A mutation in CsHY2 encoding a phytochromobilin (PΦB) synthase leads to an elongated hypocotyl 1 (elh1) phenotype in cucumber (Cucumis sativus L.)

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Key message The elongated hypocotyl1 (elh1) mutant in cucumber is due to a mutation in CsHY2, which is a homolog of the Arabidopsis HY2 encoding the phytochromobilin (PΦB) synthase for phytochrome biosynthesis

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 natural cucumber populations. Ectopic expression of CsHY2 in Arabidopsis hy2-1 long-hypocotyl mutant led to reduced hypocotyl length. The PΦB protein was targeted to the chloroplast. 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. 2020; Liu et al. 2021). Plants perceive 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...
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 chlorophylls (Li et al. 2011). The first committed step in the synthesis of PΦB is the conversion of the heme to biliverdin (BV) I\(\alpha\) by a ferredoxin-dependent heme oxygenase HO1 encoded 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 I\(\alpha\) is reduced to 3Z-PΦB by the PΦB synthase or HY2 that is encoded by the HY2 gene, and is then isomerized to 3E-PΦB and assembled into the functional holo-phys (Tanaka et al. 2011).

Mutants that fail to synthesize the PΦB 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 PΦB (Muramoto et al. 2005). Therefore, the disruption of PΦB 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., 2\(n=14\)), is 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 HYPOCOTYL1 (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 cucumber (e.g., Robinson and Shail. 1981; Koornneef and van der knaap. 1983; López-Juez et al. 1992), but the genes responsible for the long hypocotyl have not been cloned, and the molecular mechanism 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 PΦB synthase. This mutant provides a valuable tool to understand the role of phytochrome 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-d). We designated this mutant locus as elh1 (elongated hypocotyl-1) in C1238.

To study the inheritance, several segregating populations were employed by crossing 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, F\(_2\) populations were derived from the C1238 ×9930 F\(_1\) and C1238 ×Gy14 F\(_1\) plants, respectively. Since the polymorphism of molecular markers was very low between C1238 and 9930, only the C1238 ×Gy14 population was employed in molecular mapping of elh1. Recombinants among F\(_2\) plants of C1238 ×Gy14 were defined by flanking molecular markers. All the F\(_2\) plants used for primary mapping and recombinants were self-pollination to produce F\(_3\) offspring. At least 50 plants of each F\(_3\) family were examined for segregation of hypocotyl to determine F\(_2\) genotype at the elh1 locus. Segregation was tested against expected ratio with Chi-square (\(\chi^2\)) tests. All individuals were grown in the Northwest A&F University plastic greenhouses under natural sunlight (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 ℃ in a 1:1:18 solution mixed of acetic acid, formaldehyde and 70% ethanol, respectively. The hypocotyls were stained overnight at 42 ℃ with 1% safranin in 75% ethanol, subsequently dehydrated using a graded ethanol series. The samples were then treated with xylene and embedded in paraffin, 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 investigated 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 × 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). Polymorphic markers between the two parental lines were used for 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 polycrylamide gel electrophoresis were executed as described by Li et al. (2011). Linkage analysis of elh1 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://sln.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 × Gy14 and 1249 F2 plants of C1238 × 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% polycrylamide 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 (20.9 kb) with the quantitative real-time PCR (qPCR). The hypocotyl samples from C1238 and WT were collected at 3, 5, 7, 9, 11 and 13 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 phy, 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 (EXTENSIONS3), CsEXPA8 (EXPANSIN-A8), CsDWF4 (DWARF4),...
**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. The 35S:CsHY2-EGFP and the 35S:EGFP plasmid constructs were used for transient expression in tobacco (N. benthamiana) leaves following Li (2011). 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 confirmed by PCR were used for further characterization.

**RNA-Seq analysis**

Total RNA was extracted from hypocotyls of seven 5d-old seedlings each of C1238 and WT using 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 absolute log2(fold change) ≥ 1. Meanwhile, we also performed GO (gen ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway-enrichment analyses on identified DGEs at the Majorbio Cloud Platform (https://cloud.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 (Fig. 1a-d, 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 (Fig. 1a-g), 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 monochromic 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 (Fig. 2a, b). 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 (Fig. 2a, b). 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 × Gy14 F2 plants, 4012 and 1305 had normal and long hypocotyl, respectively, which was consistent with 3:1 segregation \( (p = 0.442 \text{ in } \chi^2 \text{ test}) \) (Table 1). Of 1249 F2 plants from C1238 × 9930, 949 had normal hypocotyl and 300 had long hypocotyl \( (p = 0.423 \text{ in } \chi^2 \text{ test fit 3:1}) \) (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 × 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 × 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 82.1 kb (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 20.9 kb...
interval flanked by SSR17922 and CAPS1238-1 in the Gy14 scaffold00598 (Table S1).

Cloning and identification of elh1 candidate gene

In the 20.9 kb 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 (Fig. 5b, c); 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 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 may be 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 (Fig. 4a, b).
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 species**

To better understand the phylogenetic relationships of CsHY2 protein with its orthologs in other species, a phylogenetic 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.
Sequences similarity between the cucumber CsHY2 and its homologs suggested that CsHY2 may serve conserved functions like in these species (Supplementary 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 elhl and WT hypocotyls at different time points after germination (Fig. 5a). We found that the expression level of CsHY2 was highly consistent with the hypocotyl elongation rate in the two lines (Figs. 5a, 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. 5a).

We further examined the expression of CsHY2 in seven organs from elhl and WT (Fig. 5d). 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 elhl (Fig. 5d). These results suggest that the long hypocotyl, elongated internode (stem) and yellow-green leaves of elhl 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 (Fig. 6a–e). 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–f, S3c). These data suggested that these genes are involved in hypocotyl cell elongation and the functional CsHY2 gene suppress their expression in cucumber.

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**Fig. 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 regarded 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**
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). Previous studies have shown that the HY2 protein in Arabidopsis (Kohchi et al. 2001) and Maize (Sawers et al. 2014) all are located to the chloroplast. As the homologue of AtHY2 and ZmHY2, we speculate that the CsHY2 protein may be also located to the chloroplast. 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 organelles (Fig. S10). It was predicated the CsHY2 protein sequence contains an N-terminal chloroplast transit peptide (cTP) (http://www.cbs.dtu.dk/services/ChloroP/). These findings suggest that the CsHY2 protein plays a role in the chloroplast, which is consistent with the plastid localization of PΦB 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 PΦB 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 (Fig. 2a, b). 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-I) 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. 4).

Finally, the expression pattern of Cshy2 well matched its role in hypocotyl elongation in elh1 mutant (Figs. 5a, S3). Taken together, these results provided convincing evidence in support of the Cshy2 as the best candidate gene of elh1.

However, it should be pointed out that, while the majority of the cucumber lines in the natural population examined at the SNP locus carried the wild-type G alleles, which is consistent with their normal (wild-type) hypocotyl length, a few lines carrying the G allele have long hypocotyls (for example, WT284, AM274, AM149L). Additional work in our laboratories has identified different mutations in the PhyB signaling pathway for the long hypocotyl phenotype in three lines (unpublished data). These observations suggest that multiple mechanisms may contribute to the long hypocotyl in cucumber.

The PΦB 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 phsys (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, auR and auW (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, 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 elh1 plants. Instead, unexpectedly, we found that phys might be involved in the regulation of sex determination in elh1 plants (Figs. S2a-S2b). CCMC is monocious with separate male and female flowers on the same plant. We found that elh1 mutant had fewer male but 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 change in sex expression (i.e., different female/male flower ratios). Interestingly, in this study, we found that elh1 mutant has higher female/male flower ratios than WT in both the summer (Fig. S2e) and autumn (Fig. S2f) field conditions. In Arabidopsis, phyB can regulate gynoecium formation (Foreman et al. 2011). These observations may suggest that phytochromes may be involved in sex determination in the elh1 mutant. However, more evidence is needed to elucidate this.

It is worth mentioning that despite 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 elh1 plants. The internode number, little mentioned in chomophore deficient mutants in other species, is an important determining factor for cucumber plant height. Although it is unknown how phys regulate node numbers in the elh1 mutant our findings herein extend our understanding of their roles in stem development and plant height of cucumber.

Consistently with the reduced chlorophyll (Chl) levels (Fig. S4), transcriptome analysis revealed that porphyrin and chlorophyll metabolism was profoundly altered in elh1 (Figs. S7, S8). It has been proposed that the PΦB and chlorophyll were synthesized from the common precursor ALA (Li et al. 2011). Meanwhile, previous studies have shown that the Chl biosynthesis was feedback-inhibited by the branch of the tetrapyrrole biosynthetic pathway in phytochrome chomophore-deficient mutants (Terry and Kendrick. 1999). Thus, the roles of Cshy2 in the synthesis of PΦB and Chl could explain the yellow green phenotype (Fig. 1c, d) in the elh1
plants, a phenotype 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).

Similar to hy2 mutants in Arabidopsis (Derbyshire et al. 2007; Boron and Vissenberg. 2014), the long hypocotyl phenotype of the cucumber elh1 mutant is due primarily to increased cell length rather than cell numbers (Fig. 1e-h). This is consistent with the 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).

Hypocotyl elongation involves endogenous phytohormones, such as gibberellin (GA) and auxin, which are known to promote 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, CsGA2ox-I encoding a GA-inactivating enzyme (Serrani et al. 2007) and CsGA1 functioning signal transduction repressor 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). None of these TFs was present in the DEG list of RNA-Seq data in this study. 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 involved in regulation of the hypocotyl elongation. For example, Zhou et al. (2018) reported overexpression of TCP17 leading 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 hypocotyl of Arabidopsis (Ferrero et al. 2019; Ferrero et al. 2021). Thus, we speculate that hypocotyl elongation in elh1 is mainly due to the interaction and cooperative regulation of phy, GA/Auxin, cell- elongation-related genes and these differential TFs. However, how they interact and crosstalk to control hypocotyl elongation remains to be investigated in the future.

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Author’s contribution LH performed the research and prepared a draft of the manuscript. PL, ZJ and IS 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.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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