Soybean GmHY2a encodes a phytochromobilin synthase that regulates internode length and flowering time

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Abstract

Plant height and flowering time are important agronomic traits that directly affect soybean [Glycine max (L.) Merr.] adaptability and yield. Here, the Glycine max long internode 1 (Gmlin1) mutant was selected from an ethyl methyl sulfonate (EMS)-mutated Williams 82 population due to its long internodes and early flowering. Using bulked segregant analysis (BSA), the Gmlin1 locus was mapped to Glyma.02G304700, a homologue of the Arabidopsis HY2 gene, which encodes a phytochromobilin (PΦB) synthase involved in phytochrome chromophore synthesis. Mutation of GmHY2a results in failure of the de-etiolation response under both red and far-red light. The Gmlin1 mutant exhibits a constitutive shade avoidance response under normal light, and the mutations influence the auxin and gibberellin pathways to promote internode elongation. The Gmlin1 mutant also exhibits decreased photoperiod sensitivity. In addition, the soybean photoperiod repressor gene E1 is down-regulated in the Gmlin1 mutant, resulting in accelerated flowering. The nuclear import of phytochrome A (GmphyA) and GmphyB following light treatment is decreased in Gmlin1 protoplasts, indicating that the weak light response of the Gmlin1 mutant is caused by a decrease in functional phytochrome. Together, these results indicate that GmHY2a plays an important role in soybean phytochrome biosynthesis and provide insights into the adaptability of the soybean plant.

Keywords: Flowering time, GmHY2a, internode length, phytochrome, phytochromobilin synthase, soybean.

Introduction

Soybean [Glycine max (L.) Merr.] is one of the most economically important leguminous seed crops globally, providing more than a quarter of the total protein in food and animal feed worldwide (Muramoto, 1999; Graham and Vance, 2003). Concomitant with economic development and population accretion, soybean demand is gradually increasing, requiring increased soybean yield (Liu et al., 2020). Soybean adaptability and productivity are directly linked to plant height and flowering time, these important agronomic traits depend not only on endogenous genetic manipulation but also on environmental signals (Eshed and Lippman, 2019). When plants are grown under high-density conditions, the neighbouring vegetation absorbs red (R) light and reflects or transmits far-red (FR) light, which triggers a series of characteristic...
shade-avoidance syndromes (SASs), including the elongation of the hypocotyl (or stem) and petiole, the reorientation of the growth directions of the leaves or branches, and the acceleration of flowering (Carriero et al., 2016).

Plant perception of neighbour proximity or canopy shading is primarily mediated by the phytochromes, which are R- and FR-light photoreceptors (Liu et al., 2021). Phytochromes exist in two photo-convertible forms: an inactive R-absorbing Pr form and an active FR-absorbing Pfr form (Li et al., 2011). Under dark conditions, Pr form phytochromes are synthesized in the cytoplasm; following exposure to normal light (high R: FR), the Pfr form phytochromes are photoconverted to the Pfr form and translocated to the nucleus (Franklin and Quail, 2010). The FR-rich conditions of vegetation shade shift the phytochrome equilibrium toward the inactive Pr form, leading to changes in the downstream signalling pathway, which subsequently alters plant traits and morphologies (Mawphlang and Kharsing, 2017). Phytochrome apoproteins are encoded by a small multigene family (e.g. PHYA to PHYE in Arabidopsis). Phytochrome A (phyA), which is light labile, is the primary phytochrome in etiolated seedlings but is rapidly degraded to much lower steady-state levels upon transfer to light. In contrast, phyB-phyE are light stable in the Pfr form, and phyB is the predominant phytochrome regulating de-etiolation responses in R light (Franklin and Quail, 2010; Li et al., 2011). Of the five phytochromes identified in Arabidopsis, phyB is the predominant negative regulator of SAS (Reed et al., 1993). Active phyB triggers the phosphorylation of phytochrome interacting factors (PIFs), leading to their proteosome-mediated degradation. Under shade conditions, phyB inactivation stabilizes PIFs and allows them to bind and activate downstream targets, mostly auxin biosynthetic genes and cell wall-associated genes involved in promoting stem elongation (Liu et al., 2021). Under deep canopy conditions, phyA has been shown to weaken auxin signalling to antagonize the SAS induced by phyB inactivation, thus preventing unnecessary shade-avoidance responses in severely light-deficient environments (Martínez-García et al., 2014; Yang et al., 2018).

In addition, the role of phytochromes in response to the changing light environments involves the perception of photoperiod, which has been well documented to provide important seasonal information that affects flowering and maturity (Franklin and Quail, 2010). The cultivation of soybean, which is a short-day (SD), photoperiod-sensitive plant, is limited to a narrow range of latitudes. Reducing photoperiod sensitivity to promote early flowering may allow the soybean to adapt to long-day (LD) conditions at higher latitudes. The homologues of soybean phyA, E3 and E4, were identified as flowering and maturity loci using classical genetic approaches (Liu et al., 2008; Watanabe et al., 2009). A unique component and signalling pathway that regulates photoperiodic flowering is a specific E1-mediated regulatory pathway in soybean (Xia et al., 2012). Under LD conditions, E3 and E4 promote the expression of E1 and suppress the expression of the florigen genes GmFT2a and GmFT5a (FLOWERING LOCUS T), resulting in late flowering (Lin et al., 2021). Loss of function of the E3 and E4 alleles leads to photoperiod insensitivity and earlier flowering (Xu et al., 2013).

The photoactive holo-phytochromes consist of a PHY apoprotein covalently attached to a linear tetrapyrrole chromophore (phytochromobilin, 3E-PΦB) (Terry, 1997). The ability of given phytochromes to absorb R and FR light depends on the structural isomerization of PΦB (Legris et al., 2019). The phytochrome chromophore is synthesized in the plastid from 5-aminolevulinic acid (ALA) via the heme branch of the tetrapyrrole biosynthetic pathway (Terry et al., 1993). The first committed step in the synthesis of PΦB is the conversion of heme to biliverdin (BV) IXα by the enzyme heme oxygenase (Weller et al., 1996). BV IXα is reduced to 3Z-PΦB by PΦB synthase (Weller et al., 1997), isomerized to 3E-PΦB, and assembled into the functional holo-phytochrome protein. PΦB synthase is a ferredoxin-dependent BV reductase encoded by HY2 (Kohchi et al., 2001); the HY1 gene encodes heme oxygenase (Muramoto et al., 1999). Disruption of phytochrome synthesis inactivates the entire phytochrome system because all phytochrome proteins bind to the same chromophore (Sawers et al., 2002). Chromophore-deficient mutants, including Arabidopsis hy1 and hy2 (Parks and Quail, 1991; Kohchi et al., 2001), tomato yy-2 and aurea (Muramoto et al., 2005), pea pud1 and pud2 (Weller et al., 1996, 1997), and rice se5 and se13 (Izawa et al., 2000; Yoshitake et al., 2015), are characterized by impaired photomorphogenesis due to lack of light-reversible phytochrome. Characterization of the rice se5 and se13 mutants has demonstrated that the phytochrome chromophore makes significant contributions to the regulation of flowering time (Izawa et al., 2000; Yoshitake et al., 2015).

In this study, a forward genetic strategy was employed to analyse a mutant soybean variety, herein named Glycine max long internode 1 (Gmlin1), which exhibited long internodes and early flowering. We characterized the Gmlin1 mutant and identified GmHY2a as its associated candidate gene. We found that GmHY2a encodes a PΦB synthase and plays a role in the holo-phytochrome response to light. The genetic disruption of chromophore synthesis in Gmlin1 mutants demonstrated here provides a framework for the specific inactivation of the entire soybean phytochrome system. In addition, our results help to clarify the role of phytochrome signalling in soybean development and highlight the potential agronomic importance of light-induced phenotypic plasticity.

Materials and methods

Plant materials

The soybean cultivar Williams 82 was obtained from the Chinese Academy of Agricultural Sciences (Beijing, China). Gmlin1-1 and Gmlin1-2 mutants were generated from the seeds of Williams 82 using ethyl methyl sulfonate (EMS)-induced mutagenesis and selected based on phenotype (long internodes and early flowering). Flowering time was measured at the R1 stage,
which is defined as the time from emergence to the opening of the first flower (Bernard, 1971). Plant height and internode length were recorded at maturity (R8 stage), while the seeds per plant, pods per plant, seed weight per plant, and 100-seed weight were measured after harvesting. To purify the genetic background, the Gmlin1-1 and Gmlin1-2 mutants were backcrossed for four generations in an experimental field in Changchun, Jilin Province, China (43° 88‘N, 125° 35‘E).

**Bulked segregant analysis (BSA)**

We used M2-seq (Zhou et al., 2021) to identify candidate genes in the Gmlin1-1 mutant. We collected leaves from 50 wild-type plants and 45 Gmlin1-1 mutants in the M2 segregation generation. The DNA samples were bulked into two Gmlin1-2 mutants in the M2 segregation generation. The DNA samples were subsequently extracted from the leaves using the Plant Genomic Kit (TIANGEN, China). The candidate genomic regions were identified via whole-genome sequencing, with a depth of approximately 30×, using an Illumina HiSeqX (Illumina Inc., San Diego, CA, USA). Single-nucleotide polymorphisms (SNPs) and small indels were calculated between the wild-type and mutant bulked DNA samples by aligning the sequence reads of individual bulked DNA samples to the Glycine max Wm82.a2.v1 reference genome (https://phytozome-next.jgi.doe.gov/). SNPs and indels were filtered to identify the candidate genes following Zhou et al. (2021).

The Gmlin1-2 mutants were backcrossed with Williams 82 to generate the BCF2 segregating population for re-sequencing. DNA sequences from 50 F2 individuals expressing the wild-type phenotype and 50 F2 individuals expressing the Gmlin1-2 phenotype were bulked into two pools. Candidate genomic regions were identified using a QTL-seq method developed for F2 population sequences by aligning the sequence reads of individual bulked DNA samples to the Glycine max Wm82.a2.v1 reference genome (https://phytozome-next.jgi.doe.gov/). SNPs and indels were filtered to identify the candidate genes following Zhou et al. (2021). We used M2-seq (Zhou et al., 2021) to identify candidate genes in the Gmlin1-1 mutant. We collected leaves from 50 wild-type plants and 45 Gmlin1-1 mutants in the M2 segregation generation. The DNA samples were bulked into two Gmlin1-2 mutants in the M2 segregation generation. The DNA samples were subsequently extracted from the leaves using the Plant Genomic Kit (TIANGEN, China). The candidate genomic regions were identified via whole-genome sequencing, with a depth of approximately 30×, using an Illumina HiSeqX (Illumina Inc., San Diego, CA, USA). Single-nucleotide polymorphisms (SNPs) and small indels were calculated between the wild-type and mutant bulked DNA samples by aligning the sequence reads of individual bulked DNA samples to the Glycine max Wm82.a2.v1 reference genome (https://phytozome-next.jgi.doe.gov/). SNPs and indels were filtered to identify the candidate genes following Zhou et al. (2021). The Gmlin1-2 mutants were backcrossed with Williams 82 to generate the BCF2 segregating population for re-sequencing. DNA sequences from 50 F2 individuals expressing the wild-type phenotype and 50 F2 individuals expressing the Gmlin1-2 phenotype were bulked into two pools. Candidate genomic regions were identified using a QTL-seq method developed for F2 population sequences (Takagi et al., 2013). A genomic region with delta SNP >0.5 was selected as the candidate area. All sequences are available from the BIG Data Center (https://bigd.big.ac.cn/gsa/index.jsp) under accession number CRA005967.

**Vector construction and complementation testing**

The full-length GmHY2a gene sequences, about 7.5 kb long, as well as the ∼3 kb native promoter regions, were amplified from Williams 82 using overlapping PCR. The PCR amplicons were inserted into the binary vector pCAMBIA3301T between the restriction endonuclease sites SacI and Xmal to generate the GmHY2a:pro-GmHY2a plasmid. The GmHY2a:pro-GmHY2a recombinant plasmid was introduced into Agrobacterium tumefaciens strain EHA105 and transformed in the Gmlin1-1 mutant for a complementation test following Agrobacterium-mediated transformation as previously described (Yamada et al., 2010). The primers used are listed in Supplementary Table S1.

**Phylogenetic analysis and conserved protein motif searching**

Homologues of GmHY2a were identified by searching for the full-length amino acid sequence in the Phytozome proteome database (https://phytozome-next.jgi.doe.gov/) and the NCBI database (https://www.ncbi.nlm.nih.gov/) using the BLASTp program. The identified homologues were aligned with GmHY2a using ClustalW as implemented in MEGA7 (Kumar et al., 2016). To analyse the interspecific functional conservation and phylogenetic relationships of the HY2 protein, neighbour-joining phylogenetic tree was then constructed based on alignment using MEGA7 with 1000 bootstrap iterations. Conserved protein motifs were identified using MEME (http://meme-suite.org/tools/meme) (Bailey et al., 2009), with the maximum number of motifs to be found set to 5.

**Gene duplication and expansion analysis**

We performed a synteny conserved block analysis to investigate the gene duplication pattern of the three copies of GmHY2 in soybean. Intraspecific synteny blocks were detected using the MCScanX algorithm (Wang et al., 2013a). Sequences of homologous genes were identified using BLASTp with an E-value cut-off of 1e-5 and other parameters set to default or recommended values. Non-synonymous substitutions per non-synonymous site (Ka) and synonymous substitutions per synonymous site (Ks) values for the homologues were calculated using TBtools (Chen et al., 2020). The time (T) since each duplication event was calculated using the equation T=Ks/2E, where the mean synonymous substitution rate (E) for soybean was set to 6.1 × 10−9 substitutions per synonymous site per year (Lynch and Conery, 2000; Lavin et al., 2005).

**RNA isolation and real-time quantitative PCR (RT-qPCR) analysis**

To analyse the expression patterns of the GmHY2 genes, total RNA was isolated from multiple soybean tissues, including leaves, the stem apex (SA), stems, roots at the V1 stage (first trifoliate), flowers at the R1 stage (beginning of flowering), 2 cm pods at the R3 stage (beginning pod), and seeds at 20 days after pollination, using TRNzol universal reagent (TIANGEN Biotech Co., Beijing, China) following the manufacturer’s instructions. RNA concentration and quality were determined using a Nanodrop spectrophotometer (P330; Implen, Germany). First-strand cDNA was synthesized from 2 μg of total RNA using the Trans-Script One-Step gDNA Removal and cDNA Synthesis SuperMix with Anchored Oligo(dT)18 primer (TransGen Biotech, Beijing, China) following the manufacturer’s instructions. The cDNA was diluted to 100 ng/μl in sterile water, and 2 μl diluted was used as the template for RT-qPCR. RT-qPCR was performed using 2×RealStar Green Fast Mixture (GenStar, Beijing, China) on a Stratagene MX3005P Sequence Detection System (Stratagene, La Jolla, CA, USA) following the manufacturer’s instructions. Expression levels were quantified using three biological replicates. The differences between groups were calculated with the 2−ΔΔCt method. Relative expression levels were calculated using Actin 11 (Glyma.18G229000) as an internal control (Zhang et al., 2020). The primers used for RT-qPCR are listed in Supplementary Table S1.

**Subcellular localization analysis**

To locate the GmHY2a protein, a 1-kb coding region of GmHY2a was amplified from Williams 82 and cloned into an entry vector (pDonor/zeo; Invitrogen, USA) using Gateway BP clonase II (Invitrogen, USA) recombination, following the manufacturer’s instructions. The correct Entry Clone was introduced into the destination vector pENSG-YFP using Gateway LR clonase II (Invitrogen, USA) recombination (Wenkkel et al., 2006). The coding region of RbcS (encoding a protein marker of chloroplast stroma in Arabidopsis) was amplified from Arabidopsis (Col-0) and infused into the binary vector pCAMBIA1300-mCherry. The 35S::YFP-GmHY2a and 35S::AtRbcS-mCherry plasmids were introduced into Arabidopsis (Col-0) mesophyll protoplasts as previously described (Yoo et al., 2007).

To localize the GmPHYA and GmPHYB proteins, the coding sequences of GmPHYA and GmPHYB were amplified from Williams 82 and cloned into the pA7-YFP vector between the restriction endonuclease sites SacI and SalI to generate the 35S::GmPHYA-YFP and 35S::GmPHYB-YFP plasmids, respectively. The two plasmids were transformed into soybean mesophyll protoplasts from Williams 82 and the Gmlin1-1 mutant. Soybean mesophyll protoplasts were prepared, transfected, and cultured as previously described (Xiong et al., 2019). The transformed protoplasts were kept in the dark or illuminated with white light (100 μmol m−2 s−1) for 10 mm before observation under a fluorescence microscope (ECLIPSE C2; Nikon, Japan). YFP fluorescence was visualized using excitation and emission wavelengths of 488 and 500–550 nm, respectively. mCherry fluorescence was visualized using excitation and emission...
wavelengths of 561 and 570–1000 nm, respectively. Chloroplast autofluorescence was visualized using excitation and emission wavelengths of 405 and 417–477 nm, respectively. The primers used to construct vectors for subcellular localization are listed in Supplementary Table S1.

Luciferase complementation imaging (LCI) assays
To test the interaction between GmHY2 and GmFd2, LCI assays were performed in 4-week-old Nicotiana benthamiana leaves. The coding regions of GmHY2a and GmHY2b were fused with the pCAMBIA1300-NLUC vector and GmFd2 was fused with the pCAMBIA1300-CLUC vector between the restriction endonuclease sites KpnI and SalI (Zhou et al., 2018). The recombinant plasmids were introduced into Agrobacterium tumefaciens strain EHA105 and co-infiltrated into Nicotiana benthamiana leaves as described previously (Zhou et al., 2018). LUC activity was analysed using chemiluminescence imaging (4600SF; Tanon, China) after infiltration for 48 h. The primers used for the LCI assay are listed in Supplementary Table S1.

Photomorphogenic assays and end-of-day FR (EOD-FR) treatment
Photomorphogenic assays were performed as described previously (Izawa et al., 2000; Liu et al., 2008), with few modifications. Williams 82, Gmlin1-1, Gmlin1-2, and the transgenic complementation plants were grown at 25 °C in darkness, or under continuous R, FR, blue, or white light conditions in a multicolour LED incubator (ZDN-1000; YangHui, China). Hypocotyl length was measured 7 days after sowing. For EOD-FR treatments, plants were grown in a multicolour LED incubator with a 16 h light/8 h dark cycle under normal white light (WL, 100 μmol m⁻² s⁻¹). At 3 days after emergence, half of the seedlings were treated with FR light (14 μmol m⁻² s⁻¹) for 30 min at the end of the light period for 4 days before measurements were taken. Control seedlings were grown under continuous normal WL conditions. To measure the expression levels of SAS-related genes, seedling hypocotyl tissues were harvested 1 h after the end of the final EOD-FR pulse on day 7.

Photoperiodic transfer treatment
Photoperiodic reciprocal transfer experiments were performed following the analytical model previously described (Ellis et al., 1992). Two photoperiods were established: a LD photoperiod of 16 h light/8 h dark, and a SD photoperiod of 12 h light/12 h dark. The growth chambers were kept at a constant temperature of 25 °C. A tray of 15 plants was considered a block, and each transfer treatment was assigned to one block. The trays were periodically moved randomly around the growth chambers to reduce the impact of microclimatic conditions. Transfers were made at 5, 10, 15, 20, 25, 30, 35, and 40 days after emergence (DAE). Control plants were continuously grown under LD or SD conditions. Once plants had been transferred, they were maintained in the new growth chamber. Time to first flower (R1) (Fehr et al., 1971) was noted for each plant. We performed a segmented linear regression analysis of the data obtained through the above transfer experiments with OriginPro 8.5. To analyse the expression patterns, we screened for the most likely causal variant of Gmlin1-1. The likely variant was mapped to Glyma.02G304700 on the Glyma.02G304700 map. After filtering out all undesirable variations, we screened for the most likely causal variant of Gmlin1-1. The likely variant was mapped to Glyma.02G304700 on the Glyma.02G304700 map. After filtering out all undesirable variations, we screened for the most likely causal variant of Gmlin1-1. The likely variant was mapped to Glyma.02G304700 on the Glyma.02G304700 map. After filtering out all undesirable variations, we screened for the most likely causal variant of Gmlin1-1. The likely variant was mapped to Glyma.02G304700 on the Glyma.02G304700 map.

Results
Phenotypic characterization of the soybean Gmlin1 mutant
Compared to the wild-type soybean cultivar (Williams 82), the EMS-induced soybean mutants Gmlin1-1 and Gmlin1-2 were significantly taller and flowered significantly earlier (Fig. 1A). In the field and under artificial LD conditions (16 h light/8 h dark), the Gmlin1-1 and Gmlin1-2 mutant lines flowered about 1 d earlier than Williams 82: a small, but statistically significant (P<0.01), difference (Fig. 1C). Even under the relatively extreme SD conditions (12 h light/12 h dark), the Gmlin1-1 and Gmlin1-2 mutants flowered 1 day earlier than Williams 82: a small, but statistically significant difference (Fig. 1C). At maturation, the main stems of the field-grown Gmlin1-1 and Gmlin1-2 mutants were about 15 cm taller than those of Williams 82 (Fig. 1D). This difference in plant height was reflected in the longer internodes of the Gmlin1-1 and Gmlin1-2 mutants; the Gmlin1 mutants had fewer internodes on the main stem (Fig. 1E). Genetic allelic tests were performed by crossing Gmlin1-1 with Gmlin1-2. The phenotypes of the intercrossed F1 hybrids were similar to those of their parents (Fig. 1A). Sequencing analysis indicated the sites mutated in the parent mutants were heterozygous in the F1 plants (Supplementary Fig. S1), suggesting that Gmlin1-1 and Gmlin1-2 were allelic to each other and that both phenotypes were controlled by the same gene.
other mis-splice was a 41 bp intron insertion in the coding sequence and the loss of exon 3, shifting the reading frame of the processed mRNA. The Gmlin1-2 mutant had a single-nucleotide substitution (G to A at 5862 bp) in exon 7 of the Glyma.02G304700 gene (Fig. 2C), which caused the non-synonymous substitution of Gly255 to Glu255 in the encoded protein.

To validate the candidate gene, a genetic complementation test was performed to confirm that these mutations produced the Gmlin1 mutant phenotype. We obtained 30 independent positive transgenic lines in the Gmlin1-1 background in the T0 generation; 24 of these transgenic lines had reverted to the wild-type phenotype in the T1 generation. Three independent complementation transgenic lines were selected for characterization analysis (Fig. 2D). Genetic analysis verified that transgenic plants in the Gmlin1-1 background carried both the wild-type and Gmlin1-1 mutant genotypes (Fig. 2E). We statistically analysed the flowering times and heights of the T2 generation transgenic plants grown in the field. In contrast to the Gmlin1-1 mutant phenotype and similar to Williams 82, the flowering time of the three complementation transgenic lines was about 54 days, and the plant height of these lines was 110 cm at maturity (Fig. 2F, G). These results indicated that Glyma.02G304700 was indeed the candidate gene.

**Conservation and duplication of GmHY2 in soybean**

Bioinformatics analysis indicated that the Glyma.02G304700 gene encoded a phytochromobilin synthase (PΦB synthase), with a ferredoxin-dependent biliverdin reductase (FDBR) domain. The alignment of known homologous sequences indicated that the mutations of the candidate gene in Gmlin1-1 and Gmlin1-2 were both located in the FDBR structural domain and demonstrated that these sites were highly conserved across multiple plant species (Supplementary Fig. S4). As sequence comparisons showed that the candidate gene was a homologue of the Arabidopsis HY2 (65% amino acid identity), we named the Glyma.02G304700 gene GmHY2a. Glyma.14G009100 (GmHY2b) and Glyma.14G136300 (GmHY2c) genes are likely GmHY2a homologues in the soybean genome (Glycine max Win82.a4.v1). An exon is lost in GmHY2b due to the exon skip in the coding sequence (Supplementary Fig. S5A). GmHY2c might be a pseudogene that had a truncated open reading frame length of 423 bp (Supplementary Fig. S4), and its expression level was predicted to be extremely low in the transcriptome database phytozome (https://phytozome-next.jgi.doe.gov/). Phylogenetic analysis of 14 homologues of the HY2 protein from 13 species showed that homologues of HY2 were present in a wide variety of photosynthetic organisms, including algae, basal vascular plants, monocotyledons, and...
Fig. 2. Bulked segregant analysis (BSA)-based localization of the candidate gene and functional complementation test. (A, B) Delta SNP indexes of (A) all chromosomes and (B) chromosome 02 of the F2 population of Gmlin1-2. (C) Genomic structure of the Glyma.02G304700 gene. The red vertical lines represent the sites of the two allelic mutations. (D) Phenotypes of Williams 82, the Gmlin1-1 mutant, and three independent complementary transgenic plants (Gmlin1-1\textsubscript{Com}) with the Gmlin1-1 mutant background. Plants were grown in a growth chamber under a 14 h light/10 h dark cycle. Scale bar=5 cm. (E) Sequence analysis of the Glyma.02G304700 gene showing the transgenic events. The red box represents the mutation site of the Gmlin1-1 mutant. Two product peaks can be seen in the transgenic plants corresponding to both the wild-type and the mutant sequences. (F) Flowering times and (G) plant heights of Williams 82, the Gmlin1-1 mutant, and the transgenic complementation lines. Plants were grown in the field at Changchun, China (May to August 2021). All data shown are means ±SD (at least 10 plants). Asterisks indicate statistically significant differences (**P<0.001, Student's t-test), ns, non-significant difference.
GmFd2 (Supplementary Fig. S5C). This result suggested that was significantly stronger than that between GmHY2b and indicated that the interaction between GmHY2a and GmFd2. Using the 1 Mb region on chromosome 14 around GmHY2c, we found that some sequences flanking GmHY2c had a syntenic relationship with those on chromosome 17, suggesting that chromosome 17 was the most likely origin of GmHY2c. However, the GmHY2c homologue was lost from the homologous region of chromosome 17 (Fig. 3B).

Estimations of the possible duplication times of the three GmHY2 genes based on the average synonymous substitution rate (Ks) for the homologous block suggested that GmHY2a and GmHY2b were duplicated about 11.15 Mya, GmHY2c and the corresponding region of chromosome 17 were duplicated about 12.4 Mya, corresponding to a Glycine lineage-specific duplication event. The highest levels of expression found in the leaves and stem apex (Fig. 3D). The similar expression patterns of the two genes implied that both genes have normal transcription levels in soybeans (Fig. 3A). Across plants, as expected, soybean GmHY2 was closest to its homologues from other plants in the Leguminosae (Fig. 3A). Across these 14 HY2 proteins, MEME analysis identified five significant motifs; GmHY2b lacked conserved motif 3 compared to GmHY2a and other higher plants (Fig. 3A).

Synteny analysis revealed that GmHY2a and GmHY2b, not GmHY2c, were likely duplicated genes in soybean (Fig. 3B). The similar expression patterns of the two genes implied that both of the GmHY2 homologues interacted with GmFd2 (Supplementary Fig. S5C). However, differences in LUC intensity in GmHY2a and GmHY2b were constitutively expressed in all tested tissues (leaves, SAs, stems, roots, flowers, pods, and seeds), with the highest levels of expression found in the leaves and stem apex (Fig. 3D). The similar expression patterns of the two genes suggested that the weak interaction suggests the GmHY2b proteins might have redundant functions. We thus speculated that GmHY2a plays an important role in the soybean chromophore biosynthesis pathway.

To determine the subcellular location of GmHY2a, we fused the yellow fluorescent protein (YFP) to the coding region of GmHY2a under the control of the 35S promoter, and then transferred the fusion protein to Arabidopsis protoplasts. In Arabidopsis protoplasts, YFP fluorescence signals, corresponding to the 35S:YFP-GmHY2a fusion protein, were observed in a punctate pattern inside the chloroplasts (Fig. 3E). When YFP-GmHY2a was transiently co-expressed with AtRbcS (a marker protein of the chloroplast stroma in Arabidopsis) fused with mCherry (a red fluorescent protein), the yellow and red fluorescent signals were merged within the chloroplasts (Fig. 3E), demonstrating that GmHY2a was a chloroplast-targeted protein, similar to Arabidopsis HY2 (Kohchi et al., 2001).

The phytochrome-mediated light response was disrupted in Gmlin1 seedlings

To test the response of the Gmlin1 mutants to light signals, we analysed the inhibitory effects of different light conditions on hypocotyl length, which is considered a standard test of light-responsiveness (Sawers et al., 2002; Hu et al., 2021). The phytochrome-mediated light responses were weakened in these mutants. The insensitivity of the Gmlin1 mutants to R and FR light, suggesting that the phytochrome-mediated light responses were weakened in these mutants.
Fig. 3. Functional analysis of GmHY2 and its homologues. (A) Phylogenetic analysis and motif comparisons among GmHY2a (Glyma.02G304700) and GmHY2b (Glyma.14G009100) from Glycine max and its homologues from Phaseolus vulgaris, Vigna unguiculata, Medicago truncatula, Cicer arietinum, Lotus japonicus, Arabidopsis thaliana, Oryza sativa, Zea mays, Sorghum bicolor, Physcomitrella patens, Selaginella moellendorffii, and Prochlorococcus phage. The five conserved motifs are represented by coloured boxes. (B) Syntenic relationships among homologous blocks carrying the GmHY2 sequences. The red blocks represent GmHY2, and the blue blocks represent other genes around GmHY2. Solid black lines connect homologous gene pairs. (C) The evolutionary model for GmHY2-containing genomic blocks over the course of soybean genome evolution. 0.3<Ks<1.5 represents legume WGD, Ks<0.3 represents Glycine WGD. (D) Relative expression levels of GmHY2a and GmHY2b in various tissues of Williams 82. All data shown are means ±SD. (E) Subcellular localization of GmHY2a in Arabidopsis protoplasts. Free YFP was used as the control. Scale bars=10 μm.
Fig. 4. Phytochrome responses of Williams 82 and the Gmlin1 mutants. Phenotypes and the corresponding hypocotyl lengths of Williams 82, the two Gmlin1 mutants, and the Gmlin1-1 complementation plants after 7 days of growth (A, B) in the dark, (C, D) under white light (100 μmol m$^{-2}$ s$^{-1}$), (E, F) under red light (31 μmol m$^{-2}$ s$^{-1}$), (G, H) under far-red light (14 μmol m$^{-2}$ s$^{-1}$), and (I, J) under blue light (63 μmol m$^{-2}$ s$^{-1}$). Scale bars=2 cm. Hypocotyl lengths are means ±SD (n=10). Asterisks above the graphs indicate statistically significant differences (***P<0.001, Student's t-test). ns, non-significant difference.
exposure initiated the nuclear transport of GmPHYA and GmPHYB. However, in the light-exposed Gmlin1-1 protoplasts, GmPHYA-YFP and GmPHYB-YFP primarily remained in the cytoplasm, suggesting that phytochromes could not be effectively imported into the nucleus in the Gmlin1-1 mutant (Fig. 5). These results indicated that, in Williams 82, GmPHYA and GmPHYB act as functional photoreceptors and nuclear importers, whereas in the Gmlin1-1 mutant, the nuclear transport of GmPHYA and GmPHYB is inhibited. Thus, mutations in GmHY2a may prevent the light-inducible photoconversion of Pr to the physiologically active Pfr form, leading to changes in phytochrome signalling transduction in the nucleus.

The Gmlin1 mutant exhibited constitutive SARs, causing internode elongation

We next investigated the response of the Gmlin1-1 mutant to EOD-FR treatment, which mimics shade conditions (i.e. reduced R:FR ratios) and causes similar changes in plant phenotype (Smith and Whitelam, 1997; Takemura et al., 2015). Williams 82 seedlings reacted strongly to simulated shade conditions (EOD-FR), developing significantly elongated hypocotyls (Fig. 6A, B). In contrast, the response of the Gmlin1-1 seedlings to EOD-FR was greatly reduced, and the difference in hypocotyl length between the seedlings grown under WL and under EOD-FR was slight (Fig. 6A, B). RT-qPCR analysis showed that the differences in the expression levels of three soybean genes (GmHB2, GmIAA29, and GmPIL1), which are homologues of known shade-induced markers (Yang and Li, 2017), were more marked between WL and EOD-FR in Williams 82 than in Gmlin1-1 (Fig. 6C–E). Under normal WL, the expression levels of three marker genes were significantly increased in the Gmlin1-1 mutant when compared to Williams 82 (Fig. 6C–E). These results suggested that Gmlin1 mutants might express a constitutive shade avoidance response, even

![Fig. 5. Subcellular localization of soybean phytochrome A and phytochrome B. GmPHYA-YFP and GmPHYB-YFP were expressed in soybean mesophyll protoplasts from Williams 82 and the Gmlin1-1 mutant. YFP fluorescence appears yellow, while chloroplast autofluorescence appears red. Merged images show the combination of the three channels (YFP fluorescence, chloroplast autofluorescence, and bright field). Scale bars=10 μm.](image-url)
under normal growth conditions. Moreover, they were less sensitive to shade.

Key phytohormones reported to be involved in the plant response to shading based on an increase in hypocotyl and internode length are gibberellin (GA) and auxin [indole-3-acetic acid (IAA)] (Yang and Li, 2017; Liu et al., 2021). RT-qPCR was performed to measure the relative expression of known IAA-related genes, including \textit{GmAIA9} and \textit{GmSAUR} (Small Auxin Up RNA), as well as GA-related genes, including \textit{GmGA20ox} (GA-20 oxidase) and \textit{GmGA3ox} (GA-3 oxidase), in Williams 82 and the \textit{Gmlin1} mutant. Compared with Williams 82, these genes were significantly up-regulated in the \textit{Gmlin1} mutant, especially \textit{GmSAUR} and \textit{GmGA3ox} (Supplementary Fig. S6). These results suggested that mutation in \textit{GmHY2a} might affect the levels of GA and IAA, resulting in promoting internode elongation of the \textit{Gmlin1} mutant.

Photoperiod sensitivity was reduced and flowering time was accelerated in the \textit{Gmlin1} mutant

To analyse the photoperiod sensitivity of the \textit{Gmlin1} mutant, we performed a photoperiod transfer experiment following the model of Ellis et al. (1992). Development before floral initiation in soybean consists of three phases: the pre-inductive phase (\(aI\)), the inductive (photoperiod-sensitive) phase under LD or SD conditions (\(IL/IS\)), and the post-inductive phase (\(a3\)) (Fig. 7A) (Ellis et al., 1992). Segmented linear regression analysis of the flowering times of Williams 82 and \textit{Gmlin1} revealed that the duration of the photoperiod-sensitive phase under SD conditions (\(I_S\)) was 16 DAE and 15 DAE, respectively, whereas the duration of the photoperiod-sensitive phase under LD conditions (\(I_L\)) was 37 DAE and 29 DAE, respectively (Fig. 7B, C). The slope coefficient of the regression line, which reflects photoperiod sensitivity,
was lower for the *Gmlin1-1* mutant (*I_s* slope, -0.89; *I_l* slope, 0.48) when compared to Williams 82 (*I_s* slope, -1.27; *I_l* slope, 0.58), suggesting that the *Gmlin1-1* mutant was less sensitive to photoperiod than Williams 82 (Fig. 7B, C; Supplementary Table S3).

We then measured the expression levels of key genes in the soybean photoperiodic pathway under LD and SD conditions. *E1*, which is the central gene in the soybean photoperiodic pathway (Xia *et al.*, 2012), exhibited a bimodal expression pattern and was down-regulated in *Gmlin1-1* compared to Williams 82 under both LD and SD conditions (Fig. 7D, E). In Williams 82, the transcriptional levels of *GmFT2a* and *GmFT5a*, two flowering inducers in soybean (Kong *et al.*, 2010, 2014), peaked 4 h after dawn and at dusk under LD conditions (Fig. 7D), whereas under SD conditions, these genes were up-regulated during the day and down-regulated at night (Fig. 7E). In general, both genes were up-regulated in Williams 82 under SD conditions compared to LD conditions (Fig. 7D, E). Although the expression patterns of *GmFT2a* and *GmFT5a* in the *Gmlin1-1* mutant were similar to those in Williams 82, both genes were strongly up-regulated in *Gmlin1-1* compared to Williams 82 irrespective of day length (Fig. 7D, E). *GmHY2a* expression was seen throughout the day and night, with a slight increase at 4 h after dawn, then decreased gradually during daytime and increased gradually at night under both SD and LD conditions in Williams 82. The expression levels in the *Gmlin1-1* mutant were slightly lower, but have similar patterns of expression to that of Williams 82 (Supplementary Fig. S7). These results suggest that *GmHY2a* might affect the soybean photoperiodic pathway, and that the downregulation of *E1* in

![Fig. 7. Photoperiodic response of Williams 82 and *Gmlin1-1*. (A) Schematic representation of flowering responses of plants transferred from LD conditions (16 h light / 8 h dark) to SD conditions (12 h light / 12 h dark) or vice versa at various times after emergence. Solid lines represent transfers from SD to LD; dashed lines represent transfers from LD to SD. (B) Flowering response of Williams 82. (C) Flowering response of *Gmlin1-1*. All data are means ±SD (n=15). (D, E) Diurnal expression levels of *E1*, *GmFT2a*, *GmFT5a* in Williams 82 and *Gmlin1-1* seedlings grown under (D) LD conditions and (E) SD conditions. Light periods are unshaded; dark periods are shaded. All data are means ±SD of three biological replicates.](image)
the Gmlin1 mutant may lead to the upregulation of GmFT2a and GmFT5a and the consequent acceleration of flowering.

No significant effect on yield between Williams 82 and the Gmlin1 mutant

Next, we tested whether the Gmlin1 mutation affected soybean yield. At maturity, seeds of the Gmlin1-1 and Gmlin1-2 mutants were of the same size as those of Williams 82 (Fig. 8A). We measured several yield-related parameters viz., pods per plant, seeds per plant, seed weight per plant, and 100-seed weight for Williams 82 and the two Gmlin1 mutants under normal condition in the field for 2 years, and we found no significant differences in yield-related parameters between Williams 82 and the Gmlin1 mutants (Fig. 8B-E). These observations indicated that the Gmlin1 mutation had limited influence on yield at higher latitudes.

Discussion

Mutation in soybean GmHY2a dysregulated the phytochrome response to light

Phytochromes are important photoreceptors that respond to ambient light to regulate plant growth and development (Franklin and Quail, 2010). In this study, we found that the long internodes and early flowering of the mutant line Gmlin1 were associated with the mutation of a single recessive gene, GmHY2a, which encodes the PΦB synthase known to be a key enzyme of phytochrome chromophore biosynthesis (Kohchi et al., 2001). Mutations that disrupt PΦB synthase function have been characterized in many species. The first identified and located mutant was Arabidopsis hy2, which expresses a long-hypocotyl phenotype and was shown to lack light-reversible phytochrome (Koornneef et al., 1980; Kohchi et al., 2001). Subsequently, hy2 mutants have been identified in many common crops, including tomato (aurea; Muramoto et al., 2005), maize (elh1; Sawers et al., 2002, 2004), rice (sc13; Saito et al., 2011; Yoshitake et al., 2015), and cucumber (elh1; Hu et al., 2021). These mutants, which are defined as phytochrome chromophore-deficient mutants, have similar phenotypes that are consistent with severe reductions in functional phytochromes. Similar to these previously reported species, the soybean Gmlin1 mutant responded weakly to both R and FR light and expressed a photomorphogenesis defective phenotype (Fig. 4). This indicated that, across multiple plant species, GmHY2a is functionally conserved and that HY2 proteins play a role in phytochrome biosynthesis. In the rice mutant sc5, which harbours a mutation in a heme oxygenase important for phytochrome chromophore synthesis, PHYA and PHYB were mostly found in the cytoplasm under light conditions, suggesting that phytochromes could not be effectively imported into the nucleus, where most signalling functions occur (Zheng et al., 2019). Similarly, phytochromes were not effectively imported into the nucleus in the Gmlin1 mutant exposed to light (Fig. 5). Our results indicated that the entire phytochrome system was inactivated and that the mutation in GmHY2 influenced the conversion of Pr to Pfr, leading to the accumulation of the Pfr form in the cytoplasm.

The Gmlin1 mutant exhibited constitutive SARs, and this mutation might influence the auxin and gibberellin pathways to promote internode elongation

Previous studies have shown that shade conditions or treatment with low R:FR light leads to the conversion of phytochromes from Pfr to Pr and induces many auxin-responsive genes to increase stem elongation (Müller-Moule et al., 2016). It has been shown that active phyB is translocated into the nucleus, where it acts to repress SAR by inhibiting the activities of a group of positive regulators of SAR, including PIF3, PIF4, PIF5, and PIF7 (Lorrain et al., 2008). The PIFs fine-tune the SAR by directly targeting core marker genes, including ATHB2 (HOMEBOX2) and PIL1 (PIF-LIKE 1) (Kuniihiro et al., 2011). ATHB2 is a member of the homeodomain leucine zipper (HDZip) family of transcription factors (Chen et al., 2014; Zhang et al., 2019). In Arabidopsis, ATHB2 transcript abundance increases rapidly following EOD-FR treatment, leading to the expression of a series of SAR phenotypes (Schenk et al., 1993); the basic helix-loop-helix (bHLH) transcription factor PIL1 is also rapidly up-regulated in Arabidopsis under low R:FR conditions (Hornitschek et al., 2009). PIL1 is also reported to induce the endogenous auxin biosynthesis pathway in plants exhibiting SAS via auxin signalling components, such as AUX/IAAs (Procko et al., 2016), and auxin has been implicated in hypocotyl and stem elongation in plants (Liscum et al., 2002). Consistent with these previous studies, GmHB2, GmIAA29, and GmPIL1 were significantly up-regulated in Williams 82 following EOD-FR treatment (Fig. 6C-E). Under normal WL, all three marker genes were up-regulated in the Gmlin1 mutant compared to Williams 82 and thus may play an important role in the elongation of the internode and hypocotyl in the Gmlin1 mutant. Up-regulation of the auxin-responsive genes GmIAA29, GmIAA9, and GmSAUR (Small Auxin Up RNA) in the Gmlin1 mutant suggested that one mechanism underlying the expression of the long internode phenotype in the mutant may be the promotion of auxin synthesis (Fig. 6D; Supplementary Fig. S6A, B). In addition, gibberellin also plays a major role in shade-induced elongation of the hypocotyl and stem. It has been reported that PIFs are released from repression by Pfr-form phytochromes, increasing GA levels (Liu et al., 2021). Low R/FR, conditions up-regulate the GA synthesis genes GA20ox1 and GA20ox2 (Hisamatsu et al., 2005). In a recent report, soybean GmGA3ox was found to be functionally conserved in GA biosynthesis regulating plant height and yield (Hu et al., 2022). Here, the GmGA2ox and GmGA3ox genes were significantly up-regulated in the Gmlin1 mutant, suggesting that GA was also involved in hypocotyl and internode elongation of the Gmlin1 mutant (Supplementary Fig. S6C, D). Together, our results showed that mutations in GmHY2a prevent the
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phytochrome-mediated regulation of downstream genes in the nucleus and increase the expression of auxin- and gibberellin-related genes to promote internode elongation.

The early flowering and reduced photoperiod sensitivity of Gmlin1 indicate that this mutant might successfully tolerate LD conditions at higher latitudes (Lin et al., 2021). The GmHY2 mutation affected all soybean holo-phytochromes, and the Gmlin1 mutant flowered earlier than Williams 82 under both LD and SD conditions; flowering was particularly early under LD conditions (Fig. 1C). To compare the photoperiod sensitivity of Williams 82 and the Gmlin1 mutant, we performed reciprocal transfer experiments between the LD and SD photoperiods. In chickpeas, similar photoconversion experiments demonstrated that
time to flowering is positively correlated with the duration of photoperiod sensitivity: plants with later flowering times have longer periods of photosensitivity, and plants with earlier flowering times have shorter periods of photosensitivity (Daba et al., 2016). Consistent with this, the photoperiod-sensitive phase of the Gmlin1 mutant was shorter than that of Williams 82 under both LD and SD conditions (Fig. 7B, C; Supplementary Table S3). In addition, based on the slope coefficients of the flowering responses, which can be used to estimate photoperiod sensitivity, the Gmlin1 mutant was less sensitive to photoperiod than Williams 82 (Fig. 7B, C; Supplementary Table S3).

Phytochromes are photoreceptors that mediate light signals to regulate the photoperiodic response and control flowering time (Franklin and Quail, 2010). The soybean E3 and E4 genes, which encode homologues of phytochrome A (GmphyA3 and GmphyA2, respectively), are known photoreceptors in the soybean photoperiodic response (Liu et al., 2008; Watanabe et al., 2009). In the double recessive and non-functional e3/e4 genotype soybean cultivar, early flowering was observed under both natural day length and artificial LD conditions (Xu et al., 2013; Lin et al., 2021). Similarly, we demonstrated that Gmlin1, which lacks all phytochromes, also exhibited early flowering under LD and SD conditions. The photoperiod insensitivity and early flowering of the Gmlin1 mutants were reflected in the expression patterns of genes involved in the photoperiod regulatory pathway. The expression of E1 was also suppressed in the Gmlin1 mutant, as in the soybean e3/e4 genotype (Xu et al., 2015). Early flowering is often associated with a short vegetative stage, leading to reduced soybean yield. Our results indicated that the GmHY2a mutation does not reduce weight per plant or decrease grain number per plant (Fig. 8), with Gmlin1 exhibiting a WT-like yield per plant. We identified two possible explanations for this result: first, the constitutive shade avoidance phenotype of the Gmlin1 mutant may have led to the growth of petioles or branches at more acute angles increasing sunlight capture and compensating for decreases in photosynthetic efficiency. Alternatively, the North American cultivar Williams 82, which is suitable for cultivation in the Huanghuai region of China, may not have been the most suitable choice for cultivation at higher latitudes, resulting in misleadingly low yields. A premise of soybean adaptation to long-day conditions at higher latitudes is that these adaptations should not affect normal growth. To sum up, the photoperiod-insensitive mutant Gmlin1 may represent a useful reference point for soybean cultivation under LD conditions at higher latitudes.

Supplementary data

The following supplementary data are available at JXB online.

Fig. S1. Sequence analysis of the mutation site in GmHY2a in Gmlin1-1, Gmlin1-2, and F1 (Gmlin1-1 × Gmlin1-2) plant.

Fig. S2. Bulked segregant analysis (BSA) mapping of Gmlin1-1.

Fig. S3. The transcription sequence of GmHY2a in Williams 82 and Gmlin1-1.

Fig. S4. Alignment of GmHY2 and known homologous proteins from Oryza sativa, Glycine max, Arabidopsis thaliana, Zea mays, and Cucumis sativus.

Fig. S5. Comparison of structure and function between GmHY2a and GmHY2b.

Fig. S6. Relative expression levels of plant height-related genes in Williams 82 and Gmlin1-1.

Fig. S7. Diurnal expression levels of GmHY2a in Williams 82 and Gmlin1-1.

Table S1. The primers used in this study.

Table S2. Ka/Ks values and estimation of the absolute dates of GmHY2 gene duplications.

Table S3. Sub-phase length in plants transferred from SD to LD or LD to SD conditions.

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Author contributions

SY and XF designed the research. ZZ, QW, TW, XY, and JM performed the experiments. ZZ and QW modified the images. KT and BZ analysed the sequencing data. ZZ, HY, SY, and XF discussed the results and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

The sequencing datasets generated during and/or analysed during the current study are available in the Genome Sequence Archive (GSA) database in the BIG Data Center (https://bigd.big.ac.cn/gsa/index.jsp) under accession number CRA005967.

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