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Research article

Functional characterization of *GhPHOT2* in chloroplast avoidance of *Gossypium hirsutum*

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ABSTRACT

Chloroplast movement mediated by the plant-specific phototropin blue light photoreceptors is crucial for plants to cope with fluctuating light conditions. While chloroplasts accumulate at weak light-illuminated areas, chloroplast avoidance response mediated primarily by the phototropin2 (phot2) receptor is induced by strong light illumination. Although extensive studies have been performed on phot2-mediated chloroplast avoidance in the model plant *Arabidopsis*, little is known on the role of the corresponding *PHOT2* orthologs in chloroplast movement in cotton. In this study, we found that chloroplast avoidance movement also occurs in the tetraploid *G. hirsutum* and two diploid species, *G. arboreum* and *G. raimondii*, albeit with distinct features. Further bioinformatics and genetic analysis identified the cotton *PHOT2* ortholog, *GhPHOT2-1*, which retained a conserved role in plant chloroplast avoidance movement under strong blue light. Ghphot2-1was localized in the plasma membrane and formed aggregates after high blue light irradiation. Constitutive expression of *GhPHOT2-1* restored chloroplast avoidance and accumulation response, as well as phototropism, and leaf flattening characteristics of the *Arabidopsis phot2* or *phot1 phot2* mutants. On the contrary, silencing of *GhPHOT2-1* by virus-induced gene silencing (VIGS) disrupted high blue light-induced chloroplast avoidance movement and caused photo damage in cotton leaves. Taken together, these findings demonstrated that *GhPHOT2-1* is a conserved *PHOT2* ortholog in regulating chloroplast avoidance and the other aforementioned phot2-mediated responses, implicating its potential role for improving high light tolerance in cotton cultivars.

1. Introduction

As sessile organisms, plants have evolved sophisticated strategies to cope with fluctuating environmental conditions. Light is one of the most crucial environmental signals controlling various aspects of plant development and adaption, ranging from seed germination, photomorphogenesis, stomata opening, leaf flattening, phototropism to chloroplast movement and flowering (Kami et al., 2010). Of which, chloroplast movement is essential for plants to optimize light harvesting while avoiding photodamage under fluctuating light conditions (Wada, 2013). To maximize light harvesting for efficient photosynthesis, chloroplasts will accumulate in weak light-illuminated regions of a cell (accumulation response) (Wada, 2013). In contrast, chloroplasts will move away from regions exposed to strong light (avoidance response), in order to protect themselves from excessive energy from the strong light conditions (Kasahara et al., 2002).

In terrestrial angiosperms, chloroplast movement is mediated by the phototropin blue light receptors (Kagawa et al., 2001; Sakai et al., 2001; Jarillo et al., 2001; Kong et al., 2013c). There are two phototropins in *Arabidopsis*, namely phot1 and phot2. The phototropins are consisted of two LOV (light, oxygen or voltage) light sensor domains (LOV1 and LOV2) in the N-terminus and a Ser/Thr kinase domain in the C-terminus, and they act redundantly in the regulation of a broad range of blue light-induced responses (Briggs and Christie, 2002). In chloroplast movements, phot1 and phot2 redundantly mediate the accumulation response, while phot2 primarily regulates the strong light-induced avoidance response (Kagawa et al., 2001; Sakai et al., 2001; Jarillo et al., 2001). Previous work showed that the dynamic trafficking of the phototropins may be important for their role in chloroplast movement. Upon blue light irradiation, localization of phot2 in the plasma membrane and the chloroplast outer membrane, as well as its translocation from the plasma membrane to the Golgi apparatus were reported to be involved in the avoidance response (Kong et al., 2013c; Wada, 2013; Kong and Wada, 2016).

Cotton (*Gossypium* spp.) is an economically important crop cultivated globally for both natural fibers and oil production. The simultaneous improvement of fiber quality, productivity, and stress adaption in upland cotton (*Gossypium hirsutum*) is a challenging task in

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conventional cotton breeding worldwide. Significant progress has been made recently with the complete sequencing of cotton genomes (Wang et al., 2012; Paterson et al., 2012; Li et al., 2014, 2015; Zhang et al., 2015; Liu et al., 2015), however, the decoding of cotton genomes and deciphering the function of specific genes are in their infancy. Specifically, little information is available on cotton genes responsible for growth and development under high light conditions. Plants grown under full sunlight have adopted self-defense mechanisms against strong light conditions, and different plant species have evolved sophisticated strategies to avoid photodamage under strong light conditions (Koniger and Bollinger, 2012). Extensive studies have been performed on mechanisms of high blue light-mediated chloroplast avoidance movement in the model flowering plant *Arabidopsis* (Banas et al., 2012; Wada, 2013; Kong and Wada, 2016). However, it remains elusive whether or not the response and defense mechanisms of cotton to high light are conserved as those in *Arabidopsis*.

Here, we report the identification of an evolutionarily conserved ortholog of *PHOT2* and its role in chloroplast relocation response to high light conditions in cotton. Our results suggest that chloroplast avoidance response is a strategy adopted by different cotton species to acclimate to fluctuating light conditions. Moreover, we found that these activities are regulated by *GhPHOT2*, indicating conserved mechanisms for cotton chloroplast avoidance movement. While constitutive expression of *GhPHOT2-1* restored chloroplast avoidance movement in Arabidopsis *phot2* mutant, silencing of *GhPHOT2-1* by VIGS disrupted high blue light -induced chloroplast avoidance in cotton leaves. Taken together, our findings demonstrate that *GhPHOT2-1* is conserved in mediating the chloroplast avoidance response in cotton, implicating its role for excessive light acclimation of cotton cultivars.

2. Results

2.1. Characteristics of the chloroplast movement in cotton

To investigate the strategies that cotton cultivars exhibit in response to excessive light irradiation, we analyzed the chloroplast relocation response of cotton leaves treated with different blue light intensities and compared the responses among the tetraploid species, *G. hirsutum,* and its ancestor-like diploid species, *G. arboreum* and *G. raimondii*. Detached leaves were irradiated with blue light intensities of 1 μmol m^{-2} s^{-1} to 10 μmol m^{-2} s^{-1}, or even stronger blue light intensities from 30 μmol m^{-2} s^{-1} to 200 μmol m^{-2} s^{-1}. It was reported that the irradiated leaf area become pale green (white band) as chloroplasts move to the cell side under strong, and they become dark green (green band) due to chloroplast accumulation at the cell face under weak light (Kagawa et al., 2001). Standard methods for analyzing chloroplast movement, such as band assay, microscopy observation, and light transmittance assay were adopted (Wada and Kong, 2011; Wada, 2013). Chloroplasts of cotton leaf cells exposed to high blue light irradiation exhibited the avoidance response when light intensity was above 100 μmol m^{-2} s^{-1} (Fig. 1A, Fig. S1). No significant chloroplast avoidance response was observed in *G. hirsutum* and *G. arboreum* at a blue light intensity of 30 μmol m^{-2} s^{-1} (Fig. 1A, Fig. S1), which was considered strong light that can induce the chloroplast avoidance response in *Arabidopsis* (Banas et al., 2012; Wada, 2013). However, the chloroplast avoidance response did occur in leaves of *G. raimondii* at a blue light intensity of 30 μmol m^{-2} s^{-1} (Fig. 1, Fig. S1). Chloroplast avoidance response was observed for the cotton species when the intensity of BL was at 100 μmol m^{-2} s^{-1} or higher, while chloroplast avoidance response was observable in *Arabidopsis* from 20 μmol m^{-2} s^{-1} to stronger blue light intensities (Fig. 1, Fig. S1 and Fig. S2). We also compared the chloroplast accumulation and avoidance response of cotton and *Arabidopsis* using red light transmittance method (Wada and Kong, 2011). We found that relative red light transmittance level of cotton was generally lower than that of *Arabidopsis* at the same blue light intensities (Fig. 1, Fig. S1 and Fig. S2). Taken together, these results indicated that the chloroplast avoidance and accumulation response occurs in multiple cotton species as a coping mechanism to fluctuating light conditions, while chloroplast avoidance response of *G. hirsutum* and *G. arboreum* occurred at higher blue light intensities than *G. raimondii*.

2.2. Identification of the GhPHOT2 ortholog in Gossypium hirsutum

In an effort to identify the functional *PHOT2* orthologs in regulating chloroplast movement of *Gossypium hirsutum*, we systematically investigated the *PHOT2* subfamily in cotton. Using the full-length amino acid sequence of Atphot2 as a basis of our query, we have identified *PHOT2* orthologs from different databases available in CottonFGD for the three cotton species of interest (Zhu et al., 2017). Two *GaPHOT2* orthologs were found in the BGI (Beijing Genomics Institute) *G. arboreum* (A2) protein database, Cotton_A_28225 (chromosome 13) and Cotton_A_30874 (chromosome 6), where Cotton_A_28225 had a higher total hit score (higher scores indicate greater similarity). Two *GrPHOT2* orthologs were also found in the JGI (Joint Genome Institute) *G. raimondii* (D5) protein database, namely Gorai.008G012600 (chromosome 8) and Gorai.013G065600 (chromosome 13), where Gorai.008G012600 had a higher total hit score. The following six cotton genes were predicated to encode Ghphot2 in the BGI *G. hirsutum* (AD1) protein database, namely CotAD_58277 (scaffold3276.1), CotAD_12100 (D subgenome of chromosome 13), CotAD_33403 (A subgenome of chromosome 13), CotAD_13007 (A subgenome of chromosome 8), CotAD_33323 (scaffold1088.1), and CotAD_70827 (A subgenome of chromosome 8). Of these six genes, CotAD_58277 obtained the highest total hit score. Five Ghphot2-encoding genes were found in the NAU (Nanjing Agricultural University) *G. hirsutum* (AD1) protein database, namely Gh_A12G0099 (A subgenome of chromosome 12), Gh_D13G0576 (D subgenome of chromosome 13), Gh_A13G0554 (A subgenome of chromosome 13), Gh_D11G3202 (D subgenome of chromosome 11), and Gh_D12G0111 (D subgenome of chromosome 12) where Gh_A12G0099 obtained the highest total hit score (Supplementary Table S1).

We found that CotAD_58277 of the BGI database is equal to Gh_A12G0099 of the NAU database, and both attained the highest total hit scores against AtPHOT2, and consequently, we cloned and named the gene as *GhPHOT2-1*. To further verify the identity of Ghphot2-1, we also performed a BLAST search of Ghphot2-1 against other plant species and analyzed its phylogenic relationship with genes predicted to be *AtPHOT2* orthologs according to results of PANTHER (Protein ANalysis THrough Evolutionary Relationships) plant homologs on TAIR (The Arabidopsis Information Resource). A multiple sequence alignment analysis revealed that there are two conserved LOV domains, identical amino acids for flavin mononucleotide (FMN) binding, and a Ser/Thr protein kinase domain in Ghphot2-1 (CotAD_58277/Gh_A12G0099), which resembled those of Atphot2 (Fig. 2B, Fig. S3). To reveal further the evolutionary relationships of Ghphot2-1, phylogenetic trees of the predicted *PHOT2* orthologs in *G. hirsutum* and the two ancestor-like diploid cotton species were constructed, and their gene structures were depicted. We found that *GhPHOT2-1* is an evolutionarily conserved ortholog of *AtPHOT2* and other predicted *PHOT2* orthologs (Fig. 2B). Besides the differences in their intron-exon structure, and potentially the splicing form, the basic CDS structures were similar among the different cotton *PHOT2* genes (Fig. 2C). Overall, these results indicated that CotAD_58277/Gh_A12G0099 is the most likely candidate of Arabidopsis *AtPHOT2* orthologs in *Gossypium hirsutum*.

2.3. GhPHOT2-1 is a conserved ortholog of AtPHOT2 and can mediate Atphot2-mediated responses in Arabidopsis

To determine the role of *GhPHOT2-1* in the chloroplast avoidance response, full-length cDNA of *GhPHOT2-1* was cloned and in-frame fused with a green fluorescent protein (GFP) gene to generate a

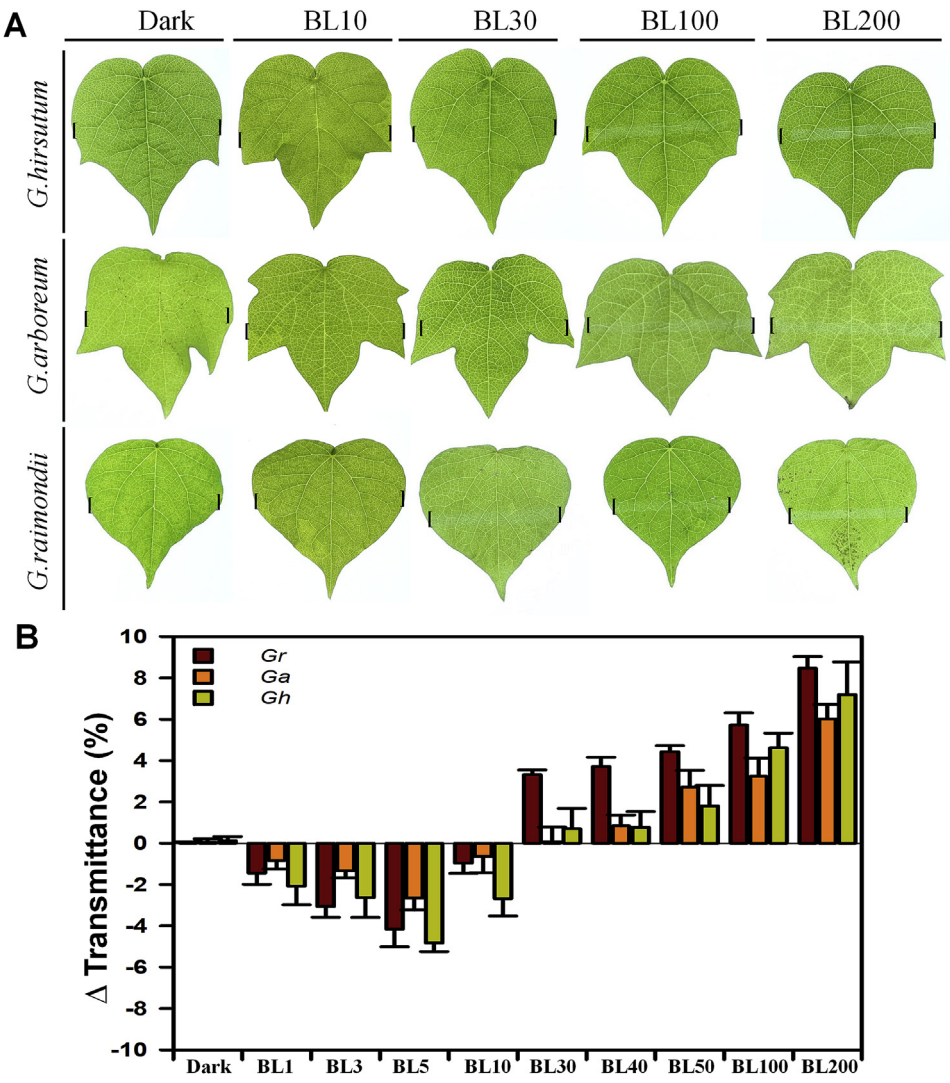

Fig. 1. Chloroplast avoidance response of *G. hirsutum*, *G. arboreum* and *G. raimondii* cotton species. (**A**) Chloroplast movement of the three cotton species to blue light irradiation of 10 μmol m^{-2} s^{-1} (BL10), 30 μmol m^{-2} s^{-1} (BL30), 100 μmol m^{-2} s^{-1} (BL100), or 200 μmol m^{-2} s^{-1} (BL200). Leaves of 4-week-old cotton plants (pre-incubated in the dark) were detached and irradiated with various blue light intensities for 3 h through the 5 mm slit (shown in brackets). Chloroplasts moved to the anticlinal walls under high blue light, and the irradiated area appeared pale green. (**B**) Red light transmittance quantification for leaves of the three cotton species under blue light intensities of 1 μmol m^{-2} s^{-1} (BL1) to 200 μmol m^{-2} s^{-1} (BL200) for 3 h. Data are means ± SD, at least 15 leaves of different species were used for each treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

35S::GhPHOT2-1:GFP construct, and the corresponding homozygous transgenic lines in *phot2* background were subjected for further chloroplast avoidance analysis (Fig. 3A). After treatment of 100 μmol m^{-2} s^{-1} of high blue light irradiation for 1 h, no obvious chloroplast avoidance response was observed in irradiated regions of *phot2* or *phot1 phot2* mutants, and the chloroplasts distributed evenly in the mesophyll cells of these mutants (Fig. 3B–D). However, introduction of the *35S::GhPHOT2-1:GFP* construct into *phot2* restored its chloroplast avoidance response when subjected to high light irradiation (Fig. 3B and C). In leaves of the transgenic lines that were exposed to high blue light of 100 μmol m^{-2} s^{-1}, we observed the accumulation of chloroplasts at the anticlinal membranes (Fig. 3B and C). The results thus indicated that *GhPHOT2-1* is likely a conserved *PHOT2* ortholog in regulating the photo-relocation response in cotton. While chloroplast movement was impaired in *phot1 phot2*, chloroplast accumulation and avoidance response were restored in the *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines (Fig. 3D). In addition, we also found that introduction of this construct into the *phot1 phot2* double mutant could partially restore its phototropic response to high blue light (Fig. 4), and the curled leaf phenotype of *phot1 phot2* was also recovered (Fig. 5). Taken together, these results suggested that *GhPHOT2-1* is a functional *PHOT2* ortholog in high blue light-mediated phototropism and avoidance response in cotton.

2.4. Subcellular localization and cytoplasmic motility of Ghphot2-1

Arabidopsis phot2 is localized in the plasma and chloroplast membranes, which is crucial for its unique role in chloroplast movement (Kong et al., 2013a, 2013c). To determine the subcellular localization of Ghphot2-1, expression of Ghphot2-1:GFP was observed in *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines, and GFP signal was detected mainly in the plasma membrane (Fig. 6A). To further confirm the subcellular localization of Ghphot2-1:GFP, *35S::GhPHOT2-1:GFP* construct was transiently expressed in *Arabidopsis* protoplasts and *Nicotiana benthamiana*. The Ghphot2-1:GFP signal was also detected in the plasma membrane (Fig. 6B and C). We also examined the cytoplasmic motility of Ghphot2 in the transient expression system upon high blue light irradiation. In *Arabidopsis*, Atphot2 forms dot-like aggregates in the cytosol (or Golgi apparatus) in response to high blue light irradiation (Kong et al., 2013c). In *Nicotiana* pavement cells, Ghphot2-1:GFP formed aggregates in the cytoplasm of all irradiated cells after continuous irradiation with blue light of 100 μmol m^{-2} s^{-1}, and it was diminished after dark adaption (Fig. 6C), similar with the pattern of Atphot2 observed in *Nicotiana benthamiana* leaves (Aggarwal et al., (2014)) . Thus, the results showed that subcellular localization of Ghphot2-1 resembles that of Atphot2, and provided additional support to the conclusion that *GhPHOT2-1* is a *PHOT2* ortholog in cotton.

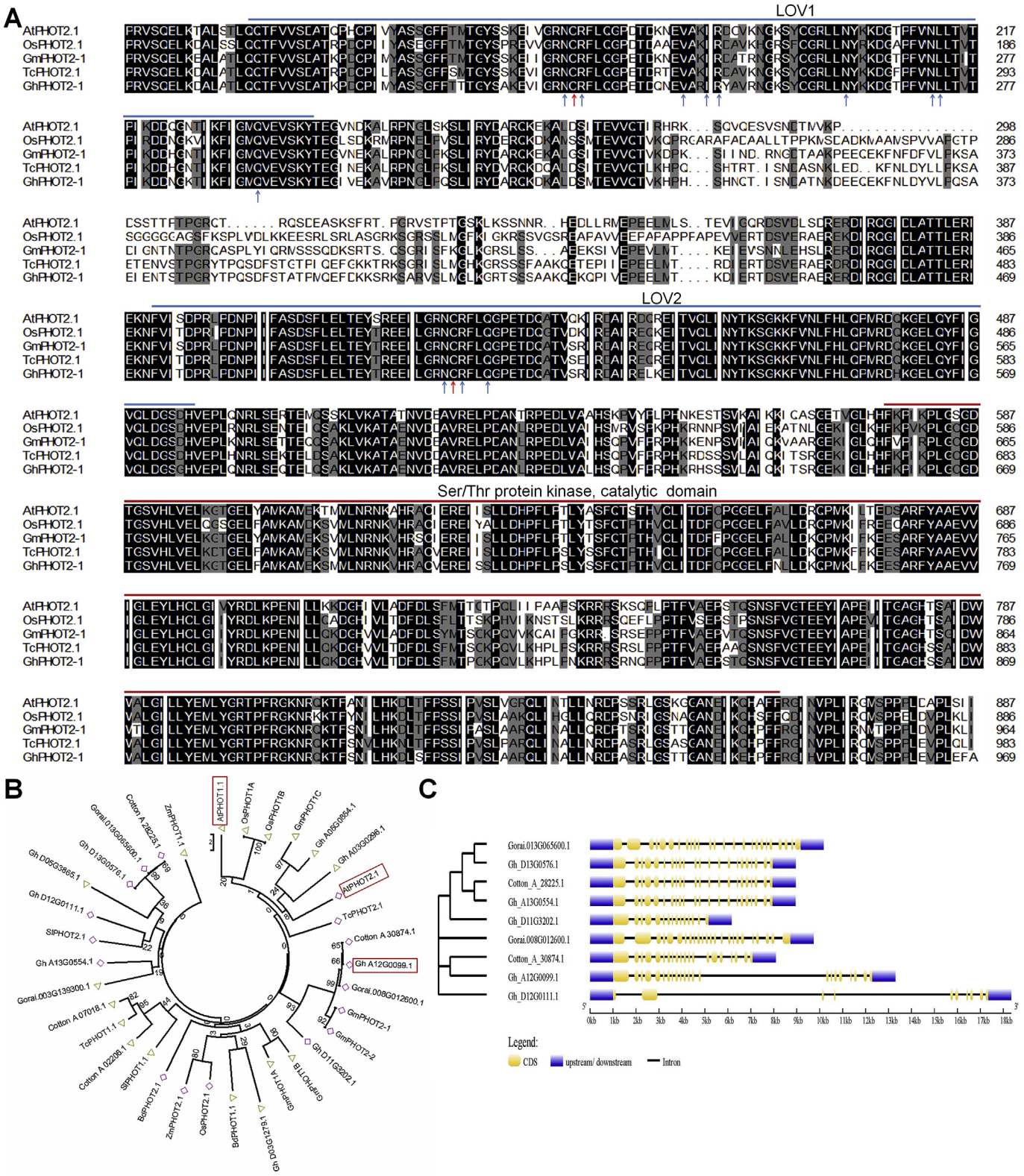

Fig. 2. Sequence alignment and phylogenic analyses of *GhPHOT2-1* with other orthologs. (**A**) Amino acid alignment of Ghphot2-1 with other phot2 orthologs. Residues identical to those of the consensus sequence derived from the sequence alignment are enclosed in dark boxes. Conserved domains of LOV1, LOV2, and Ser/Thr protein kinases are underlined in blue and red lines. Red arrows indicated the cysteine residues of GRNCRFLQG region in LOV domains essential for FMN–cysteinyl adduct formation, and blue arrows indicated other FMN-interacting residues in LOV1 domain. (**B**) Phylogenic analysis of *GhPHOT2-1* with other *PHOT1* or *PHOT2* orthologs. Phylogenic analysis was performed using the Neighbor-Joining method with 1000 bootstrap replicates. Sequences of representative PHOT1 or PHOT2 orthologs of Glycine max (I1MDL2, I1M4Q3, I1LR23, K7L915) Theobroma cacao (XP_017983114.1, A0A061E9J3), Oryza sativa (Q2QYY8, Q2RBR1, Q9ST27), Brachypodium distachyon (I1IVB6, I1IWY7), Solanum lycopersicum (K4DAX8, K4B0G4), and Zea mays (A0A1D6NP39, A0A1D6KQ74) were retrieved from UniProt accessions based on results of PANTHER plant orthologs. Triangle and diamond indicated the predicted *PHOT1* or *PHOT2* orthologs, respectively. (**C**) Schematic representation of the structures of candidate *GhPHOT2* genes. The *GhPHOT2-1* gene (Gh_A12G0099.1) contains 22 exons and 21 introns. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

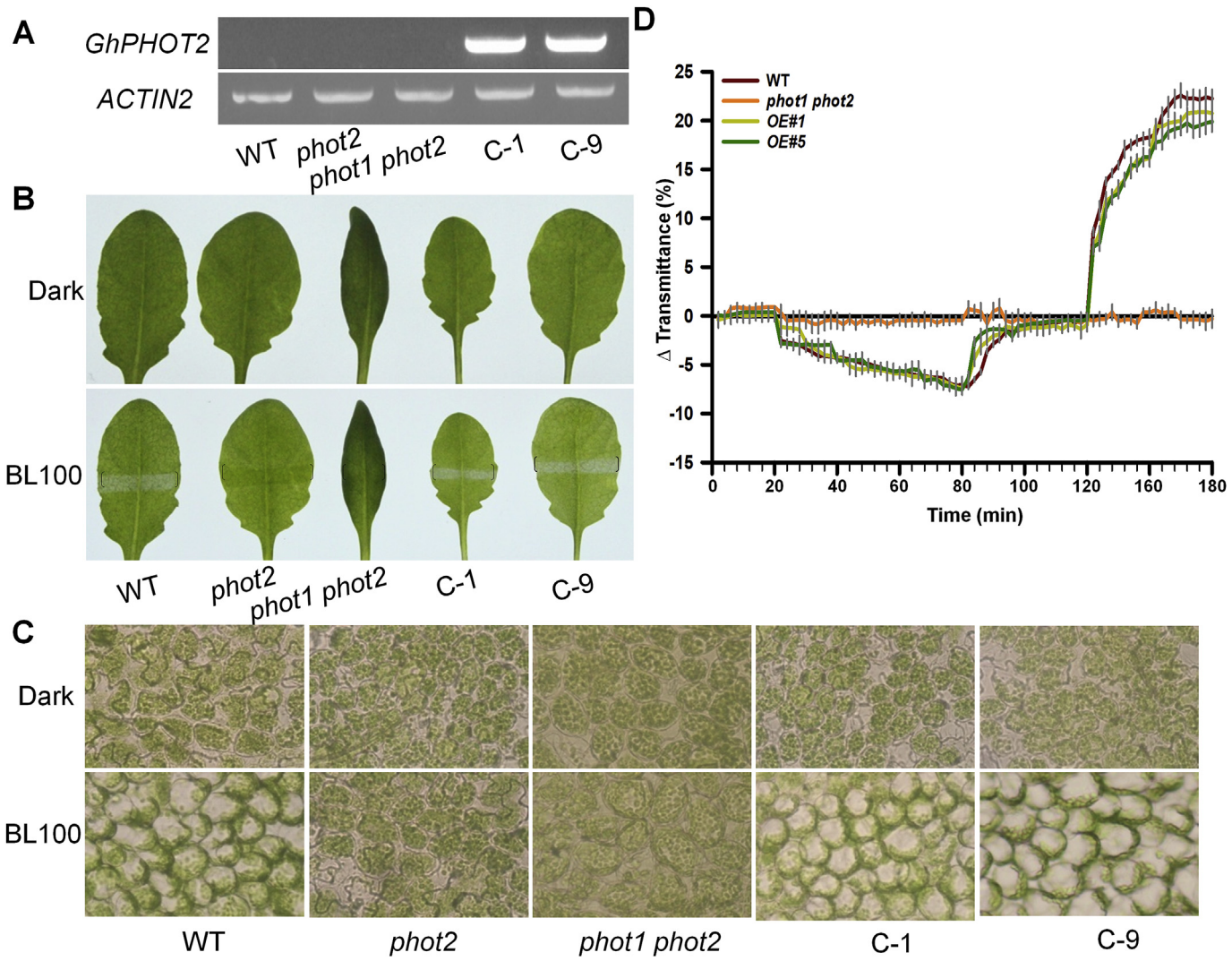

Fig. 3. GhPHOT2 restored chloroplast avoidance response of *Arabidopsis phot2*. (**A**) Expression level of *GhPHOT2* transcripts in WT, *phot2*, *phot1 phot2*, and *35S::GhPHOT2:GFP phot2* transgenic lines. Results represented one of the three biological replicates. (**B and C**) Chloroplast avoidance response of leaves (**B**) and mesophyll cells (**C**) of WT, *phot2*, *phot1 phot2*, and *35S::GhPHOT2:GFP phot2* transgenic lines (C1 and C9). The third leaf of 3-week-old *Arabidopsis* seedlings, pre-incubated in the dark, was detached and irradiated with high blue light of 100 μmol m^{-2} s^{-1} for 1 h through the 3 mm slit. Chloroplasts moved to the anticlinal membranes under high blue light, and the irradiated area appeared pale green (As shown in brackets). At least 10 leaves were used for each biological replicate. (**D**) Quantification of chloroplast accumulation and avoidance response in leaves of WT, *phot1 phot2*, and *35S::GhPHOT2:GFP phot1 phot2* transgenic lines (OE#1 and OE#5). Dark-adapted Arabidopsis leaves were excised and placed in a 6-well microplate reader, and red light transmittance was recorded for 20 min in dark. Red light transmittance Leaves were sequentially measured under low blue light of 3.2 μmol m^{-2} s^{-1} for 60 min, high blue light of 25 μmol m^{-2} s^{-1} for 40 min and 60 μmol m^{-2} s^{-1} for 40 min. Each 2-min Blue light irradiation was given at the intervals after red light transmittance measurements. Data are means ± SD, at least 15 leaves were used for each background and treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.5. *GhPHOT2-1 mediates chloroplast avoidance response and high-light tolerance in cotton*

To determine the role of *GhPHOT2-1* in high light tolerance of cotton cultivars, we analyzed the maximum photochemical efficiency of PSII (Fv/Fm) for *35S::GhPHOT2-1:GFP* transgenic lines, which is a crucial parameter of photosynthetic apparatus (Takahashi and Badger, 2011). The results showed that no significant difference in Fv/Fm was found among WT, *phot1 phot2*, and *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines under growth light of 80 μmol m^{-2} s^{-1} (Fig. 7A and B). However, after irradiation under high light of 1200 μmol m^{-2} s^{-1} for 3 h, Fv/Fm value of *phot1 phot2* was lower than WT, and introduction of *35S::GhPHOT2-1:GFP* into *phot1 phot2* restored the phenotype (Fig. 7A and C). Furthermore, constitutive expression of *GhPHOT2-1* could also restored the capacity of Arabidopsis *phot2* to eliminate photodamage (Fig. S4). Taken together, the results indicated that *GhPHOT2-1* play roles in protection of photosynthetic apparatus and prevent plant from photodamage.

To further explore the role of *GhPHOT2-1* in chloroplast movement, virus-induced gene silencing (VIGS) was used to repress the target gene *GhPHOT2-1*, and *GhCLA1* was used as a visual marker to monitor VIGS efficiency in cotton germplasm CCRI16 (Gao et al., 2011). We found that leaves of *GhCLA1*-silenced lines were albino, and the expression of *GhPHOT2-1* was verified to be knocked down (Fig. 8A and B). While the chloroplast avoidance response was observed in the leaf blade of CCRI16 cultivar or vector control-inoculated plants, silencing of *GhPHOT2-1* disrupted the chloroplast avoidance response under high blue light of 100 μmol m^{-2} s^{-1} (Fig. 8C and D). In addition, we found that Fv/Fm value of *GhPHOT2*-silenced lines was lower than CCRI16 and vector control-inoculated lines after high light irradiation of

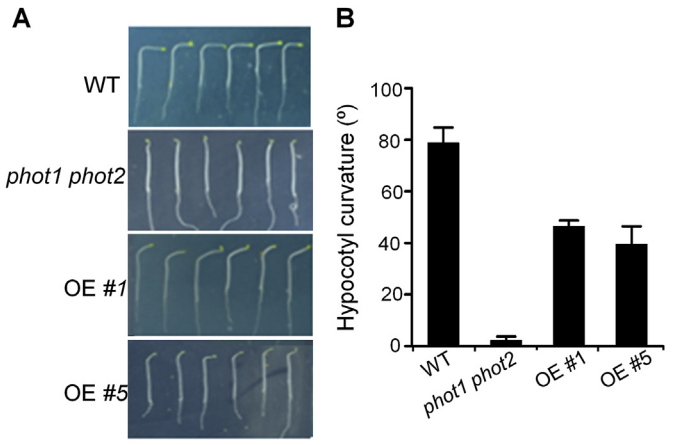

Fig. 4. GhPHOT2-1 restored the phototropism of *Arabidopsis phot1 phot2*. (**A and B**) Hypocotyl phototropism response (**A**) and quantification of hypocotyl curvature (**B**) for *Arabidopsis* WT, *phot1 phot2*, and *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines. Seven-day-old etiolated seedlings of WT, *phot1 phot2*, and *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines were irradiated under high blue light (100 μmol m^{-2} s^{-1}) for 12 h. At least 10 seedlings were used for each biological replicates. Data are means ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

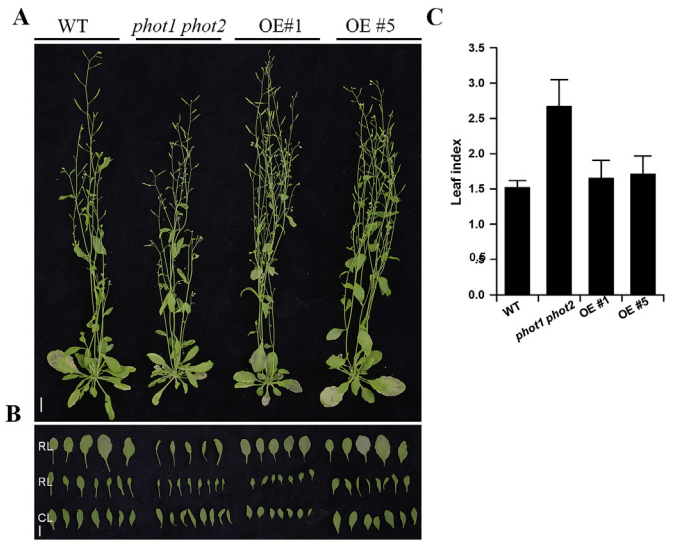

Fig. 5. *GhPHOT2-1* restored the leaf flattering phenotype of phot1 *phot2*. (**A and B**) Plant phenotype (**A**) and leaf shape (**B**) of 50-day-old WT, *phot1 phot2*, and *35S::GhPHOT2:GFP phot1 phot2* transgenic lines (OE#1 and OE#5). Representative rosette leaves (RL) and cauline leaves (CL) were detached for photograph. Scale bars: 1 cm. (**C**) Leaf index for the fifth leaves of WT, *phot1 phot2*, and *35S::GhPHOT2:GFP phot1 phot2* transgenic lines (OE#1 and OE#5). Data are means ± SD of at least 10 plants.

1200 μmol m^{-2} s^{-1} for 3 h, even though the preliminary values were identical to all the seedlings at growth light of 80 μmol m^{-2} s^{-1} (Fig. 8E and F, Fig. S5). Taken together, these results demonstrated that *GhPHOT2-1* is involved in the regulation of cotton chloroplast avoidance response and photoprotection of cotton cultivars under high light.

3. Discussion

Plants have evolved sophisticated mechanisms to cope with fluctuating light conditions, and when exposed to high light irradiation, chloroplast avoidance response is an important photoprotective strategy (Kasahara et al., 2002; Wada et al., 2003; Takahashi and Badger, 2011). However, there is also report that chloroplast avoidance movement is not functional in climbing plants grown under strong sunlight (Higa and Wada, 2016). To date, little research has been focused on the characteristics of cotton chloroplast avoidance movement and no candidate photoreceptors in cotton have been reported to be functionally relevant to adaption to high light stress. In this study, we have revealed that chloroplast avoidance occurs in a tetraploid, *G. hirsutum*, and the two ancestor-like diploid cotton species to cope with high light conditions (Fig. 1, Fig. S1). Our results showed that *G. raimondii* is more sensitive to high blue light irradiation than *G. hirsutum* and *G. arboretum* (Fig. 1, Fig. S1), indicating that different strategies and mechanisms have likely evolved in the three cotton species to adapt to their habitats. We have identified *GhPHOT2-1* as a conserved ortholog of *AtPHOT2*, and it is crucial to the functioning of the chloroplast avoidance response in *G. hirsutum* (Figs. 2, Figs. 3 and 8), indicating that the mechanisms conferring light stress tolerance is conserved between *Arabidopsis* and cotton. Our findings not only revealed the conserved role of a *GhPHOT2* ortholog in directing chloroplast avoidance response, but also may inform future molecular studies that aim to increase production of cotton yield by improving coping mechanisms of cotton to environmental stresses in light of global climate challenge.

Phototropins are plant-specific blue light photoreceptors that are found in various organisms, ranking from unicellular algae to higher plants (Suetsugu and Wada, 2007; Christie, 2007). It was reported that two *Arabidopsis* phototropins, phot1 and phot2, are functionally redundant in mediating chloroplast photo-relocalization response (Sakai et al., 2001; Luesse et al., 2010). In this work, we found that the chloroplast avoidance response of the *Arabidopsis phot2* mutant was restored by the *GhPHOT2-1* ortholog, and chloroplast avoidance response of cotton was disrupted in the VIGS lines of *GhPHOT2-1* (Figs. 3 and 8). Furthermore, maximum quantum yield of PSII (Fv/Fm) of *GhPHOT2-1* VIGS lines was lower than that of the control lines (Fig. 8 and Fig. S5), indicating that silencing of *GhPHOT2*-1 result in damage of the photosynthetic machinery. Previous researches have reported that Atphot2 is localized in the plasma membrane in dark, and the relocalization of Atphot2 to the cytoplasm and the Golgi apparatus is correlated with its role in chloroplast avoidance upon high light irradiation (Kong et al., 2006, 2013a, 2013c). The subcellular localization features of Ghphot2-1 resembles that of Atphot2 (Fig. 6), further proved that Ghphot2-1 is a conserved phot2 receptors. However, given that there are likely redundant members of the phototropin subfamily in cotton (Supplementary Table S1), it remained to be verified on their potential conserved roles as *GhPHOT2* orthologs and their unique contribution to the chloroplast relocation mechanism has yet to be verified. Additionally, several other factors (Kong and Wada, 2016), such as chloroplast actin (cp-actin) filaments (Kadota et al., 2009; Kong et al., 2013b), Chloroplast Unusual Positioning1 (CHUP1) (Oikawa et al., 2008), and PLASTID MOVEMENT IMPAIRED 1 (PMI1) was identified as necessary components in the phot2-mediated chloroplast avoidance response (DeBlasio et al., 2005; Suetsugu et al., 2015). It remains to be elucidated whether the mechanisms are conserved in cotton and whether modification of these genes in cotton has the potential to improve irradiation stress tolerance in the future.

4. Material and methods

4.1. Plant materials and growth condition

Cotton (*Gossypium hirsutum*) line CCRI16 was grown in pots in a growth room at 28 °C, 60% relative humidity, and 80–90 μmol m^{-2} s^{-1} light with a 16-h light/8-h dark photoperiod. The *Arabidopsis* Col-0 ecotype was used as wild-type (WT), and the *phot2* and *phot1 phot2* mutants were described previously (Kinoshita et al., 2001). *Arabidopsis* seedlings were grown in a growth room at 23 °C, 60% relative humidity, and 80–90 μmol m^{-2} s^{-1} light intensity with a 16-h light/8-h dark photoperiod.

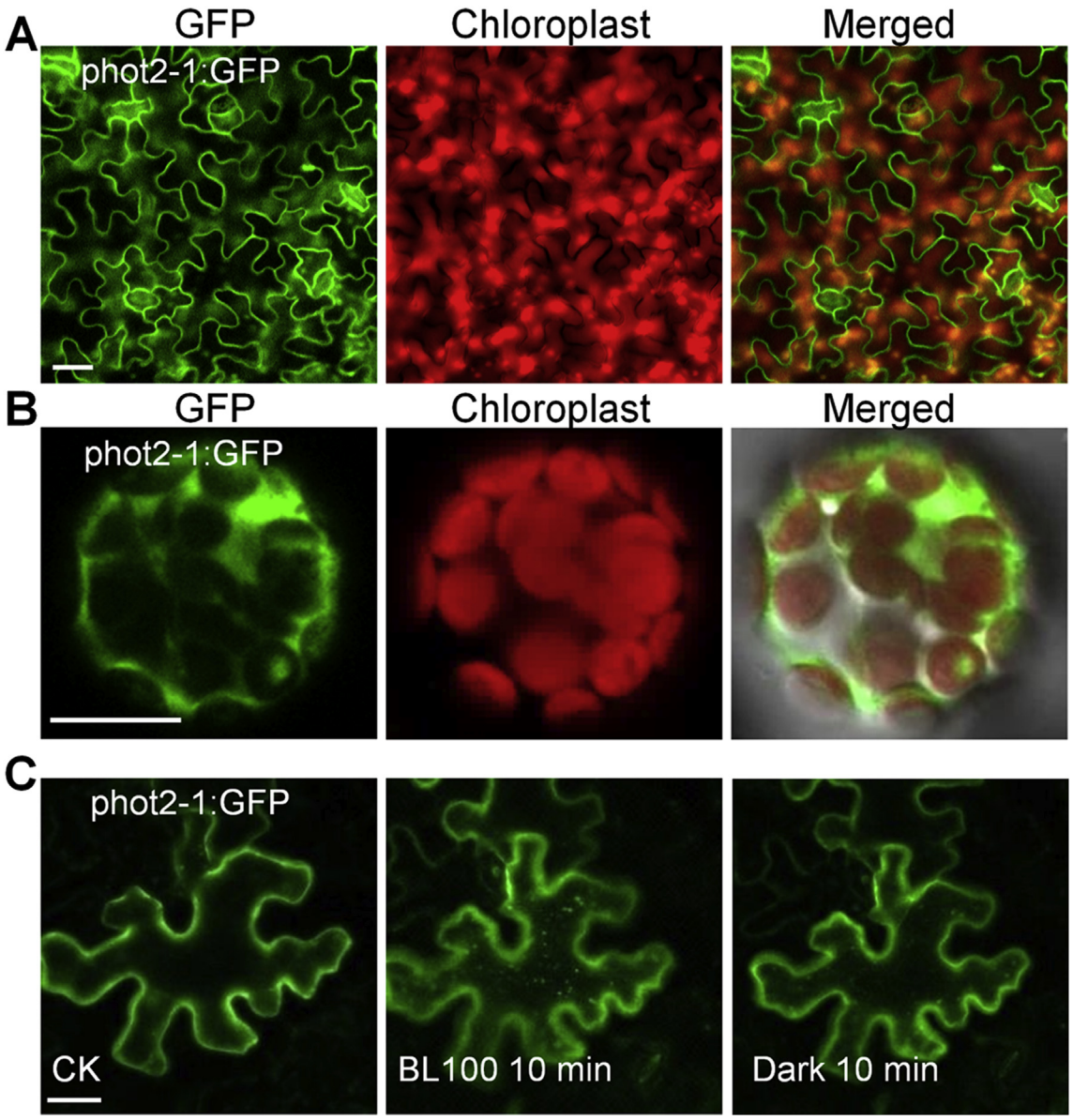

Fig. 6. Subcellular localization of Ghphot2. (**A and B**) Subcellular localization of Ghphot2:GFP in *Arabidopsis* epidermal cells (**A**) and protoplasts (**B**). (**C**) Subcellular localization of Ghphot2:GFP in tobacco pavement cells. Chloroplast aggregates were formed or diminished in the tobacco cytoplasm after high blue light (100 μmol m^{-2}·s^{-1}) irradiation for 10 min and then dark-adapted further for 10 min, leaves without high blue light irradiation were used as controls. Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. Bioinformatics analysis of the cotton PHOT2 gene family

To retrieve the cotton *PHOT2* genes, a full length *AtPHOT2* cDNA and Atphot2 amino acid sequences were used as a query to conduct a BLAST search against *G. hirsutum*, *G. raimondii*, and *G. arboretum* cDNA and protein databases at CottonFGD (https://cottonfgd.org/). Sequences of representative *PHOT1* or *PHOT2* orthologs of *Glycine* max (I1MDL2, I1M4Q3, I1LR23, K7L915), *Theobroma cacao* (XP_017983114.1, A0A061E9J3), *Oryza sativa* (Q2QYY8, Q2RBR1, Q9ST27), *Brachypodium distachyon* (I1IVB6, I1IWY7), *Solanum lycopersicum* (K4DAX8, K4B0G4), and *Zea mays* (A0A1D6NP39, A0A1D6KQ74) were retrieved from UniProt accessions. The amino acid sequence alignment of phot2 between cotton and the above-mentioned species was performed using ClustalW (http://www.genome.jp/tools/clustalw/), and the phylogenetic tree was constructed using the Neighbor-Joining method by MEGA7 (Kumar et al., 2016). Gene structure was displayed with the gene structure display server 2.0 (http://gsds.cbi.pku.edu.cn/).

4.3. Vector construction and transformation

The full-length cDNA of *GhPHOT2-1* was cloned using the primers *GhPHOT2*-1 F: 5′-GGGTCTAGAATGGCTGGGGGACTGCAGG-3′ and *GhPHOT2*-1 R: 5′-CCCGGTACCTTAAAAAATATCCATGTCA-3′, and in-frame fused with the GFP-encoding gene using the XbaI and KpnI restriction sites of the Super1300GFP vector to generate a *35S::GhPHOT2-1:GFP* construct. The construct was transformed into *Arabidopsis phot2* or *phot1 phot2* plants using the floral-dip method (Clough and Bent, 1998), and homozygous transgenic lines were confirmed by RT-PCR analysis.

4.4. VIGS assay

GhCLA1 was cloned into the pYL156 (pTRV-RNA2) vector as previously described (Gao et al., 2011). *GhPHOT2-1* was cloned from cDNA of *G. hirsutum* leaf tissues with primers *GhPHOT2-1*-VIGS F, 5′-CGGAA TTCGCACACTCGGAGCTGCAAG G-3′, and *GhPHOT2-1*-VIGS R, 5′-

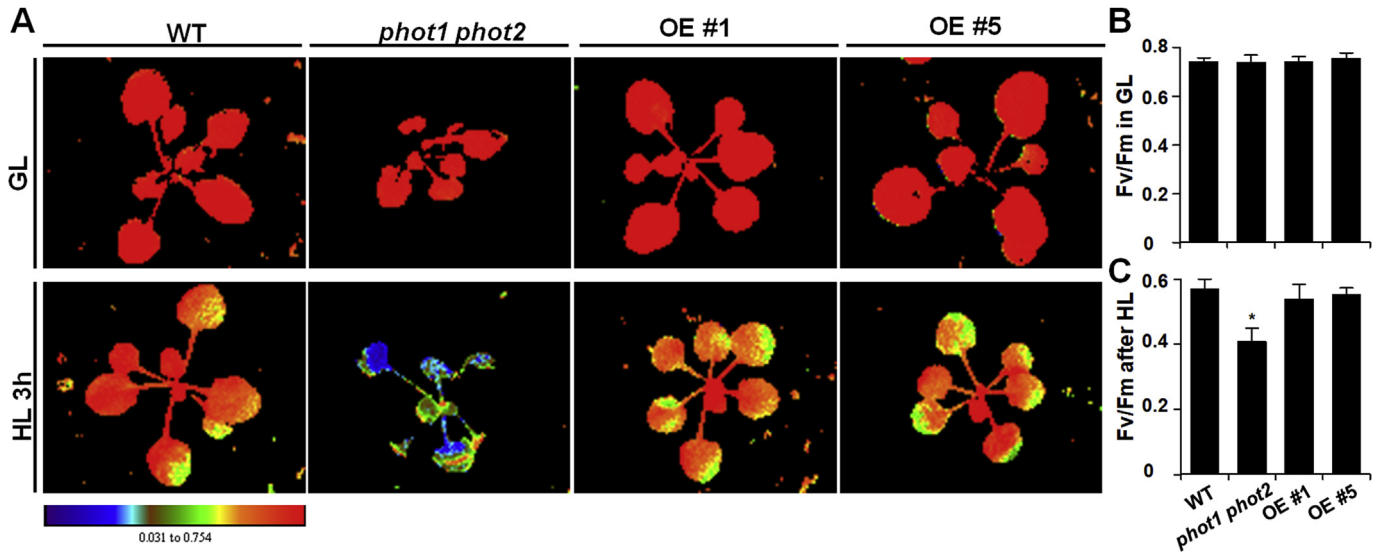

Fig. 7. Chlorophyll fluorescence parameter of *GhPHOT2* transgenic lines. (**A and B**) False-color images (**A**) and quantification (**A**) of Fv/Fm in three-week-old WT, *phot1 phot2*, and *35S::GhPHOT2-1:GFP phot1 phot2* under growth light (GL) or high light (HL). Seedlings of WT, *phot1 phot2*, and *35S::GhPHOT2-1:GFP phot1 phot2* transgenic lines (OE#1, OE#5) were irradiated under growth light of 80 μmol m^{-2} s^{-1} or high light of 1200 μmol m^{-2} s^{-1} for 3 h, and maximal photochemical efficiency of PSII (Fv/Fm) was measured after dark-adapted for 30 min. Range of the false-color was indicated at the bottom. At least 10 seedlings were used for each biological replicates. Data are means ± SD. Significance was determined by Student's *t*-test. *P < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GGGGTACCGAGTGCACAACAGAGCC-3′, and inserted into the pYL156 vector with restriction enzymes BamHI and KpnI. Plasmids containing binary TRV vectors of *pTRV-RNA1* and *pTRV-RNA2* (*pYL156*), as well as *pYL156-GhPHOT2-1* construct were transformed into *Agrobacterium tumefaciens* strain GV3101, respectively. *Agrobacterium* cultures were harvested and infiltrated into two fully expanded cotyledons of 2-week-old plants as previously described (Gao et al., 2011). Virus-induced gene silencing experiments were repeated at least three times with more than 15 plants for each construct per replicate, and *GhCLA1* was used as a visual marker to monitor the efficiency of VIGS in cotton germplasm CCRI16 (Gao et al., 2011).

4.5. RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the materials mentioned above using TRIzol reagent kit (Invitrogen) according to the manufacturer's instruction. First-strand cDNA was synthesized from 5 μg of total RNA with SuperScript II Reverse Transcriptase (Invitrogen). Real time quantitative RT-PCR (qRT-PCR) was performed using Applied Biosystems 7500 Real-Time PCR system, and the expression levels of *AtACTIN2* (*AtACTIN2*-RT F: 5′-AGTGGTCGTACAACCGGTATTGTGCT-3′ and *AtACTIN2*-RT R: 5′- TCCCGCTCTGCTGTTGTGGTG-3′) or *GhUbiquitin7* (*GhUBQ7*, *GhUBQ7*-qRT F: 5′-GAAGGCATTCCACCTGAC CAAC-3′ and *GhUBQ7*-qRT R: 5′-CTTGACCTTCTTCTTCTTGTGCTTG-3′) were used as internal controls for RT-PCR and qRT-PCR, respectively. Expression level of *GhPHOT2* was determined using the *GhPHOT2*-1 RT F: 5′-CAGACTCCCACCCACGTTTGTT-3′ and *GhPHOT2*-1 RT R: 5′-TCT GCCTGTTTTTACCCCTGA-3′, *GhPHOT2*-1 qRT F: 5′-CAGACTCCCACCC ACGTTTGTT-3′ and *GhPHOT2*-1 qRT R: 5′- ACCAATGACAACCTCTGC TGCAT-3′ primer pairs. All of the analyses were performed with three independent biological replicates.

4.6. Chloroplast movement analysis

Leaves were dark-adapted for at least 3 h and detached from seedlings and placed on 1% (w/v) gellan gum. Slit band assays for chloroplast relocations were performed and detached leaves were irradiated with various blue light intensities of 1, 3, 5, 10, 20, 30, 40, 50,100, 200 μmol m^{-2} s^{-1} for 1–3 h through a 3∼5-mm-width slit (Wada, 2013). Chloroplast distribution patterns were observed under bright field microscopy using mesophyll cells of leaf discs under different light conditions. For the measurement of leaf transmittance, dark-adapted excised leaves were placed in a 6-well microplate reader and red light transmittance was recorded using BioTek Epoch before or after irradiation of various blue light intensities for different duration according to Wada et al. (Wada and Kong, 2011; Wada, 2013). High power LEDs with peak wavelength at 470 nm were used as blue light resource. The microplate reader was programmed to eject automatically and to be irradiated with various blue light intensities at designated duration, and red light transmittance was automatically measured.

4.7. Chlorophyll a fluorescence

Chlorophyll fluorescence parameters were measured with the CF Imager chlorophyll fluorescence system (ecotec). Plants irradiated with growth light of 80 μmol m^{-2} s^{-1} or high light of 1200 μmol m^{-2} s^{-1} for 3 h were dark-adapted for 30 min, and maximal photochemical efficiency of PSII (Fv/Fm) was measured.

CRediT authorship contribution statement

Baoshuan Shang: Formal analysis, Formal analysis, Writing – original draft. **Yihao Zang:** Formal analysis, Formal analysis, Writing – original draft. **Xiang Zhao:** Formal analysis, Formal analysis, Writing – original draft, Writing – original draft. **Jindong Zhu:** Formal analysis, Formal analysis. **Cheng Fan:** Formal analysis, Formal analysis. **Xining Guo:** Formal analysis, Formal analysis. **Xiao Zhang:** Formal analysis.

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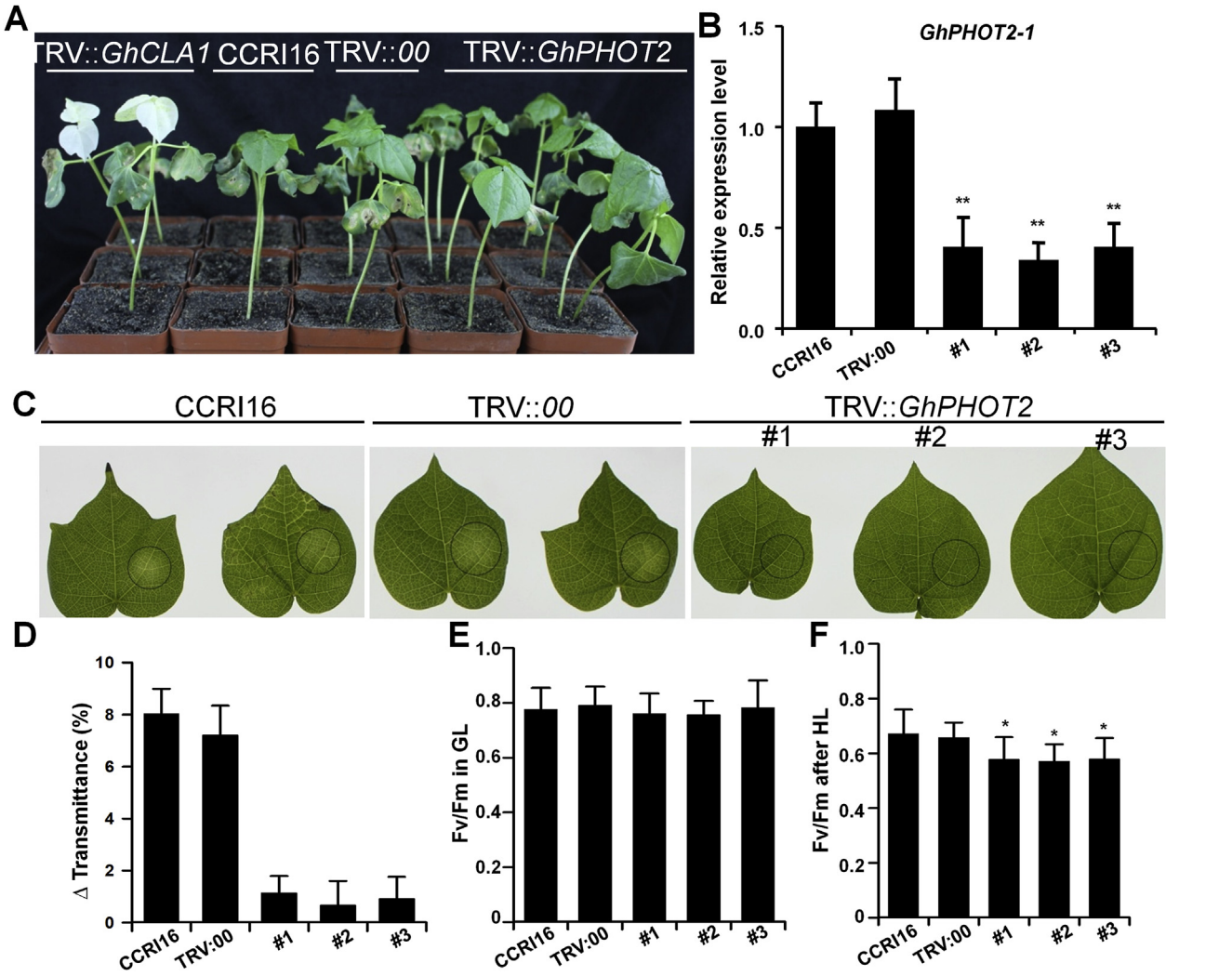

Fig. 8. Silencing of *GhPHOT2* repressed chloroplast avoidance response of cotton leaves. (**A and B**) Morphology (**A**) and qRT-PCR (**B**) analyses of CCRI16, TRV:00, and TRV:*GhPHOT2* lines. TRV:*GhCLA1* was used as a positive control. Expression level of *GhPHOT2* in leaves of CCRI16, TRV:00, and TRV:*GhPHOT2* lines was analyzed. Data are means ± SD of three biological replicates. Significance was determined by Student's *t*-test. **P < 0.01. (**C**) Chloroplast avoidance response in cotton leaves of CK, TRV:00, and TRV:*GhPHOT2* lines under high blue light. The leaves were pre-incubated in the dark and then detached and irradiated with 100 μmol m^{-2} s^{-1} high blue light for 1 h through a 7-mm-diameter hole. Chloroplasts moved to the anticlinal walls under high blue light, and the irradiated area appeared pale green (open circle). (**D**) Red light transmittance quantification for leaves of CCRI16, TRV:00, and TRV:*GhPHOT2* lines under blue light intensities of 100 μmol m^{-2} s^{-1} (BL100) for 3 h. Data are means ± SD of three biological replicates. (**E and F**) Maximal photochemical efficiency of PSII (Fv/Fm) of CCRI16, TRV:00, and TRV:*GhPHOT2* lines under growth light (GL) of 80 μmol m^{-2} s^{-1} (**E**) or high light (HL) of 1200 μmol m^{-2} s^{-1} (**F**) for 3 h. Four-week-old seedlings were irradiated at GL or HL for 3 h and maximal photochemical efficiency of PSII (Fv/Fm) was measured after dark-adapted for 30 min. At least 9 seedlings were used for each biological replicates. Data are means ± SD. Significance was determined by Student's *t*-test. *P < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.11.027.

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