alga C. reinhardtii to higher plants (Fig. 3C; Halavaty and Moffat, 2007). We also ascertained the presence of a mutation in phot1 by using CEL I endonuclease-mediated digestion of mismatch in heteroduplexes between wild-type and Nps1 mutant CDNA using the standard protocol of TILLING (Till et al., 2006). In conformity with the expected location of the mutation, CEL I digestion generated two bands of approximately 1,350 and 250 bp only when cDNA from the wild type and Nps1 were combined, whereas with either Nps1 or wild-type cDNA, no cDNA bands were seen (Fig. 3D).

Figure 3. Localization of the mutation in the Aα region of phot1. A, Expression of SIPHOT1 and SIPHOT2 transcripts in dark-grown seedlings (D) or BL-exposed (2 h; 25 μmol m⁻² s⁻¹) seedlings (L). ACTIN was used as an internal standard for cDNA amounts. B, Chromatogram of the sequence of wild-type (WT) tomato PHOT1 and Nps1 PHOT1 depicts the single-base-pair change of Gly-1484 to Ala (arrow). C, Alignment of the Hinge1 region of phototropins. The arrow indicates the Aα-to-His substitution in the predicted Aα region of Nps1 mutant phot1. The locations of the Aα region and the LOV2 region are shown by red and green lines above the protein sequences, respectively. Alignment of Hinge1 and LOV2 domains of phototropins was carried out on protein sequences from tomato (phot1 and phot2), Arabidopsis (phot1 and phot2), pea (Pisum sativum; phot1), Vicia faba (phot1a and phot1b), maize (Zea mays; phot1), bean (Phaseolus vulgaris; phot1a, phot1b, and phot2), rice (Oryza sativa; phot1 and phot2), oat (phot1a and phot1b), Physcomitrella patens (phot1a, phot2A, and phot2B), Adiantum capillus-veneris (phot1 and phot2), Mougetia scalaris (photA and photB), and C. reinhardtii (phot1). Sequence alignments were carried out using the program BOXSHADE 3.21 (www.ch.EMBnet.org). D, Mutation detection in Nps1 by CEL I cleavage. The cartoon on the left side of the gel shows the likely site of CEL I cleavage, and green and blue stars indicate the positions of IRD700- and IRD800-labeled primers. The cDNA (1,597 bp) from the wild type and the Nps1 mutant was PCR amplified, denatured, and renatured to make heteroduplexes prior to CEL I digestion. The image of the cleavage reaction is collected from the individual wells as the wild type (lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, and M). M denotes dye-labeled DNA size ladders whose molecular weights are indicated on the sides of the gels. The sizes of the cleavage products (boxes) from the individual dye-labeled DNA strands add up to the size of the full-length PCR product (boxes) on top of the gel. [See online article for color version of this figure.]
The R495H Substitution Possibly Alters the Conformation of the Ja Helix in the Mutant Protein

Three-dimensional (3-D) models of wild-type and Nps1 phot1-LOV2 domains against the oat phot1-LOV2 crystal (Protein Data Bank identifier 2v1b; Halavaty and Moffat, 2007), although nearly identical in the protein backbone structures, indicated a shift in the interaction between Asp-517 and His-495 in the mutant protein, as compared with that with Arg-495 in the wild type (Fig. 4A). Oat phot1 contains four bound water molecules between Arg-410 and Asp-432, which correspond to Arg-495 and Asp-517 in the predicted protein structures for tomato phot1. Similar to oat, in tomato too, four water molecules can reside between Arg-495 and Asp-517 in the wild type phot1 but not in the Nps1 phot1 (Fig. 4B). The mutant phot1 does not have enough space for the water molecules, as displayed by a space-filling model, where the water molecule H2O-1 in the mutant is partly overlapped with His-495 (Fig. 4C, right, red arrow). The altered interaction between H2O-1 and His-495 and/or H2O-1 and Asp-517 in the mutant may result in the displacement of the water molecule and/or conformational modification at around His-495 and/or Asp-517 (Fig. 4). Since Asp-517 is located at the N-terminal end of the Ca helix in the rigid core of the LOV domain, protein conformation is more likely to be modified at around His-495 in the A’α helix. Since the A’α helix also interacts with the Ja helix (Fig. 4D, top, white circles), the modification at His-495 may alter the interaction of the A’α helix with the Ja helix. This altered interaction could lead to partial unfolding and displacement of the Ja helix away from the LOV2 core in the mutant (Fig. 4D, bottom right, green arrows), as has been observed in the mutated oat phot1 (Harper et al., 2004). Moreover, this displacement could cause the observed instability of the mutant phot1.

Nps1 Is Inhibited in phot1-Specific Chloroplast Accumulation But Shows Normal phot2-Mediated Chloroplast Movement

The partial phototropic defect was observed in Nps1 at 10 μmol m−2 s−1 (Fig. 1D). At this intensity, phot2 can elicit at least a partial response in Arabidopsis. Hence, we further examined whether the other phot2-mediated responses are altered in the Nps1 mutant. The BL-induced movements of chloroplasts in plants have been shown to be differentially regulated by

Figure 4. Models of wild-type (WT; left, green) and Nps1 (right, cyan) phot1-LOV2 regions. A, At top, ribbon models of Slphot1-LOV2 show the wider space between Arg-495 and Asp-517 in the wild type compared with a narrow space between His-495 and Asp-517 in Nps1. At bottom is an enlarged view of the space between Arg-495 and Asp-517 of the wild type and between His-495 and Asp-517 of Nps1. B, Four water molecules between Arg-495 and Asp-517 in the wild type and Nps1 are displayed. Yellow spheres indicate the oxygen atom of the water molecules. C, Residues Arg-495, His-495, and Asp-517 from B are indicated with space-filling models. The red arrow indicates that one of the overlaid water molecules, H2O-1, is partly overlapped with the imidazole ring of His-495. D, Models viewed in the direction highlighting the interactions between the A’α helix and the Ja helix. The A’α helix, the Ja helix, and the four water molecules are shown in purple, orange, and yellow, respectively. At top, white circles on the surface models indicate the interactions between the A’α helix and the Ja helix (the red arrow is as in C). At bottom, four water molecules, Arg-495 of the wild type, and His-495 of Nps1 are shown by the space-filling model in the cartoon representation (above). Green arrows indicate the path of conformational alteration relay from the A’α helix to the Ja helix due to the mutation of Arg-495 to His-495 in Nps1.
phototropins in Arabidopsis (Sakai et al., 2001). While phot1 mediates only the chloroplast accumulation response to a wide range of BL (0.1–100 μmol m⁻² s⁻¹), phot2 elicits both accumulation at around 2 to 16 μmol m⁻² s⁻¹ and avoidance at more than 32 μmol m⁻² s⁻¹. When wild-type tomato cotyledons/leaves were exposed to BL, the chloroplasts showed normal light-induced movements, as reported earlier in Arabidopsis and also in tomato (Srinivas et al., 2004). However, in the Nps1 mutant subjected to low-fluence BL (0.6 μmol m⁻² s⁻¹), the chloroplasts remained fixed on the anticlinal walls of the mesophyll cells of leaves while those of the wild type were spread evenly over the entire surface (Fig. 5A), indicating loss of the phot1-mediated accumulation response in Nps1.

We then examined the chloroplast positions at higher fluence (60 μmol m⁻² s⁻¹). In both the wild type and Nps1, chloroplasts were positioned on the anticlinal side of the cell, suggesting that the phot2-mediated avoidance response was functional in Nps1. However, because of the lack of accumulation at 0.6 μmol m⁻² s⁻¹ in Nps1, it is possible that chloroplasts remained in the dark position regardless of the light conditions in this mutant. Indeed, examination of vertical sections of dark-adapted cells showed that chloroplasts accumulate at the bottom of these cells both in darkness and at 60 μmol m⁻² s⁻¹ BL (Fig. 5B). On exposure to 0.6 μmol m⁻² s⁻¹ BL, while some chloroplasts in wild-type cells rose to the top of cells and spread around, this movement was absent in the Nps1 mutant.

In view of the unusual positioning of chloroplasts in the Nps1 mutant, it was difficult to discriminate between the accumulation and avoidance responses. Since phot1 is mutated in Nps1, in view of redundancy in the action of phot1 and phot2, we considered the possibility that chloroplast accumulation can be mediated by phot2 at a little higher fluence of BL (Sakai et al., 2001). Consistent with this, increase in BL fluence to 60 μmol m⁻² s⁻¹ triggered the chloroplast accumulation in Nps1 leaf cells, albeit less than in the wild type (Fig. 5C). The time-lapse imaging of chloroplast movement after irradiation with a microbeam (Kong et al., 2007) with 2.4 or 5 μmol m⁻² s⁻¹ BL showed distinct accumulation of chloroplasts in the mutant in the illuminated section of the cell (Supplemental Fig. S2). These experiments indicated that the Nps1 mutant did retain the chloroplast accumulation response but that it was mediated by phot2. To further ascertain whether Nps1 retains normal chloroplast avoidance, after chloroplast accumulation we irradiated a section of the cell with a range of high-fluence BL (10–40 μmol m⁻² s⁻¹; Supplemental Fig. S2). Consistent with active phot2 function, the accumulated chloroplasts in Nps1 moved away from the irradiated area.

The Nps1 Mutant Lacks phot1-Induced Stomatal Opening

Stomatal opening is regulated by many external stimuli, such as light, temperature, and humidity, of which only the light receptors have been identified to date (Kinoshita et al., 2001), including both phot1 and phot2. Using plants grown under normal day/night conditions, we examined whether the phot1 mutation affected the rhythm of stomatal opening. Interestingly, Nps1 plants grown under normal day/night conditions display an unaltered rhythm of stomatal movement, while the extent of opening of the stomatal pores was reduced compared with the wild type under the same conditions (Fig. 6A). We next examined stomatal opening in the Nps1 mutant and the wild type using a gas-exchange method to determine on-vine stomatal conductance in the leaflets. We used two different intensities of BL to distinguish between phot1- and phot2-mediated

Figure 5. Chloroplast relocation in wild-type (WT) and Nps1 plants. The plants were dark adapted for 12 h before BL treatment. Chlorophyll autofluorescence was detected by confocal microscopy. A, Surface view of mesophyll cells exposed to 0.6 and 60 μmol m⁻² s⁻¹ BL. B, Transverse section of cells prepared from A. C, Surface view of mesophyll cells exposed to 6 and 30 μmol m⁻² s⁻¹ BL. [See online article for color version of this figure.]

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stomatal opening. The dark-adapted leaves were exposed to red light (RL) to saturate the photosynthesis and achieve a steady level of stomatal conductance. A superimposition of BL (2 μmol m⁻² s⁻¹) on the RL background linearly increased stomatal conductance in the wild type for 20 min and then attained steady state (Fig. 6B). In contrast, in the Nps1 mutant, the above BL exposure did not alter stomatal conductance. Increasing BL fluence to 100 μmol m⁻² s⁻¹ further increased stomatal conductance in wild-type leaflets, whereas in the Nps1 mutant a paltry increase was observed. (Fig. 6C). These results were consistent with the loss of phot1-specific responses in the Nps1 mutant but were equivocal for the role of phot2 in stomatal conductance.

Cotyledon Opening Is Delayed in the Nps1 Mutant

One of the characteristic phenotypes of the light-grown Nps1 seedlings was sluggish opening of cotyledons compared with the wild type. While phytochromes have been suggested to regulate cotyledon opening in Arabidopsis (Hennig et al., 2002), there is no report of phototropins playing a role in this process. Time-lapse imaging of seedlings grown under white light (WL) revealed that the opening of both cotyledons and petiole was extremely delayed in the Nps1 seedlings compared with the wild type as well as in fri mutant seedlings (Fig. 7A). Even after 120 h of light exposure, the cotyledons and petioles of Nps1 seedlings showed reduced curvature compared with the wild type and the fri mutant (Fig. 7, B and C). Nps1 also showed slightly reduced cotyledon expansion under WL (Fig. 7, D–F). Our results substantiate that, similar to Arabidopsis, phytochrome A plays a role in regulating cotyledon opening in tomato seedlings. Additionally, the lesser amplitude of opening of both cotyledons and petiole in the Nps1 seedlings suggests the involvement of phot1 in concert with phytochrome responses during the deetiolation of tomato seedlings.

Nps1 Seedlings Display Altered Hypocotyl Growth Orientation in Weak BL

In young seedlings of Arabidopsis grown under omnilateral weak BL, the normal gravitropic growth orientation of the hypocotyl response is seemingly lost in the phot1-deficient mutants (Lariguet and Fankhauser, 2004). The lack of a phototropic response in the Nps1 mutant raised the question of whether a similar response would occur in the Nps1 seedlings. When dark-germinated wild-type and mutant seedlings were grown under continuous omnilateral low-fluence BL, the mutant seedlings showed altered hypocotyl growth orientation very similar to that of the phot1 seedlings of Arabidopsis (Fig. 8A). The orientation of the hypocotyl of the Nps1 seedlings varied from around 70° to 110° with respect to the vertical plane (Fig. 8, B and C). When BL was supplied from underneath, all wild-type seedlings aligned themselves to the light stimulus against the gravity vector. On the other hand, the mutant seedlings did not align to the direction of BL but showed altered hypocotyl growth orientation similar to BL exposure from above (Fig. 8, D–G). These results suggest that the Nps1 mutant may be defective in a component that determines hypocotyl growth orientation during the early stages of photomorphogenesis of tomato seedlings. Although Nps1 mutant seedlings lacked phototropin-mediated responses, they displayed BL-mediated inhibition of hypocotyl growth similar to wild-type seedlings (Fig. 8H). Considering that Arabidopsis mutants deficient in phot1 display a similar orientation of hypocotyl under comparable conditions, the above results are consistent with the Nps1 mutant bearing a defect in tomato phot1.

DISCUSSION

The Nps1 Mutant Is Impaired in Responses Involving Phot1

In plants, phot1 and phot2 redundantly mediate low- and high-fluence BL responses. The phot1 mutant of Arabidopsis, which is a null mutant of PHOT1, retains phototropic responses at high light intensities, while the phot2 mutant, containing a deletion of seven nucleotides in PHOT2 (Kinoshita et al., 2001), is not
impaired in its phototropic responses. Even though both genes function redundantly, they have also been described to have separate and distinct pathways. Like the phot1 mutant of Arabidopsis, our physiological and sequencing data conclusively show that the mutation in PHOT1 of the Nps1 mutant of tomato disrupts the responses that are principally controlled by phot1 in Arabidopsis (Kinoshita et al., 2001). However, unlike any known phototropin mutant, the mutation in the Nps1 locus dominantly impairs most of the phototropin-mediated responses. The loss of low-fluence BL responses in Nps1 seedlings, therefore, provides an interesting insight into the role for phot1 in the general phototropic signaling pathway of tomato seedlings.

One of the enigmatic features of the Nps1 mutant was very strong impairment of chloroplast accumulation, which consequently affected the chloroplast avoidance movement. It is believed that low-fluence light inducing chloroplast accumulation is perceived by plasma membrane-localized phot1 and phot2 (Sakai et al., 2001), whereas chloroplast envelope-localized phot2 triggered the high-fluence light-mediated avoidance response (Kong et al., 2013). Considering that Nps1 seedlings lack low-fluence (0.6 μmol m$^{-2}$ s$^{-1}$) BL-mediated phototropic bending of seedlings, the observed impairment of chloroplast accumulation appears to be related to the loss of phot1 function. Consistent with this, phot2-mediated chloroplast accumulation in Nps1 seedlings can be observed, albeit at a higher fluence of BL (6.0 μmol m$^{-2}$ s$^{-1}$; Sakai et al., 2001). Since the accumulated chloroplasts show avoidance movement away from the zone of high-fluence BL (10–40 μmol m$^{-2}$ s$^{-1}$) exposure, it supports the view that the Nps1 mutant retains phot2 functions, although at reduced magnitude compared with the wild type.

Stomatal opening is regulated by many external stimuli, such as light, temperature, and humidity, where phot1 and phot2 mediate stomatal opening at low-fluence and high-fluence BL, respectively (Kinoshita et al., 2001). Consistent with the loss of phot1 function,
the stomata of *Nps1* plants are seemingly unresponsive to low-fluence BL and lack the BL-induced increase in transpiration and increase in stomatal aperture. Since only a minor increase in stomatal conductance is observed in *Nps1* under high-fluence BL, the participation of phot2 in this increase remains equivocal. At the same time, retention of the rhythm of stomatal movement in the *Nps1* mutant implies that it is not affected in stomatal responses mediated by cryptochrome and phytochrome (Somers et al., 1998; McClung, 2001; Boccalandro et al., 2012). The *Nps1* seedlings grown under omnilateral BL showed altered hypocotyl growth orientation very similar to that of the *phot1* seedlings of Arabidopsis. The lack of all these photoresponses suggests that the *Nps1* mutation affects those signaling pathways wherein phot1 participates.

Our results also reveal an additional role of phot1 in cotyledon opening, where it may act in concert with other photoreceptors. So far, phytochromes have been implicated in regulating cotyledon opening in Arabidopsis (Hennig et al., 2002). Considering that the *Nps1* seedling shows sluggish opening of cotyledons and reduced petiole angle compared with the wild type and the phytochrome A (phyA)-deficient *fri* mutant, it is likely that phot1 along with phyA regulates the cotyledon opening and petiole angle during deetiolation of seedlings. Cotyledon expansion is another growth response reportedly regulated by the interaction among phyA, phyB, cryptochrome1, and phototropins (Neff and Chory, 1998; Ohgishi et al., 2004; Ward et al., 2005). The *Nps1* mutant also shows slightly reduced cotyledon expansion, implying that reduced expansion of cotyledons in *Nps1* seedlings may be similarly related to the loss of phot1 function. Additionally, *Nps1* also shows a decrease in root elongation both in etiolated and deetiolated seedlings, and it is plausible that the observed reduction in root length may reflect an interaction of phot1 with plant hormones such as auxin.
The Dominance of the Mutant phot1 May Result from Its Intermolecular Interactions

The loss of the low-fluence BL-mediated response in the Nps1 mutant indicated that the mutant may be either defective in phot1 protein or a closely associated signaling partner. While wild-type tomato seedlings show normal phosphorylation (Reymond et al., 1992; Srinivas et al., 2004), the seedlings of the Nps1 mutant lacked BL-induced phosphorylation. The absence of phosphorylation may result either from a lack of phosphorylation or a constitutive phosphorylation of phototropin in the Nps1 mutant. Immunodetection showed very low levels of the phot1 protein compared with the wild type, indicating that diminished phot1 levels in Nps1 were insufficient to show a BL-mediated phosphorylation response or that constitutive phosphorylation may have led to the degradation of protein. Our gene expression analyses for PHOT1 and PHOT2, however, did not reflect significant differences in transcript levels between the Nps1 seedlings and wild-type seedlings, indicating that a loss of phot1 protein stability may have led to a reduction in the phot1 protein level in the mutant seedlings. Consistent with this, near abolition of phototropic responses in heterozygous F1 plants is associated with a reduced level of phot1 protein. It is known that the dominant negative alleles have a very strong impact on the phenotype of heterozygotes, and most often the phenotype closely resembles that of a knockout mutation (Meinke, 2013). The loss of phot1 responses in Nps1 is consistent with the view that mutant phot1 protein affected the function of wild-type phot1 protein in a dominant-negative fashion.

The molecular basis of the dominant-negative function of the mutated phot1 protein in the Nps1 mutant is not yet known and, hence, can only be speculated. A survey of 200 dominant or semidominant mutants of Arabidopsis indicated that the observed dominant phenotypes following chemical mutagenesis are predominantly caused by missense mutations that either modify protein function or confer a dominant-negative effect on protein-protein interactions (Meinke, 2013). Among the two LOV domains of phototropins, the LOV1 domain has been thought to be a dimerization site in the full-length phototropin (Nakasako et al., 2008). Recently, it was shown that the phot1LOV1 (180G–329K) polypeptide (prepared by using a bacterial overexpression system and purified for in vitro experiments) exists as a dimer in the dark state and that the photoexcited dimer associates with another dimer to form a tetramer (Nakasone et al., 2013). Since the LOV1 region is not disrupted in the Nps1 mutant, it seems likely that mutated phot1 protein of the Nps1 mutant might form dimers or tetramers with wild-type phot1, abolishing its activity (for possible mechanisms, see Veitia [2007]). Such a reduction in activity has been shown for the K⁺ uptake channel KAT1 (the potassium uptake channel from Arabidopsis) in Arabidopsis, where mutated forms of KAT1 reduced the activity of the wild-type guard cell channel function by forming heteromeric complexes (Kwak et al., 2001).

The strong reduction in phototropic responses in F1 plants indicates that the mutated phot1 protein likely interacts with wild-type phot1, hindering phot1-mediated signal transduction. Considering that the phot1 protein may function as dimer, it is also likely that F1 plants may also have some residual level of wild-type phot1 homodimers. Consistent with this view, in F1 plants exposed to low-fluence BL, some residual phototropism eliciting less than 5° curvature is observed. The observed suppression of phot1 function is similar to that reported for the ethylene-insensitive mutants of ETR1 (Chen and Bleecker 1995), wherein the dominance of the ETR1 mutant is likely derived in part from its ability to associate with other ethylene receptors and conferring upon them a similar conformation (Gao et al., 2008).

The Aα Region May Have an Essential Role in phot1 Function

The translated protein of the mutant phot1 of Nps1 harbors a single Arg-495-to-His substitution located in a highly conserved Aα helix in the Hinge1 region located between the conserved LOV1 and LOV2 domains of both phot1 and phot2. The sequences in the Aα helix region are strikingly conserved across phototropins reported from various species. Molecular studies have revealed that the functional LOV2 domain is essential for the biological activity of phototropin (Demarsy and Fankhauser, 2009). In oat, the LOV2 domain is flanked by a conserved N-terminal turn-helix-turn motif containing the Aα helix and a C-terminal flanking region containing the Ja helix, which plays an important role in phototropin signal transduction. Modeling of the mutated phot1 in the Nps1 mutant indicates a conformational modification in the Aα helix that may cause partial unfolding and displacement of the Ja helix from the LOV2 core. This may loosen the packing of the tertiary structure of phot1 in the LOV2 domain and its vicinity and may make it susceptible to proteolysis.

Several recent studies have suggested the importance of the Aα helix as the signaling route from the LOV2 domain to the kinase domain of phototropin (Halavaty and Moffat, 2007; Zayner et al., 2012). Similar to the Ja helix, the Aα helix is also amphipathic in nature and in the dark state binds to the LOV2 domain via hydrophobic interactions. A combination of biophysical measurements using a variety of oat Phot1-LOV2 domain mutants indicated that the N-terminal Aα helix plays a role in the light-activated dissociation of the C-terminal Ja helix during activation of the LOV2 domain (Zayner et al., 2012). Similar conformation changes in the Aα helix were detected by Fourier transform infrared spectroscopy (Pfeifer et al., 2009). Molecular dynamics simulations using oat phot1 indicated that the Aα helix shifts to a position
where it interacts more closely with Ja, leading to the formation of a salt bridge between residues Arg-410 (Arg-495 of tomato) and Glu-545 (Freedolino et al., 2013). Furthermore, point mutations along the Ja helix of oat and Arabidopsis phototropin have been shown to displace the Ja helix from the LOV2 domain in a manner analogous to light activation independently of illumination (Harper et al., 2004; Jones et al., 2007). Since the amino acid sequence of the Aα helix is better conserved than that of the Ja helix, it is suggested that the Aα helix provides robust regulation of the kinase activity and the Ja helix contributes to signal transduction. Considering that modeling indicates partial unfolding and displacement of the Ja helix from LOV2, it could be speculated that this conformational change may induce a down-regulation of the mutated phot1 protein independently of illumination. Taking together the above biophysical studies demonstrating the seminal role of the Aα helix in the activation of the kinase domain and the loss of phot1-specific activity in the Nps1 mutant, it is reasonable to assume that the Aα region has an essential role in phot1 function.

Based on in vitro studies involving protein fragments derived from Arabidopsis phot2, it has been proposed that the LOV2 region of phototropin acts as a dark state inhibitor of phototropin kinase activity and that light relieves this inhibition (Matsuoka and Tokutomi, 2005). In accordance with this view, the same amino acid substitution at the corresponding site in C. reinhardtii phototropin (R210H) was recently shown to exhibit constitutive kinase activity in vitro (Aihara et al., 2012). In Arabidopsis, the constitutive active kinase fragment of phot2 elicits phot2 responses without light stimulus in planta (Kong et al., 2007). It could be possible that the instability of the mutant phot1 in Nps1 could be due to its constitutive activation, via the R495H mutation, resulting in its degradation even in the absence of light. Recent reports suggesting phot1 exists as a dimer in the dark (Nakasone et al., 2013) lend further support to the observed decrease in phot1 levels in the F1 seedlings. In essence, our results showing abolishment of the regulatory activity of phot1 highlights the functional importance of the Aα helix domain in phototropic signaling. At present, limited information is available about role of the Hinge1 region that encompasses the Aα helix in the functioning of phototropins. It is known that autophosphorylation in response to BL occurs on at least three Ser residues, Ser-350, Ser-376, and Ser-410, in the Hinge1 region (Salomon et al., 2003; Inoue et al., 2008a; Sullivan et al., 2008). It is also known that a 14-3-3 protein specifically binds to autophosphorylated phot1 protein in a BL-dependent manner, to the binding motifs located in the Hinge1 region of phot1, which include the three Ser residues mentioned above (Kinoshita et al., 2003; Inoue et al., 2008a; Sullivan et al., 2009), although the functional significance of this binding has not yet been reported. Considering that Ser-747 of PHOT2 in vivo interacts with 14-3-3α protein and that this interaction is presumably required for BL-activated stomatal opening (Tseng et al., 2012), the interaction of 14-3-3 protein with the Hinge1 region of phot1 may have some functional significance.

In summary, in this study, we report the characterization of a dominant-negative nonphototropic mutant of tomato that lacks phot1 responses due to a single mutation in the PHOT1 homolog. These findings indicate the importance of the Aα helix domain in the Hinge1 region for the functional regulation of the phot1 molecule, which could be a key step in phot1-mediated responses. Molecular characterization of the nature of the dominance of the phot1 mutation and the mechanism by which the mutation hinders native phot1 signaling would be the next step, which would lend more insight into the phototropin signaling pathway in plants.

MATERIALS AND METHODS

Plant Growth Conditions

The tomato (Solanum lycopersicum) genotypes used were Nps1, fri (van Tuinen et al., 1995), and its isogenic wild type in the Alisa Craig background. Seedlings were grown on wet germination papers in the dark for 3 d at 25°C ± 1°C. The age of seedlings was counted from the time of radicle emergence. For time-lapse videography, after germination, seeds were transferred to Eppendorf tubes filled with Soilrite or 0.8% (w/v) agar. For measuring root and hypocotyl length and hypocotyl orientation studies with BL from below, the seedlings were grown on vertically placed petri plates. The cotyledons from 7- or 8-d-old WL-grown seedlings were scanned using a UMAX scanner, and cotyledon size was calculated using ImageJ. All experimental data were repeated at least three times and are presented as means ± SE.

Light Sources

BL and RL were obtained using blue (λmax = 470 ± 30 nm) and red (λmax = 660 ± 20 nm) light-emitting diodes, respectively. For autophosphorylation, high-fluence BL was obtained by filtering the output of a 1,000-W halogen lamp through a 10-cm layer of water and blue cellophane (αmax = 450 ± 30 nm). Unless mentioned otherwise, the light treatments were as follows: low-fluence BL (0.5 μmol m−2 s−1), high-fluence BL (100 μmol m−2 s−1), and WL (100 μmol m−2 s−1).

Isolation of Mutants and Genetic Analysis

Ethyl methanesulfonate-mutagenized M2 seeds bulked from 1,500 M1 plants were screened to identify nonphototropic mutants. One-week-old etiolated M2 seedlings were exposed to continuous unidirectional BL (0.1 μmol m−2 s−1) for 12 to 16 h. Out of 16 putative nonphototropic mutants isolated, only two retained their nonphototropic nature by the M5 generation. Backcrosses of these mutants with the wild type resulted in selection of the Nps1 line, where the F1 seedlings retained the nonphototropic phenotype.

Time-Lapse Video Imaging and Analysis

Time-lapse images were captured using Quickcam-Pro 4000 (Logitech; http://plantsinmotion.bio.indiana.edu/plantsmotion/starthere.html). For the time-course study, frames at defined time points were extracted from the movie using MGI VideoWave4 PC video editing. The curvature angles were calculated after subtracting the zero-point image from the images at defined time points. For time-lapse imaging in the dark, the background illumination was provided by using infrared light-emitting diodes (λmax = 940 nm). All measurements were performed using NIH ImageJ software and e-Ruler (http://www.mycnknow.com/). For measuring phototropic curvature, 35 ± 0.3-cm-tall etiolated seedlings were used. Gravitropic response was measured after horizontally orienting 3-d-old etiolated or deetiolated seedlings. For angle measurements, the images of WL-grown seedlings were recorded at 12-h intervals. The angle of the petiole and cotyledon was calculated with ImageJ.
Determination of Stomatal Conductance by Gas-Exchange Measurement

Tomato plants were grown under continuous WL for 4 weeks and thereafter under 12 h of light and 12 h of dark for 1 week at 23°C. Stomatal conductance was measured after a 12-h dark period using an open gas-exchange system (LI-6400; Li-Cor). The leaf-to-air vapor pressure difference was controlled using a dew point generator (LI-610; Li-Cor). The gas exchange was measured at a leaf temperature of 25°C to 25°C, 36 Pa of CO₂, 2% (v/v) oxygen, and vapor pressure difference of 1 to 1.2 kPa. The uppermost fully expanded leaf was clamped in the leaf chamber. After 30 min of dark incubation, the leaf was illuminated with 200 μmol m⁻² s⁻¹ RL for 2 h to saturate the photosynthetic effect on stomatal conductance. Thereafter, low-fluence BL (2 μmol m⁻² s⁻¹) was supplemented to RL for 1 h followed by high-fluence BL (100 μmol m⁻² s⁻¹) to analyze the contribution of phot1 and phot2 to the stomatal conductance, respectively. Gas-exchange parameters were calculated according to the equations of von Caemmerer and Farquhar (1981).

Chloroplast Relocation Response

Chloroplast relocation was observed in mesophyll cells using the Aihara et al. (2008) protocol with modifications. Prior to the relocation experiment, leaflets from 4-week-old plants were placed on wet papers under RL (16 μmol m⁻² s⁻¹) for 12 h. The intact leaf discs were irradiated with varying fluences of BL to induce the accumulation or avoidance movement of chloroplast. For the dark-positioning experiments, 28-d-old plants were placed in the dark for 28 hours, the leaf was illuminated with 200 μmol m⁻² s⁻¹ for 2 h to induce the accumulation or avoidance movement of chloroplast. For the BL experiments, TL was set at the light source for 12-hour periods and the stomata were imaged with a confocal microscope.

In Vitro Phosphorylation and Immunodetection of Phot1 and Phot2

Hypocotyls of 3-d-old etiolated seedlings were homogenized in buffer containing 50 mM HEPES/KOH, pH 8.0, 5 mM MgSO₄, 0.5% (w/v) Triton X-100, and 2 μL of protease inhibitor cocktail (P-9599; Sigma). The phosphorylation reaction was carried out as described by Sakamoto and Briggs (2002). Immunodetection of phot1 and phot2 was carried out as described by Sakamoto and Briggs (2002). Hypocotyls from 4-d-old etiolated seedlings were homogenized in buffer containing 0.1 M Tris-HCl, pH 8.5, 4% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (P-9599; Sigma) in a ratio of 1:1 (tissue : buffer). The homogenate was then heated at 80°C for 3 min and centrifuged at 17,500g for 15 min. Total protein concentration was determined by a modified Lowry method (Sandyberg and Strominger, 1972). Western blotting was carried out as described by Towbin et al. (1979) using anti-phot1 (Doi et al., 2004) and anti-phot2 (Kong et al., 2007) antibodies.

Slphot1 and Slphot2 Cloning and Sequencing

The tomato seedling cDNA library in λ ZAP II (Stratagene) was screened for putative PHOT2 with radiolabeled EST clone BG 126772 using conditions described by Kulshreshtha et al. (2005). Subsequent sequencing was performed using commercially available T3 and T7, with primers resulting in the amplification of putative cDNAs from the wild type and mutant using the same coordinates as in 2v1bA. The 3-D models of both the wild type and mutant using the same co-ordinates as in 2v1bA. The 3-D models were drawn with the software PyMol (http://www.pymol.org).

Supplemental Data

The following materials are available in the online version of this article. Supplemental Figure S1. Characterization of phototropic curvature and the phot1 protein in F1 plants. Supplemental Figure S2. Time-lapse imaging of chloroplast movement in wild-type and Nps1 plants.

ACKNOWLEDGMENTS

We thank Ken-Ichiro Shimazaki, S.-G. Kong (both Kyushu University), and Winslow Briggs (Carnegie Institution of Washington) for kindly providing Aph1 and Aph2 antibodies, and Akhlesh Tyagi (University of Delhi) and Moritoshi Iino (Osaka City University) for providing their facilities. Received November 11, 2013; accepted February 9, 2014; published February 10, 2014.

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Transcript Analyses

Transcript analyses were performed by RT-PCR (Invitrogen) on cDNA synthesized from total RNA isolated from 3-d-old wild-type and Nps1 seedlings (Qiagen). First-strand cDNA was synthesized, and levels of PHOT1 and PHOT2 were then determined by PCR with the PHOT1- and PHOT2-specific oligonucleotides as described above. The effect of BL on transcript levels was ascertained by irradiating 3-d-old etiolated seedlings with the unilateral BL (25 μmol m⁻² s⁻¹) for 2 h prior to the isolation of total RNA.

Mismatch Cleavage by CEL I

First-strand cDNA was synthesized from total RNA of wild-type and Nps1 mutant seedlings (as described above). PCR amplification was performed in 15-μL volumes containing a 2-μL cDNA pool of the wild type or Nps1, 1× Taq buffer, 2.6 mM MgCl₂, 0.3 mM deoxynucleotidetriphosphates, 0.3 μM primers (OlmiteX Biosolutions), and 1 unit of Taq DNA polymerase. Heteroduplex formation, CEL I digestion, and electrophoresis on the LICOR-4300 analyzer (Li-Cor) were carried out using the protocol of Till et al. (2006). The images in 700- and 800-nm channels were analyzed using Adobe Photoshop, and the digested DNA fragments and wild-type PCR products were identified.

3-D Modeling of Phot1-LOV2 Regions

3-D modeling of the wild type and mutant phot1-LOV2 regions of tomato phot1 was performed using the automated comparative protein-modeling server, Swiss model (http://swissmodel.expasy.org/), in which no template was specified. The modeling program chose data from one protein (Protein Data Bank identifier 2v1bA) among the four registered crystal structures of oat (Avena sativa) phot1-LOV2 regions as a loading template. The crystal structure (2v1bA) has 129 oxygen atoms of the internal water; however, these are not included in the 3-D modeling of the tomato protein. Four water oxygen atoms between Arg-410 and Asp-432 in oat phot1-LOV2 (2v1bA) were overlaid onto the 3-D models of both the wild type and mutant using the same co-ordinates as in 2v1bA. The 3-D models were drawn with the software PyMol (http://www.pymol.org).

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