A Mutation in CsHY2 Encoding a Phytochromobilin (PΦB) Synthase Leads to an Elongated Hypocotyl 1(elh1) Phenotype in Cucumber (*Cucumis sativus* L.)

Liangliang Hu

Northwest Agriculture and Forestry University College of Horticulture

Peng Liu

Northwest Agriculture and Forestry University College of Horticulture

Zhuoshuai Jin

Northwest Agriculture and Forestry University College of Horticulture

Jing Sun

Northwest Agriculture and Forestry University College of Horticulture

Yiqun Weng

USDA-ARS Vegetable Crops Research Unit, Horticulture Department, University of Wisconsin, Madison

Peng Chen

Northwest Agriculture and Forestry University College of Life Sciences

Shengli Du

Tianjin Vegetable Research Center

Aimin Wei

Tianjin Vegetable Research Center

Yuhong Li (✉ liyuhong73@nwsuaf.edu.cn)

Northwest Agriculture and Forestry University <https://orcid.org/0000-0002-9852-8107>

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Abstract

Hypocotyl length is a critical determinant in establishing high quality seedlings for successful cucumber production, but knowledge on the molecular regulation of hypocotyl growth in cucumber is very limited. Here we reported identification and characterization of a cucumber *elongated hypocotyl 1 (elh1)* mutant. We found that the longer hypocotyl in *elh1* was due to longitudinal growth of hypocotyl cells. With fine mapping, the *elh1* locus was delimited to a 20.9-kb region containing three annotated genes; only one polymorphism was identified in this region between two parental lines, which was a non-synonymous SNP (G28153633A) in the third exon of *CsHY2 (CsGy1G030000)* that encodes a phytochromobilin (PΦB) synthase. Uniqueness of the mutant allele at *CsHY2* was verified in 515 cucumber lines. Ectopic expression of *CsHY2* in Arabidopsis *hy2-1* mutant led to reduced hypocotyl length. The PΦB protein was targeted to chloroplasts. The expression levels of *CsHY2* and five phytochrome genes, *CsPHYA1*, *CsPHYA2*, *CsPHYB*, *CsPHYC* and *CsPHYE* were all significantly down-regulated while several cell elongation related genes were up-regulated in *elh1* mutant compared to wild type cucumber, which are correlated with dynamic hypocotyl elongation in the mutant. RNA-seq analysis in the WT and mutant revealed differentially expressed genes involved in porphyrin and chlorophyll metabolisms, cell elongation, and plant hormone signal transduction pathways. This is the first report to characterize and clone the *CsHY2* gene in cucumber. This work reveals the important of *CsHY2* in regulating hypocotyl length and extends our understanding of the roles of *CsHY2* in cucumber.

Introduction

Plant development not only depends on endogenous genetic manipulation, but also their modulation in response to environmental factors (Song et al. 2019). Light is among the most important environmental cues controlling plant growth and development such as seed germination, cotyledon expansion, hypocotyl elongation, root growth, flowering and fruit set (Pham et al. 2018; Seo et al. 2019; Huai et al. 2020; Liu et al. 2021). Plant perceives lights by a suite of photoreceptors including phytochromes (for red and far-red light) (Oh et al. 2020; Kahle et al. 2020), cryptochromes and phototropins (for blue/UVA light) (Sztatelman et al. 2016; Fantini et al. 2019; Lin et al. 2019) and UVR8 (for UVB) (Zhao et al. 2016; Tavridou et al. 2020; Yang et al. 2020). Among them, the best characterized are the phytochromes (phys), which regulate a wide range of light responses from seed germination to flowering (Josse et al. 2008; Montgomery. 2009; Song and Choi. 2019)

In Arabidopsis, five phytochromes, PHYA to PHYE, have been identified (Quail. 2002). A phytochrome consists of an apoprotein covalently attached to the linear tetrapyrrole chromophore, (3E)-phytochromobilin (PΦB) (Montgomery. 2008). Phytochrome apoproteins in all plants are encoded by a small multigene family. The apoproteins are synthesized in the cytosol, where they assemble autocatalytically with the plastid-derived chromophore PΦB (Li et al. 2011). The synthesis of PΦB is accomplished by an enzymatic cascade in the plastid that begins with the 5-aminolevulinic acid (ALA) which is a common precursor for the biosynthesis of tetrapyrrole compounds such as PΦB and chlorophyll (Li et al. 2011). The first committed step in the synthesis of PΦB is the conversion of the

heme to biliverdin (BV) IX α by a ferredoxin-dependent heme oxygenase HO1 encoding by the *HY1* gene (Kraepiel et al. 1994; Terry and Kendrick. 1996; Izawa et al. 2000; Linley et al. 2006; Zhu et al. 2017). Subsequently, the BV IX α is reduced to 3Z-P Φ B by the P Φ B synthase or HY2 that is encoded by the *HY2* gene, and is then isomerized to 3E-P Φ B and assembled into the functional holo-phys (Tanaka et al. 2011).

Mutants that are unable to synthesize the P Φ B have been instrumental in improving our understanding of the roles of phys in light-regulated plant development and morphogenesis (Linley et al. 2006). All plant phys bind the same P Φ B (Muramoto et al. 2005). Therefore, the disruption of P Φ B synthesis could inactivate the entire phytochrome system and impairs photomorphogenesis (Sawers et al. 2004). For example, all *hy2* mutants in different plants, such as Arabidopsis *hy2-1*, tomato *au*, pea *pcd2*, tobacco *pew2*, and maize *elm1* are characterized by an elongated hypocotyl and yellow-green phenotype due to a deficiency in photoactive phys (Weller et al. 1997; Kraepiel et al. 1994; Kohchi et al. 2001; Sawers et al. 2004; Muramoto et al. 2005; Wu et al. 2020).

Cucumber (*Cucumis sativus* L., 2n=14), an economically important vegetable crop worldwide (Li et al. 2013; Chen et al. 2017). In many regions, cucumbers are produced from transplanted seedlings in both open field and protected cultivation. The use of high quality seedlings is a key factor for the success of cucumber production. Hypocotyl length is a critical determinant in establishing strong seedlings for safe handling, seedling transportation, and survival rate after seedling transplanting, and eventually cucumber yield (Ming et al. 2011). Further, cucumber hypocotyl is an ideal system for understanding the mechanism of light-regulated cell elongation and seedling morphogenesis (Bo et al. 2016; Song et al. 2019). However, little is known about the genetic and molecular control of hypocotyl growth in cucumber. So far, only one *SHORT HYPOCOTYL 1* (*Sh1*) gene has been cloned and characterized (Bo et al. 2016), but the molecular mechanisms of *sh1*-regulated hypocotyl growth is unknown. In addition, several long hypocotyl mutants have been characterized in a number of papers (Robinson and Shail. 1981; Koornneef and van der knaap. 1983; López-Juez et al. 1992) However, the gene responsible for the long hypocotyl have not been cloned and the molecular mechanisms of hypocotyl growth is also unclear.

In the present study, we reported and characterized a novel *elongated hypocotyl* mutant (*elh1*) in cucumber. Map-based cloning of *elh1* revealed that this mutant phenotype was due to single nucleotide polymorphism in *CsHY2*, a homolog of Arabidopsis *HY2* gene for the P Φ B synthase. This mutant provides a valuable tool to understand the role of phys signaling in cucumber development and to explore the degree, and potential agronomic importance, of light-induced phenotypic plasticity.

Materials And Methods

Plant materials and mapping populations

The long hypocotyl mutant C1238 was identified from an EMS-induced mutagenesis population of the cucumber inbred line CCMC with normal hypocotyl and green leaves (Chen et al. 2017). The mutant

C1238 exhibits apparently elongated hypocotyl and yellow-green leaves at the seedling stage (Figs. 1A-1D). We designated this mutant locus as *elh1* (*elongated hypocotyl-1*) in C1238.

To study the inheritance of *elh1*, several segregating populations were employed by crossing of the C1238 with the original parental line CCMC, the American pickling cucumber line Gy14 and the North China fresh marker type cucumber line 9930, respectively (Table 1). For linkage mapping and cloning of the *elh1* gene, F₂ populations were derived from the C1238 x 9930 F₁ and C1238 x Gy14 F₁ plants, respectively. Since the polymorphism of molecular markers was very low between C1238 and 9930, only the C1238 x Gy14 population was employed in molecular mapping of *elh1*. Recombinants among F₂ plants of C1238 x Gy14 were defined by flanking molecular markers. All the F₂ plants used for primary mapping and recombinants were self-pollination to produce F₃ offspring. At least 50 plants of each F₃ family were examined for segregation of hypocotyl to determine F₂ genotype at the *elh1* locus. Segregation was tested against expected ratio with Chi-square (χ^2) tests. All individuals were grown in plastic greenhouses under natural sunlight at the Northwest A&F University (Yangling, China). The length of hypocotyl of all plants was visually scored at the cotyledon stage as either WT or mutant.

Microscopic observation of the hypocotyl cells

Hypocotyls of 10d-old seedlings of C1238 and CCMC were cut into small sections and fixed for 24h at 4°C in a 1:1:18 solution mixed of acetic acid, formaldehyde and 70% ethanol, respectively. The hypocotyls were stained overnight at 42°C with 1% safranin in 75% ethanol, subsequently dehydrated using a graded ethanol series. And then the samples were treated with xylene and embedded in paraffin. The samples were then sectioned using the vibrating microtome and washed with 100% ethanol for 3min, after which they were stained with toluidine blue for 20min. Paraffin sections were visualized using the BX63 microscope (Olympus, Tokyo, Japan). The cell length and cell number of longitudinal sections from *elh1* mutant and WT samples were measured using the software ImageJ after photographing (<https://imagej.nih.gov/ij/>).

The dynamic change of hypocotyl elongation under the white light was also evaluated. Hypocotyl length and its cell elongation rate were evaluated every two days for 13d.

Effects of light quality on hypocotyl elongation

To investigate the effect of light quality on hypocotyl elongation, the seedlings from mutant C1238 and WT were cultured in the growth chambers with red (R), far red (FR) and blue LED light sources with a peak wavelength of 620, 740 and 420nm, respectively. The 28°C day and 18°C night with a 14 h photoperiod were set in the growth chambers. Dark treatment was also employed in parallel with these experiments. Hypocotyl length was measured at 13 d after germination.

Fine genetic mapping of *elh1* gene

Bulked segregation analysis (BSA) was used for the initial mapping of the *elh1* locus with the C1238 x Gy14 F₂ population. Two DNA pools, LH-bulk (long hypocotyl bulk) and NH-bulk (normal hypocotyl bulk), were constructed by pooling equal amount of DNA from five mutant and five WT plants, respectively. SSR markers (1680 total) evenly distributed across the seven cucumber chromosomes were screened for polymorphism between the parental lines C1238 and Gy14 (Ren et al. 2009; Cavagnaro et al. 2010). The polymorphic markers in the two parental lines were used to the two pools, and then applied to 96 F₂ individuals for linkage analysis.

For fine mapping of the *elh1* gene, additional markers were developed from SNPs (single nucleotide polymorphisms) and indels (insertion or deletion of base) in the target region by aligning resequencing reads of C1238 against the Gy14 draft genome (v2.0) with DNAMAN V10.0 (<http://www.lynnon.com/>). The SNPs were converted to CAPS or dCAPS markers using dCAPS Finder 2.0 (<http://helix.wustl.edu/dcaps/dcaps.html>). Primers were designed with Primer Premier 5.0 (<http://www.premierbiosoft.com/primerdesign/>). Information of all linked markers and primers for various purposes in the present study is provided in Supplemental Table S1.

DNA extraction, PCR amplification of molecular markers, and polyacrylamide gel electrophoresis was executed as described by Li et al. (2011). Linkage analysis of *lh1* locus with molecular markers was performed using JoinMap 4.0 at an LOD threshold of 5.0.

Gene prediction and candidate gene identification

The gene prediction was performed with the online program FGENESH from the SoftBerry (<http://sunl.softberry.com/>). Gene function prediction was performed using the BLASTP tool at NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All predicted genes in the candidate gene region were Sanger sequenced from the C1238 and WT, to confirm the polymorphisms.

dCAPS assay with the causal SNP of *elh1* candidate gene

A causal SNP inside the *elh1* candidate gene was identified between C1238 and WT. A dCAPS marker dCAPS1238-1 was developed from this SNP and used in dCAPS assays among plants from the 5317 F₂ individuals of C1238 x Gy14 and 1249 F₂ plants of C1238 x 9930. The uniqueness of this causal SNP was checked among 115 re-sequenced cucumber lines by sequences alignments (Qi et al. 2013). This dCAPS marker was also used to examine allelic diversity among 400 cucumber lines according to Bo et al (2016). For dCAPS assay, the PCR amplicons were digested with the restriction enzyme *Taq*I for 65°C for 14h, separated with 9% polyacrylamide gel electrophoresis and visualized with silver staining. In the WT, the 253bp target fragment was cut into two bands with 233bp and 20bp in size by *Taq*I, but there was only one 253-bp band in the mutant.

Cloning and sequencing of *elh1* candidate gene

Total RNA was extracted from the hypocotyls of C1238 and WT plants using the BioFast BIOZOL Total RNA Extraction Reagent (Bioer, China). The first-strand cDNA was synthesized using the 5x All-In-One RT MasterMix (ABM, Canada). The full length cDNA of *elh1* candidate gene (*CsHY2*) was amplified by gene-specific primers (Table S1) as described in Wang et al. (2017) and sequenced by TsingKe Biological Technology (China).

Expression analysis of the *elh1* and other functionally related genes

We examined the time-course expression level of the *elh1* candidate gene and other genes in the candidate gene region (20.9kb) with the quantitative real-time PCR (qPCR). The hypocotyl samples from C1238 and WT were collected 3, 5, 7, 9, 11, and 13d days after germination. We also examined the expression pattern of *elh1* gene in the roots, stem, cotyledon, true leaf, female flowers, male flowers and fruit. Total RNA extraction and the first-strand cDNA synthesis followed Wang et al. (2017).

To investigate the effect of the mutation in *CsHY2* on the function of phys, the expression dynamics of cucumber PHYs genes (*CsPHYA1*, *CsPHYA2*, *CsPHYB*, *CsPHYC* and *CsPHYE*) were analyzed. We also conducted qPCR on six cell elongation-related genes including *CsEXT3* (*EXTENSIN3*), *CsEXPA8* (*EXPANSIN-A8*), *CsDWF4* (*DWARF4*), *CsXTH22* (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE /HYDROLASE PROTEIN22*), *CsXTR6* (*XYLOGLUCAN ENDOTRANSGLYCOSYLASE6*), and *CsIAA29* (*AUXIN-RESPONSIVE PROTEIN IAA29*). Relative expression level of the target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen. 2001) with *UBI-ep* (*ubiquitin extension protein*) as the internal reference (Wan et al. 2010). There were three biological and three technical replications for each sample. Information of all the primers used in qPCR in the study is provided in the Supplemental Table S3.

Phylogenetic analysis of CsHY2 homologs from different plants

The phylogenetic relationships of HY2 proteins from 11 species were investigated. The NCBI accession numbers of these sequences are listed in the Supplemental Table S4. Multiple protein sequence alignment was performed with Clustal W and the phylogentic tree was constructed with neighbor-joining method (Saitou and Nei. 1987) based on distance calculation with 1000 bootstrap replications in MEGA 7.0 (<https://www.megasoftware.net/>).

Subcellular localization of CsHY2 protein

The coding region of the *CsHY2* gene, without the stop codon, was cloned with the gene-specific primers 35S-CsHY2-L and 35S-CsHY2-R (Table S5). The fragment was then cloned into the *Nco*I/*Bgl*II site of the overexpression vector pCAMBIA3301-EGFP. The empty pCAMBIA3301-EGFP (*35S:EGFP*) vector was used as the negative control. And then the constructed plasmid *35S:CsHY2-EGFP* and the *35S:EGFP* were used for transient expression in tobacco (*N. benthamiana*) leaves following Li (2008). Fluorescence was observed using an Olympus BX63 fluorescence microscope.

Functional complementation of *hy2* mutant in Arabidopsis

The above-mentioned *35S:CsHY2-EGFP* plasmid construct was also transformed into *Agrobacterium tumefaciens* GV3101. Arabidopsis *hy2-1* recessive homozygous mutants were transformed by the floral dip method (Clough and Bent. 1998). To identify the transgenic lines, T1 generation seedlings were selected by spraying Basta solution (0.0015%) (Bayer, Germany) every three days. The survived plants were further confirmed by PCR and used for further characterization.

RNA-Seq analysis

Total RNA was extracted from hypocotyls of seven 5d-old seedlings of C1238 and WT using the Trizol. The cDNA libraries were constructed with the TruSeq™ RNA sample prep kit (Illumina) and sequenced on the Illumina HiSeq-2500 machine by Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China). After removing low-quality and poly-N reads, the clean reads were mapped to the 9930 v3.0 reference genome using TopHat version 2.0.13. FPKM (fragments per kilobase of transcript sequence per million base pairs sequenced) were calculated by Stringtie (<https://ccb.jhu.edu/software/stringtie/>) to estimate transcript abundance. Differentially expressed genes (DEGs) were identified using cutoff of FDR (false discovery rate) <0.05 and $\log_2(\text{fold change}) \geq 2$. Meanwhile, we also performed GO- (gen ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway-enrichment analyses on identified DEGs at the Majorbio Cloud Platform (www.majorbio.com).

Results

Phenotypic characterization of *elh1* mutant

Under natural light conditions, as compared with the WT (CCMC), the *elh1* mutant showed a much longer hypocotyl, more elongated internode and yellow-green leaf (Figs. 1A-1D, S1, S2), indicating that this mutant might be defective in light perception or signal transduction due to the similar morphological phenotypes of *elh1* with some previously characterized mutants in Arabidopsis (Kohchi et al. 2001). However, under field conditions, the *elh1* mutant also exhibited fewer numbers of internodes than the WT. Therefore, there was no significant difference in plant height between the mutant and WT in adult plants (Figs. S2A-S2D).

Under a microscope, the hypocotyl cells of the mutant were significantly longer than those of the WT (Figs. 1E-1G), but the total cell numbers of the whole hypocotyl longitudinal section were not significantly different between *elh1* and WT (Fig. 1H). These results suggested that the longer hypocotyl in the mutant is due primarily to increased cell elongation.

Hypocotyl elongation of mutant under different light conditions

We examined hypocotyl growth dynamics of the mutant and WT under white light. The hypocotyl length of both *elh1* and WT increased gradually until 13d after germination (Figs. S3A, S3B). The hypocotyl elongation rate in *elh1* was significantly higher than that of WT at any time point, which reached its peak at 5d after germination (Fig. S3C). We further investigated hypocotyl growth of the mutant and WT under

continuous monochromatic red (R), far-red (FR), blue lights, as well as in the dark. The hypocotyl length of *elh1* was significantly longer than that of WT under both R and FR (Figs. 2A, 2B). In other words, elongation was strongly inhibited by both R and FR in WT seedlings, suggesting that the *elh1* plants were blind to both R and FR. In contrast, the WT and *elh1* seedlings showed similar elongation of the hypocotyl in the dark or blue light (Figs. 2A, 2B). These results suggest that *elh1* is defective in the repression of the photomorphogenesis during development in either R or FR light, indicating that light responses mediated by phytochromes are missing in *elh1*.

Inheritance analysis of the *elh1* locus

We examined segregation of hypocotyl length in different F₂ populations from crosses of the C1238 mutant with CCMC, Gy14 and 9930 (Table 1). All F₁ plants from different crosses had the same hypocotyl length as the WT parents. Among 5317 C1238 x Gy14 F₂ plants, 4012 and 1305 had normal and long hypocotyl, respectively, which was consistent with 3:1 segregation ($p=0.442$ in χ^2 test) (Table 1). Of 1249 F₂ plants from C1238 x 9930, 949 had normal hypocotyl and 300 had long hypocotyl ($p=0.423$ in χ^2 test fit 3:1) (Table 1). These results indicated that the long hypocotyl mutation in C1238 was controlled by a single recessive gene, *elh1*.

Linkage mapping of the *elh1* locus

Among 1680 SSR markers screened between C1238 and Gy14, 113 were polymorphic, 9 of which were also polymorphic between the LH-bulk and NH-bulk. All the nine polymorphic SSRs were located on cucumber chromosome (Chr) 1. Indeed, linkage analysis in 96 F₂ plants of C1238 x Gy14 revealed that all the nine SSR markers were linked with the *elh1* locus with the two closest markers. UW032400 and UW031473 being 4.7 and 0.5cM away from the *elh1* locus, respectively (Fig. 3A).

To narrow down the mapping region, 14 new SSR markers were developed in this region, and five UW032647, UW033042, UW009754, UW069958, and SSR17922 exhibited polymorphisms between C1238 and Gy14 and the two bulks. All five markers were linked with the *elh1* locus (Fig. 3B). We further phenotyped 5317 F₂ plants from C1238 x Gy14 cross using the two flanking markers SSR17922 and UW009754, and two recombinants were identified between them. Physically, the *elh1* region flanked by SSR17922 and UW009754 was 82.1kb (Table S1).

From sequence alignment of the CCMC re-sequencing reads against the Gy14 v2.0 draft genome assembly, we identified three NSPs, which were converted to CAPS or dCAPS markers (dCAPS1238-3, CAPS1238-1 and dCAPS1238-1). Genotyping of the recombinants and linkage analysis allowed establishment of the genetic and physical orders of all linked markers in relation to the *elh1* locus (Fig. 3B). The *elh1* locus was finally delimited to the 20.9kb interval flanked by SSR17922 and CAPS1238-1 in the Gy14 scaffold00598 (Table S1).

Cloning and identification of *elh1* candidate gene

In the 20.9kb genomic DNA region, three genes were predicted using the FGENESH program. The predicted functions and relevant information of the three genes are presented in Table S2. We compared the genomic DNA sequences and expression of the three genes between the mutant and WT. No sequence or expression level differences were found in the first and third genes (*CsGy1G029990* and *CsGy1G030010*) between C1238 and WT (Figs. 4B, 4C); in contrast, a non-synonymous G to A mutation (G28153633A) was identified in the CDS (coding sequence) region of the second gene, *CsGy1G030000* (*CsHY2*). We further checked the uniqueness of the mutant allele of *CsHY2* by sequence alignment in this region between C1238 and 115 resequenced cucumber lines (Qi et al. 2013). We found that the mutant A allele at this SNP locus only occurred in C1238, while all the 115 lines carried the WT G allele.

Based on this SNP, a dCAPS marker, dCAPS1238-1 was developed and used to genotype the 5317 C1238 × Gy14 F₂ plants and 1249 C1238 × 9930 F₂ plants, which confirmed the co-segregation of phenotypes and the two SNP alleles in both populations (Figs. 3B, S5). To further verify the uniqueness of the SNP, we performed dCAPS assay with dCAPS1238-1 among 400 cucumber lines, and found that all the 400 WT lines shared the same G SNP allele as Gy14 and CCMC (Fig. S6). These results all supported the conclusion that this SNP in the *elh1* gene is responsible for the long hypocotyl phenotype in mutant C1238.

To confirm the identity between *elh1* and *CsHY2*, we performed a complementation test by introducing the *CsHY2* cDNA driven by the CaMV35S promoter into Arabidopsis *hy2-1* mutant. Ten independent transgenic plants carrying the *35S:CsHY2* construct were analyzed for hypocotyl length. All of them exhibited normal hypocotyl length similar to those of the wild-type Arabidopsis plants (Figs. 5A, 5B) further confirming that *elh1* encodes *CsHY2*, a cucumber homologue of Arabidopsis HY2.

We cloned the full-length cDNA sequence of *CsHY2* from WT and C1238. Alignments of cDNA sequences and deduced amino acid sequences between WT and mutant are shown in Supplemental files 1 and 2, respectively. There was a SNP at position 28153633 (G in WT to A in mutant) in third exon of *CsHY2* in *elh1*, which results in an amino acid substitution from Asp¹⁰⁴ (D) in WT to Asn¹⁰⁴ (N) (Fig. 3D; Supplemental file 2). Annotation of *CsHY2* gene showed that it was 5701bp in length and consisted of eight exons and seven introns (Fig. 3D), which was predicted to encode a PΦB synthase with 322 amino acid residues (Supplemental file 2).

Phylogenetic relation among *CsHY2* and its homologs in other sepecies

To better understand the phylogenetic relationships of *CsHY2* protein with its orthologs in other species, a phylogenic tree was constructed by use of the amino acid sequences of HY2 from 11 species (Table S4; Fig. S7). The results manifested that the mutation site occurred in the *CsHY2* was highly conserved in different species (Supplementary file 3). Sequences similarity between the cucumber *CsHY2* and its homologs suggested that *CsHY2* may serve conserved functions like in these species (Supplemental file 3). This could be reflected from the very similar phenotypes of mutant of the HY2 gene in different plant

species such as long hypocotyl and yellow-green leaves (e.g., Parks and Quail. 1991; Terry and Kendrick. 1996; Kohchi et al. 2001; Muramoto et al. 2005; Fig. 1 of this study).

Spatiotemporal expression patterns of *CsHY2*

We investigated the expression dynamics of *CsHY2* with qPCR in *elh1* and WT hypocotyls at different time points after germination (Fig. 4A). We found that the expression level of *CsHY2* was highly consistent with the hypocotyl elongation rate in the two lines (Figs. 4A, S3C). In the mutant, the fastest elongation rate occurred around 3-5d after germination (Fig. S3C). This was consistent with the highest expression of *CsHY2* at this time (Fig. 4A).

We further examined the expression of *CsHY2* in seven organs from *elh1* and WT (Fig. 4D). The expression level of *CsHY2* in mutant was the highest in male and female flowers, followed in order by stem, true leaves, root, cotyledon and fruit. However, there was no significant difference in *CsHY2* expression in all these organs except the cotyledon, true leaves and stem between the WT and *elh1* (Fig. 4D). These results suggest that the long hypocotyl, elongated internode (stem) and yellow-green leaves of *elh1* may be the result of reduced expression of *CsHY2*.

Expression analysis of phys and cell elongation-related genes

We also compared time-course expression of five phytochrome genes including *CsPHYA1*, *CsPHYA2*, *CsPHYB*, *CsPHYC* and *CsPHYE* in the mutant and WT. We found that each of the expression of all five genes was peaked at fifth day after germination (Figs. 6A-6E). This was consistent with hypocotyl elongation rate (Fig. S3C), supporting early findings that phys genes play an important role in the regulation of hypocotyl cell elongation. We also observed coordinate changes in the levels of *CsHY2* and five cucumber phys in mutant indicating that *CsHY2* affects the expression levels of all the phys.

In order to explore the underlying molecular mechanism of *CsHY2*-regulated hypocotyl elongation, time-course expression of several cell-elongation-related genes was tested including *CsDWF4*, *CsEXT3*, *CsEXPA8*, *CsXTH22*, *CsXTR6* and *CsIAA29*. All of them showed their highest expression at the fifth day, which was consistent with the hypocotyl elongation rate (Figs. 7A-7F, S3C). These data suggested that these genes are involved in hypocotyl cell elongation and the functional *CsHY2* gene suppress their expression in cucumber.

Comparative transcriptome profiling analysis

To understand the *CsHY2*-mediated gene network for hypocotyl growth, we conducted RNA-seq analysis with hypocotyls of 5d-old seedlings of the *elh1* mutant and WT. We identified 1251 DEGs, of which 414 and 837 were up- and down-regulated, respectively in the mutant as compared with the WT. GO enrichment analysis showed these DEGs were mainly involved in tetrapyrrole binding, chlorophyll binding and photoreceptor activities in the molecular function category, chloroplast structure (chloroplast thylakoid membrane and chloroplast envelope) in the cellular component and tetrapyrrole metabolic, chlorophyll biosynthesis and response to red or far-red light in the biological process category (Fig. S8).

KEGG pathway enrichment analysis indicated that these DEGs were enriched in pathways involved of porphyrin and chlorophyll metabolism, photosynthesis, as well as phytohormone signal transduction (Fig. S9). Thus, RNA-seq data strongly supported that the *CsHY2* involves in the tetrapyrrole chromophore biosynthesis through the porphyrin and chlorophyll metabolism pathway.

In the mutant and WT transcriptomes, all five phytochrome genes including *CsPHYA1*, *CsPHYA2*, *CsPHYB*, *CsPHYC* and *CsPHYE* were down-regulated, whereas many cell elongation related genes such as *CsDWF4*, *CsEXT3*, *CsXTH22*, *CsEXPA8*, *CsXTR6* and *CsIAA29* were up-regulated in the mutant. The expression trend of these 11 genes in RNA-seq analysis was consistent with the RT-qPCR results (Figs 6 and 7) further supporting their important roles in *CsHY2*-regulated hypocotyl elongation in cucumber.

Subcellular localization of *CsHY2* protein

The subcellular location of a protein is pivotal to understand its function and associated biological processes (Briesemeister et al. 2010). We performed subcellular localization of the *CsHY2* protein in tobacco leaves cells. We found that, while the negative control (EGFP fluorescence) was present throughout the cytomembrane and nucleus, the fusion protein *35s:CsHY2-EGFP* was localized to chloroplast (Fig. S10). These findings confirmed that the *CsHY2* protein played a role in the chloroplast, which was consistent with the plastid localization of P Φ B synthesis and the RNA-seq data suggesting that tetrapyrrole chromophore biosynthesis and chlorophyll metabolism were mainly in the chloroplast.

Discussion

In this report, we cloned *elh1* by fine mapping for the *elh1* mutant and provided evidence to support *CsHY2* as the candidate gene which encodes the P Φ B synthase known to function in chromophore biosynthesis. First, like Arabidopsis *hy2* mutant, the cucumber *elh1* mutant is deficient in phytochrome responses such as hypocotyl elongation under R and FR (Figs. 2A, 2B). Second, in the 20.9 kb region harboring the *elh1* locus, there was only one non-synonymous SNP between the mutant and WT, which was located in the third exon of *CsHY2* resulting in an amino acid substitution from Asp¹⁰⁴ to Asn¹⁰⁴ (Fig. 3C-D; Supplemental file 1-3). Third, this SNP-derived dCAPS marker (dCAPS1238-1) showed complete co-segregation with the long hypocotyl mutant phenotype in two large F₂ segregating populations (Figs. 3B, S5B). Allelic analysis among 515 cucumber lines revealed the unique A allele in *elh1* (Fig. S6). Further, ectopic expression of *CsHY2* in Arabidopsis can rescue the phenotype of *hy2* mutant phenotype (short hypocotyl) in transgenic plants (Fig. 5). Finally, the expression pattern of *CsHY2* well matches its role in hypocotyl elongation in *elh1* mutant (Figs. 4A, S3). Taken together, these results provided convincing evidence in support of the *CsHY2* as the best candidate gene of *elh1*.

The P Φ B synthase/HY2 is characterized by a conserved Fe_bilin_red (FBR) domain with catalytic activity (Frankenberg et al. 2001; Rockwell and Lagarias. 2017; Zhang et al. 2019). The FBR domain is crucial for catalytic performance, and mutations in the domain would lead to loss-of-function of HY2 and thus severely deficient in photoreversible phys (Terry and Kendrick. 1996; Kohchi et al. 2001; Sawers et al.

2002; Sawers et al. 2004; Muramoto et al. 2005; Wu et al. 2020; Reig-Valiente et al. 2020). For example, all reported mutants with mutations in the FBR domain, such as Arabidopsis *hy2-1*, *hy2-104*, *hy2-101*, and *hy2-103* (Kohchi et al. 2001), tomato *au*, *au^{ls}* and *au^w* (Terry and Kendrick. 1996; Muramoto et al. 2005; Wu et al. 2020), maize *elm1*, and rice *se13* (Sawers et al. 2002; Sawers et al. 2004; Saito et al. 2011; Reig-Valiente et al. 2020), display common defective phenotypes with long hypocotyl and yellow green leaves as in the cucumber *elh1* mutant identified herein that also carried an amino acid substitution (D104N) in the FBR domain (Figs. 1A-1D, 3D, S1; Supplemental file 3). These data further support the cucumber CSHY2 with conserved function as other plant species for hypocotyl growth.

However, unlike most *hy2* mutants with early flowering phenotype in other species such as maize *elm1* and rice *se13* (Sawers et al. 2002; Saito et al. 2011; Reig-Valiente et al. 2020), mature *elh1* plants had normal flowering time under field condition (data not shown). This might be due to the relatively small contributions of phys on the control of flowering time in cucumber plants. Instead, unexpectedly, we found that phys might be involved in the regulation of sex determination in cucumber (Figs. S2A, S2B). CCMC is monoecious with both separate male and female flowers on the same plant. We found that *elh1* mutant had fewer male and more female flowers compared with the WT plants (Figs. S2A, S2B) which may suggest involvement of phys in regulating cucumber sexual development. The basis for such phenomenon is unknown, but one possibility concerns the genotype used in this study, CCMC, which like most of cucumber germplasms (Lai et al. 2018), is sensitive to seasonal or photoperiodic change in sex expression (i.e. different female/male flower ratios). Interestingly, in this study, we found the *elh1* mutant has higher female/male flower ratios than WT either the summer (Fig. S2E) or autumn (Fig. S2F) under the field condition. It has been verified that phyB can regulate gynoecium formation in Arabidopsis (Foreman et al. 2011). Based on the role of phys in regulating flower development, together with the phenomenon of the photoperiodic response of sex type in cucumber, we speculated that phys might involve with the photoperiod-mediated sex determination in cucumber. More evidence is still needed to elucidate this.

It is worth mentioning that despite of the long hypocotyl and elongated internodes in the *elh1* mutant, the plant height was normal due to the reduced internode number under field condition (Fig. S2), indicating that phys participate in the control of internode number in cucumber. The internode number, little mentioned in chlorophore deficient mutants in other species, is an important determining factor for cucumber plant height. Although it is unknown how phys regulate node number of cucumber, the result presented here might extend our understanding in stem development and plant height of cucumber.

Consistently with the reduced chlorophyll (Chl) levels (Fig. S4), the transcriptome analysis revealed that porphyrin and chlorophyll metabolism was profusely altered in *elh1* (Figs. S7, S8). It has been proposed that the P Φ B and chlorophyll were synthesized from the common precursor-5-aminolevulinic acid (ALA) (Li et al. 2011). Meanwhile, previous studies have shown the Chl biosynthesis was feedback-inhibited by the branch of the tetrapyrrole biosynthetic pathway in phytochrome chromophore-deficient mutants (Terry and Kendrick. 1999). Therefore, this is not surprising that the role of CSHY2 in the synthesis of P Φ B and Chl could explain the yellow green phenotype (Figs. 1C, 1D) in the *elh1* plants, a phenotypic trait observed

in most chromophore-deficient mutants (Weller et al. 1997; Kraepiel et al. 1994; Kohchi et al. 2001; Sawers et al. 2002; Sawers et al. 2004; Muramoto et al. 2005; Zhu et al. 2017; Wu et al. 2020).

Here, similar to *hy2* mutants in *Arabidopsis* (Derbyshire et al. 2007; Boron and Vissenberg. 2014), the long hypocotyl phenotype of *elh1* mutant is due primarily to increased cell length rather than cell numbers (Figs. 1E-1H). This is consistent with the by time-course expression patterns of six cell-elongation-related genes from both qPCR (Fig. 7) and transcriptome data from RNA-Seq analysis where all these genes were significantly up-regulated in the mutant (Table S6).

The mechanism underlying hypocotyl elongation also involves endogenous phytohormones, especially the gibberellin (GA) and auxin which are known to be involved in promoting cell elongation in excised stem and hypocotyl segments (Qu et al. 2017; Du et al. 2018; Liu et al. 2018; Bawa et al. 2020; Jiang et al. 2020). In this study, transcriptome analysis showed that the *CsKAO* and *CsGA20ox2*, two key genes responsible for bioactive GA biosynthesis, were dramatically up-regulated in the mutant *elh1* (Table S6). On the contrary, *CsGA20ox-1* encoding GA-inactivating enzyme (Serrani et al. 2007), and *CsGAI* functioning signal transduction repressors of GA (Eckardt. 2007), were down-regulated in the mutant (Table S6). Meanwhile, some auxin responsive (*IAA4*, *IAA14*, *IAA29*, *IAA32*, *SAUR23*, *SAUR32*) and induced (*AUX22*, *AUX28*) genes were also up-regulated. These results suggested that GA and auxin might regulate photomorphogenesis-related hypocotyl elongation in cucumber through modulating the biosynthesis or signaling of auxin and GA.

In *Arabidopsis*, it has been well documented that a number of transcription factors (TFs) promoting photomorphogenesis, such as PIFs (phytochrome-interacting factors), HY5 (elongated hypocotyl 5), HFR1 (long hypocotyl in far-red), LAF1 (long after far-red light 1) and HYH (hy5 homolog), can physically interact with phytochromes to regulate hypocotyl growth (Castillon et al. 2007; Jang et al. 2013; Dong et al. 2020). But none of the TFs was present in the DEG list of the mutant and WT transcriptomes. Meanwhile, many other TFs like members of TCP, AFR, ERF and WRKY gene families, were up-regulated in the mutant (Table S6); some of them have been shown to be involve in regulation of the hypocotyl elongation. For example, Zhou et al (2018) reported overexpression of *TCP17* leads to a long hypocotyl phenotype in *Arabidopsis*. *TCP14* and *TCP15* are required for optimal elongation and for the appropriate gene expression responses to a group of auxin inducible genes and the GA biosynthetic gene *GA20ox1* in hypocotyls of *Arabidopsis* (Ferrero et al. 2019; Ferrero et al. 2021). Based on the transcriptome data, combined the knowledge from scientific literature, we speculate that the hypocotyl elongation in *elh1* is mainly due to the interaction and cooperative regulation of phys, GA/Auxin, cell cell-elongation-related genes and these differential TFs. However, whether and how they make interactions and crosstalk to control hypocotyl elongation remains to be investigated in the future.

Declarations

Author Contribution Statement

LH performed the research, prepared a draft of the manuscript. PL, ZJ and JS participated in the research. PC participated in data analysis and provided technical help. YW participated in data analysis and manuscript writing. AW and YL designed the experiments, supervised this study and wrote the manuscript. All authors have read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declare that there is no conflict of interest.

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Tables

Table 1. Segregation analysis of *elongated hypocotyl-1*(*elh1*) in F₁ and F₂ populations from various crosses.

Populations	Total	Wild Type	Mutant	Expected ratio	<i>P</i> in χ^2 tests
C1238 × CCMC F ₁	63	63	0	1:0	/
C1238 × Gy14 F ₁	72	72	0	1:0	/
C1238 × 9930 F ₁	56	56	0	1:0	/
C1238 × 9930 F ₂	1249	949	300	3:1	0.423
C1238 × Gy14 F ₂	5317	4012	1305	3:1	0.442

Figures

Figure 1

Phenotypic characterization of elongated hypocotyl-1 (*elh1*) mutant C1238 and wild type (WT) CCMC plants. A-D are hypocotyls (A and B) and leaf colors (C and D) of the mutant (left), WT (middle) and their

F1 (right) at cotyledon and first true leaf stages, respectively. Scale bar = 2cm. Microscopic observation and measurement of the hypocotyl cells of 10d-old seedlings of WT (E) and mutant (F) indicate that the mutant has significantly longer cell length (G) but similar cell numbers (H) as compared with the WT. E and F: scale bar=100 μ m. Each data point is mean $\pm$ SD. ** means $P < 0.01$ from Student's t-test.

Figure 2

Hypocotyl growth of mutant (C1238) and WT (CCMC) at different light conditions (a). A and B show hypocotyl elongation and comparison of C1238 (left) and CCMC (right) under dark, red, far-red and blue lights, respectively. Hypocotyl length data in A were collected at 10d old seedlings. Each data point is mean $\pm$ SD. ** indicates $P < 0.01$ significant level from comparisons between mutant and WT under each light condition from Student's t-tests.

Figure 3

Map-based cloning of *elh1* locus in cucumber. (a) Initial mapping with 96 $F_{2:3}$ families placed at *elh1* locus between UW032400 and UW031473 at cucumber Chromosome 1; (b) Fine mapping with 5317 $F_{2:3}$ plants narrowed down *elh1* to a ~20.9kb region delimited by two markers SSR17922 and CAPS1238-1. In (a) and (b), numbers are genetic distances (cM) between adjacent markers; (c) Three genes were predicted in the 20.9kb region with *CsHY2* (second) as the candidate gene, which has eight exons (d). The SNP in the third exon results in a Aspartic acid (D) at 104 position (GAT) substitution to a Asparagine codon (AAT). The black, red boxes and black lines indicate UTR zone, exons and introns, respectively.

Figure 4

Spatial-temporal expression of *CsHY2* in the WT and mutant. (a) Expression dynamics of *CsHY2* candidate gene within 13d after germination; (b-c) Expression patterns of other two genes in the 20.9-kb region, the expression of all the genes in third day of mutant C1238 is regard as the standard of “relative” expression. (d) Transcriptional level of *CsHY2* in seven organs (FF/MF: female/male flower; CL: cotyledon; TL: true leaf), the expression of *CsHY2* in cotyledon of mutant as the standard of “relative” expression. Datas are means $\pm$ SD of three biological and three technical replicates. Asterisks indicate statistically significant differences compared with the wild type at $**p < 0.01$ and $*p < 0.05$ by Student’s t-test.

Figure 5

Map-based cloning of *elh1* locus in cucumber. (a) Initial mapping with 96 F2:3 families placed at *elh1* locus between UW032400 and UW031473 at cucumber Chromosome 1; (b) Fine mapping with 5317 F2 plants narrowed down *elh1* to a ~20.9kb region delimited by two markers SSR17922 and CAPS1238-1, In (a) and (b), numbers are genetic distances (cM) between adjacent markers; (c) Three genes were predicted in the 20.9kb region with *CsHY2* (second) as the candidate gene, which has eight exons (d). The SNP in the third exon results in a Aspartic acid (D) at 104 position (GAT) substitution to a Asparagine codon (AAT). The black, red boxes and black lines indicate UTR zone, exons and introns, respectively.

Figure 6

Expression dynamics of cucumber phytochrome genes *CsPHYA1* (a), *CsPHYA2* (b), *CsPHYB* (c), *CsPHYC* (d) and *CsPHYE* (e) in mutant C1238 and wild type CCMC hypocotyls at different days after germination. The expression of all the genes in third day of mutant C1238 is regard as the standard of “relative” expression. Asterisks indicate statistically significant differences compared with the wild type at **p < 0.01 and *p < 0.05 by Student’s t-test.

Figure 7

Expression dynamics of six cell-elongation related genes in mutant C1238 and wild type CCMC. For all six genes, the expression level was significantly up-regulated in mutant C1238, which is consistent with increased hypocotyl elongation under white light. Data are means $\pm$ SD of three biological and three technical replicates. Asterisks indicate statistically significant differences compared with the wild type at **p < 0.01 and *p < 0.05 by Student's t-test.

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