



Identification of a putative candidate gene encoding 7-dehydrocholesterol reductase involved in brassinosteroids biosynthesis for *compact plant architecture* in Cucumber (*Cucumis sativus* L.)

Mengru Zhang¹ · Mengfei Song¹ · Feng Cheng¹ · Zhige Yang¹ · Marzieh Davoudi¹ · Jinfeng Chen¹ · Qunfeng Lou¹

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Abstract

Key message By the strategy of bulked segregant analysis sequencing combined with genetic mapping, *CsDWF5*, which encodes 7 dehydrocholesterol reductase that involved in brassinosteroids biosynthesis, was identified as the candidate gene for *cpa*.

Abstract Dwarf architecture is one of the most important breeding goals in crops. The biosynthesis and signal transduction of brassinosteroids (BRs) have a great impact on plant growth and development including plant architecture. Here, we identified a *compact plant architecture* (*cpa*) mutant from an EMS-induced cucumber population. *cpa* displayed the extremely dwarf phenotype with shortened internode and petiole, darkened and wrinkled leaf. Genetic analysis revealed that *cpa* was caused by a single recessive gene. By the strategy of bulked segregant analysis sequencing combined with genetic mapping, *CsDWF5*, encoding a 7-dehydrocholesterol reductase that involved in sterol biosynthesis, was identified as the candidate gene for *cpa*. One single nucleotide mutation (G→A) in splicing site causing 3-bp insertion (TAG) was found in the first base of the sixth intron of *CsDWF5* in *cpa*, which furtherly resulted in the frameshift mutation and got a premature stop codon. The expression of *CsDWF5* gene was significantly down regulated in different tissues of the *cpa* mutant compared with that in wild type. The phenotype of *cpa* could be partially recovered by exogenous BR treatment. Transcriptome analysis identified 1096 genes that exhibited differential expression between the *cpa* mutant and wild type. KEGG enrichment analysis indicated that differentially expressed genes were significantly enriched in BR biosynthesis and plant–pathogen interaction pathways. These results provide perspectives on the molecular mechanisms underlying the dwarfing phenotype in cucumber.

Introduction

Plant architecture is one of the most important determinants for photosynthesis and nutrient distribution of plants, and it has great impact on cultivation management of plants and the final yield. The concept of ideotype was first proposed

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✉ Jinfeng Chen
jfchen@njau.edu.cn

✉ Qunfeng Lou
qflou@njau.edu.cn

Mengru Zhang
2019204035@njau.edu.cn

Mengfei Song
2017204016@njau.edu.cn

Feng Cheng
2018204029@njau.edu.cn

Zhige Yang
2018104066@njau.edu.cn

Marzieh Davoudi
2018204052@njau.edu.cn

¹ State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Horticulture, Nanjing Agricultural University, Weigang Street No. 1, Nanjing 210095, China

by Donald (1968), and plants with model characteristics improve production by influencing photosynthesis and growth. Plant architectures including dwarf or compact growth habits are closely related to lodging resistance and crop productivity (Hedden 2003). Those phenotype characters are vital traits of ideotype for many crops. Cloning of key gene for dwarf and compact trait is of great significance for further elucidating the regulation mechanism of plant architecture formation and ideotype breeding.

Various factors are responsible for the dwarf phenotype in plants, and a large part of them were due to the inhibition of the biosynthesis or signal transmission process of phytohormones. For instance, gibberellins (GA) were studied deeply on the regulation of plant height. *Dwarf3* gene in maize, *srd-1*, *d1* and *gid1* in rice, *CsCLAVATA1* in cucumber and *Rht1* in wheat were reported to be involved in the GA biosynthesis and signaling pathway (Gale et al. 1976; Winkler et al. 1995; Tanaka et al. 2010; Lei et al. 2015; Xu et al. 2018). Rice dwarf mutant *d1*, which is defective in the alpha subunit of the heterotrimeric G protein, affects gibberellin signal transduction. *Semi-dwarf1*, which contains a defective gibberellin 20-oxidase gene, brought the ‘green revolution’ in rice characterized by semi-dwarf breeding, and doubled rice yield (Ashikari et al. 2002). The dwarf mutants *d27*, *d14* and *d53* in rice were reported to be blocked in the biosynthesis or signaling of strigolactones (SLs) (Lin et al. 2009; Song et al. 2017; Yao et al. 2018). The flavin monooxygenase (FMO) acts at the key step in the biosynthesis of auxin (IAA) and the lesion of that in *yuc1D* in Arabidopsis led to abnormal development phenomena such as the loss of apical dominance and decrease of height (Mano and Nemoto 2012). The blocking of polar auxin transport in Arabidopsis *pin* and *pgp* mutants all displayed dwarf phenotype (Friml 2010; Peer et al. 2011). BRs are new but important hormones for the regulation of plant architecture (Wang et al. 2017b). A large number of mutants related to BR biosynthesis or perception have been isolated in Arabidopsis, rice, tomato and cucumber (Clouse et al. 1996; Mori et al. 2002; Tanabe et al. 2005; Hong et al. 2005; Ren et al. 2014; Wang et al. 2017a). BR-related mutants such as *scp-1*, *scp-2* (Wang et al. 2017a; Hou et al. 2017) in cucumber exhibited dwarfism with extremely short internodes and petioles, dark-green leaves, dysplastic female flower and de-etiolation characteristic in dark condition. The phenotype of the BR-deficit mutant could be rescued by the supplementary with exogenous hormones. Those phenotypes have been the typical characteristics to identify the mutants referring to BR biosynthesis.

Most cucumber varieties have vine growth habits including long internodes, large leaf angles and multiple branches, and all of those traits increase the difficulty of cultivation management and labor cost, and are not suitable for dense-planting as well. The discovery of semi-dwarf, compact materials with little branches is of great significance

for germplasm improvement. Some traits related to plant architecture had been reported in cucumber in recent years. Determinate growth is important for the improvement of cucumber breeding, and it was attributed to a single recessive gene (Sato et al. 2009). The *determinate* (*de*) locus on chromosome 6 was first proposed to control determinate growth (Weng et al. 2010). *CsTFL1* inhibited determinate growth and terminal flower formation by competing with FT for the interaction with CsNOT2a-CsFDP in cucumber (Wen et al. 2019). The branching of shoots also has a great influence on plant architecture. *Cucumber Lateral Suppressor* gene (*CLS*) affects the initiation of axillary meristems and the formation of lateral branches (Yuan et al. 2010). *CsBRC1* had been reported to inhibit lateral bud outgrowth by influencing the accumulation of auxin in axillary buds in cucumber (Shen et al. 2019). Besides, dwarf or compact plant architecture traits are meaningful for increasing planting density and production. A few compact or dwarf mutants have been reported. *cp* was the first one identified with the shortened internodes in cucumber in 1976 (Kauffman and Lower 1976), of which the gene mapping was carried out in 2011. *cp-2* then was identified keep the similar phenotype with *cp*, but the allelic relationship between them is unclear (Li et al. 2011). The EMS-induced dwarf mutant was firstly proposed in *scp*, of which the main stem was dramatically shortened and with few branches (Niemirowicz-Szczytt et al. 1996). Recently, *scp-1* and *scp-2* mutants have been characterized related to *CYP85A1* and *CsDET2*, and both of them participated in BR biosynthesis (Wang et al. 2017a; Hou et al. 2017). Another dwarf mutant, *Csdw*, causes a reduction of height by affecting GA₃ synthesis and SAM differentiation (Xu et al. 2018).

In this study, we identified a *cpa* mutant from the EMS-induced population of ‘Changchunmici’ (CCMC, wild type, WT, a common inbred line with normal plant architecture), which displayed a super compact phenotype with shrunk and dark-green leaf, shortened internode and petiole, and abortive female flower. All those phenotypes are similar with the typical characteristics of BR biosynthesis-deficient mutants. BRs are synthesized with sterols as precursor, and sterols such as stigmasterol, sitosterol and campesterol are all biosynthesized with the precursor of mevalonic acid. Sterols play a vital role in cell division, embryogenesis and development in plants. The lesion of intermediates or key enzymes involved in the sterol synthesis pathway can also cause a similar phenotype with BR-related mutants. *dwf1*, which was reported with the dwarf phenotype, was identified to be defective in C-24 reduction during the synthesis of BR in Arabidopsis, and *DWF1* encodes a FAD-binding oxidoreductase (Feldmann et al. 1989; Klahre et al. 1998; Takahashi et al. 1995). *dwf7*, another dwarf mutant in Arabidopsis, was identified to be deficient in the Δ^7 sterol C-5 desaturase gene related to sterol synthesis pathway (Choe et al. 1999;

Husselstein et al. 1999; Gachotte et al. 1995). The sterol Δ^7 reductase (S7R) mediates the enzymatic step between DWF7 and DWF1 (Choe et al. 2000). *dwf5* in *Arabidopsis* which disrupted in S7R displayed abnormal growth and development including small and dark-green leaves, short stems, petioles and pedicels (Choe et al. 2000), but no genes related to sterol biosynthesis have been reported in cucumber. Our study described the results on the phenotypic, cytological observation, genetic identification of the dwarf mutant and identified the candidate gene, *Csa7G447780* (a cucumber orthologue of *Arabidopsis* brassinosteroid biosynthesis enzyme gene *DWF5*), encoding 7-dehydrocholesterol reductase (DHCR7) and named it *CsDWF5*. These findings are helpful for understanding underlying molecular mechanism and further exploring and manipulating the BRs potential to increase agricultural yields.

Materials and methods

Plant materials

The super compact phenotype (*cpa*) mutant was identified from an M_2 family derived from an EMS-mutagenized cucumber inbred line CCMC. Two segregating populations from the crosses of *cpa* (male) with the wild type line CCMC and an inbred line Hazerd with normal phenotype were developed for genetic pattern analysis, gene mapping and cloning. All plants were planted in the greenhouse of Bai Ma farm of Nanjing Agricultural University, Nanjing, China.

Characterization and morphological identification

Phenotypes of the *cpa* and wild type cucumber were recorded. And, the data for plant height and internode number of wild type and *cpa* were collected. Each test was measured with ten plants, and each measurement was performed with three replications. Subsequent data were analyzed using Microsoft Excel 2013. Pigment contents measurement was conducted with leaf samples at the flowering stage from *cpa* and CCMC according to the procedure from Soto-Zamora et al. (2005), and the calculation formula was based on the method described by Song et al. (2020). The epidermal cells of the second internodes of main stem were performed by scanning electron microscopy (SEM).

Mapping strategy and identification of the candidate gene of *CsDWF5*

A modified MutMap method was adopted for mapping the candidate gene for *cpa* (Abe et al. 2012). The young leaves of WT and *cpa* plants were sampled for DNA extraction

following CTAB method as described previously (Edwards 1997; Song et al. 2018). Equal amounts of DNA with 21 dwarf plants and 22 wild type plants were bulked to generate the mutant type pool (aa) and normal pool (AA or Aa). Pair-end sequencing libraries with a read length of 150 bp and insert sizes of approximately 400 bp were subjected to whole-genome re-sequencing with Illumina HiSeq 2500. Short reads obtained from two DNA-pools were aligned against the cucumber genome sequence (the 9930 reference genome) to obtain the consensus sequence using BWA software (Li et al. 2009; Huang et al. 2009). The consensus sequence reads were used to call SNPs with SAM tools software (Li and Durbin 2009). SNP calling was conducted with GATK (McKenna et al. 2010). High-quality polymorphic SNPs of two pools were aligned to the reference to calculate the SNP indexes. All SNP indexes were spotted onto seven chromosomes. We calculated the SNP index and Δ (SNP index) to determine causal SNPs.

The strategy of bulked segregation analysis (BSA) was applied for quick identification of the linkage markers with *cpa* locus. The WT pool and *cpa*-pool were generated respectively through mixing equal amounts of DNAs of eight corresponding individuals from the F_2 population of *cpa* \times Hazerd. The markers of Indels and SSRs were firstly screened for polymorphism between two parental lines; then, the polymorphic markers were used to determine the genotypes of two pools. Indel and SNP markers were developed based on the re-sequencing results of CCMC and Hazerd, and the SSR markers were detected based on 450 markers distributed on 7 chromosomes (Cavagnaro et al. 2010). The framework map for *cpa* was constructed with the polymorphic markers in 96 F_2 plants. A larger F_2 population containing 907 individuals were applied to further narrow down the candidate region. Besides, SNP markers were designed based on the re-sequencing results of two pools and used for genotypic assay and linkage analysis. All primer sequences are listed in Table S1.

Cloning and the expression analysis of the *CsDWF5* candidate gene

Total RNA was extracted from the root, stem (the second internode), leaf, male flower (anthesis), ovary (one day before anthesis), cotyledon of the wild type and *cpa* mutant plant by TRIzol (Invitrogen) for cloning the gene and analyzing the relative expression level of *CsDWF5*. The first-strand cDNA was synthesized by PrimeScript TM RT reagent Kit (TaKaRa).

The cDNA from the leaf of WT and mutant plants was used as a template for cloning the target gene. Primers used for amplification of the full-length cDNA of the *CsDWF5* gene are listed in the supplemental Table S1.

qRT-PCR was performed in a 96-well plate using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with SYBR Green PCR Master Mix (TaKaRa, China). The cucumber *CsACTIN2* was used as the reference gene to normalize the gene expression results. The amplification was initiated by heating to 94 °C for 10 min, followed by 40 cycles of 94 °C for 5 s and 65 °C for 30 s. Gene expression level was calculated by the basis of the $2^{-\Delta\Delta C_t}$ with three biological and three technical replicates.

Subcellular localization of CsDWF5

To investigate the distribution of CsDWF5 in cells, the coding sequence of *CsDWF5* was firstly amplified with pMD19-*CsDWF5*-T plasmid, then fused with pGreen vector which contains green fluorescent protein (GFP) to generate p35s::CsDWF5-GFP vector. The recombinant expression vector was injected into tobacco (*Nicotiana benthamiana*) leaves for subcellular localization, and the pGreen vector was used as a negative control. The infected tobacco plants were kept in darkness for 72 h to allow the transient expression of transgenes. Besides, we extracted the protoplasts of tobacco, and transformed the recombinant plasmid and pGreen vector plasmid into the protoplast. All green fluorescence signals were observed under the laser scanning confocal microscope.

Relative analysis of BR

Since the phenotype of *cpa* mutant is consistent with the BR deficiency mutants which showed typical dwarfing shrinkage characteristics, we conducted BR response experiment to analyze the connection between *cpa* and BR biosynthesis. Seeds were germinated in an incubator with 28 °C day and 20 °C night and the 16 h day / 8 h night photoperiod. The mutants were sprayed with 0 μM, 0.2 μM, 2 μM epibrassinolide (EBR) respectively once a day when the cotyledons were fully expanded, and the treatments were ended at two true leaves stage (Wang et al. 2017a; Hou et al. 2017). For the endogenous BR content, 1.0 g tissues of *cpa* and wild type plant were collected for the measurement with Elisa kit, and each measurement had three biological repeats.

Protein sequence alignment and phylogenetic analysis

Based on the results of gene cloning, the cDNA and protein sequences alignments were conducted with the DNAMAN software to detect variations between *cpa* and WT. Protein sequences of CsDWF5 and its homologous in the other 10 species were downloaded from NCBI database to explore the phylogenetic relationship. The names of species and accession numbers of protein sequences in the NCBI database

were as follows: *Arabidopsis thaliana* NP_001077693.1; *Brassica oleracea* var. *oleracea* XP_013622239.1; *Capsicum baccatum* PHT58847.1; *Cucumis melo* XP_008462206.1; *Carica papaya* XP_021889020.1; *Cucurbita pepo* subsp. *pepo* XP_023513993.1; *Cucumis sativus* XP_011659586.1; *Glycine max* NP_001276192.1; *Ipomoea nil* XP_019196970.1; *Raphanus sativus* XP_018477406.1; *Solanum lycopersicum* NP_001353044.1. Multiple sequences were aligned by Clustal W, and the neighbor-joining tree was constructed using MEGA X based on a bootstrap test of 1000 replicates.

RNA-seq analysis

Total RNA was extracted using Trizol method from leaves of *cpa* mutant and CCMC plants with three replications. The integrity of RNA is accurately detected by Agilent 2100 Bioanalyzer. The libraries were generated by NEBNext® Ultra™ RNA Library Prep Kit (Illumina, San Diego, CA, USA). The assay was then performed on an Illumina HiSeq 4000 platform, and 150 bp pair-end read was produced (Novogene Co., LTD). The image data of the sequencing fragment detected by the high-throughput sequencer are converted into sequence data by CASAVA base recognition. To ensure the quality and reliability of data, the reads with adapter and low quality or containing N-poly were filtered out. Q20, Q30 and GC content were calculated. All subsequent analyses were based on clean data. Then, paired-end clean data were aligned to the cucumber ‘9930’ reference genome by Hisat2 v2.0.5 (Mortazavi et al. 2008). Gene expression levels were estimated by calculating the FPKM of each gene using feature Counts (1.5.0-p3) (Bray et al. 2015). DESeq2 was used to compare the DEGs between two combinations. The Benjamini and Hochberg tool was used to adjust the *P* value. $\text{padj} < 0.05$ and $\log_2(\text{Fold Change}) > 1$ was defined as the screening criterion for differential expressed genes selection. GO and KEGG enrichment analysis of DEGs were performed by cluster profiler based on the hypergeometric distribution principle (Anders and Huber 2010; Robinson et al. 2010; Love et al. 2014). The raw data of RNA-seq have been deposited in the NCBI database (SRA accession: PRJNA669004).

Results

Phenotypic characterization of the *cpa* mutant

A dwarf mutant was discovered from the M₂ families derived from an EMS-mutagenized cucumber CCMC population. Compared with wild type, the mutant displayed shorter hypocotyl, smaller leaf with wrinkled and dark-green color, and shorter petiole. The mutant had a significantly dwarfed

phenotype due to shortened internodes (Fig. 1a–d). This mutant could produce normal male flowers (except smaller size) with the fertile pollen. The ovary could grow into enlarged fruit, but the embryo could not develop sufficiently to form fertile seeds. The whole plant of the mutant was compact, so we named this mutant as *cpa*.

We conducted the microscopic observation for the stems in the longitudinal section and leaves of the *cpa* and WT plants by SEM. Both the number and length of cells

in stem were significantly reduced in *cpa* compared with those in WT plants. The cell number of leaves in single section of *cpa* is increased than that of WT (Fig. 1e, f and S1). We hypothesized that the abnormalities of cell division and cell differentiation cooperatively account for the dwarf phenotype. Pigment measurement results indicated that the contents of both Chlorophyll a and Chlorophyll b in *cpa* were significantly (P value < 0.05) higher compared with those in CCMC, but there was no significant difference in carotenoid (Table S2).

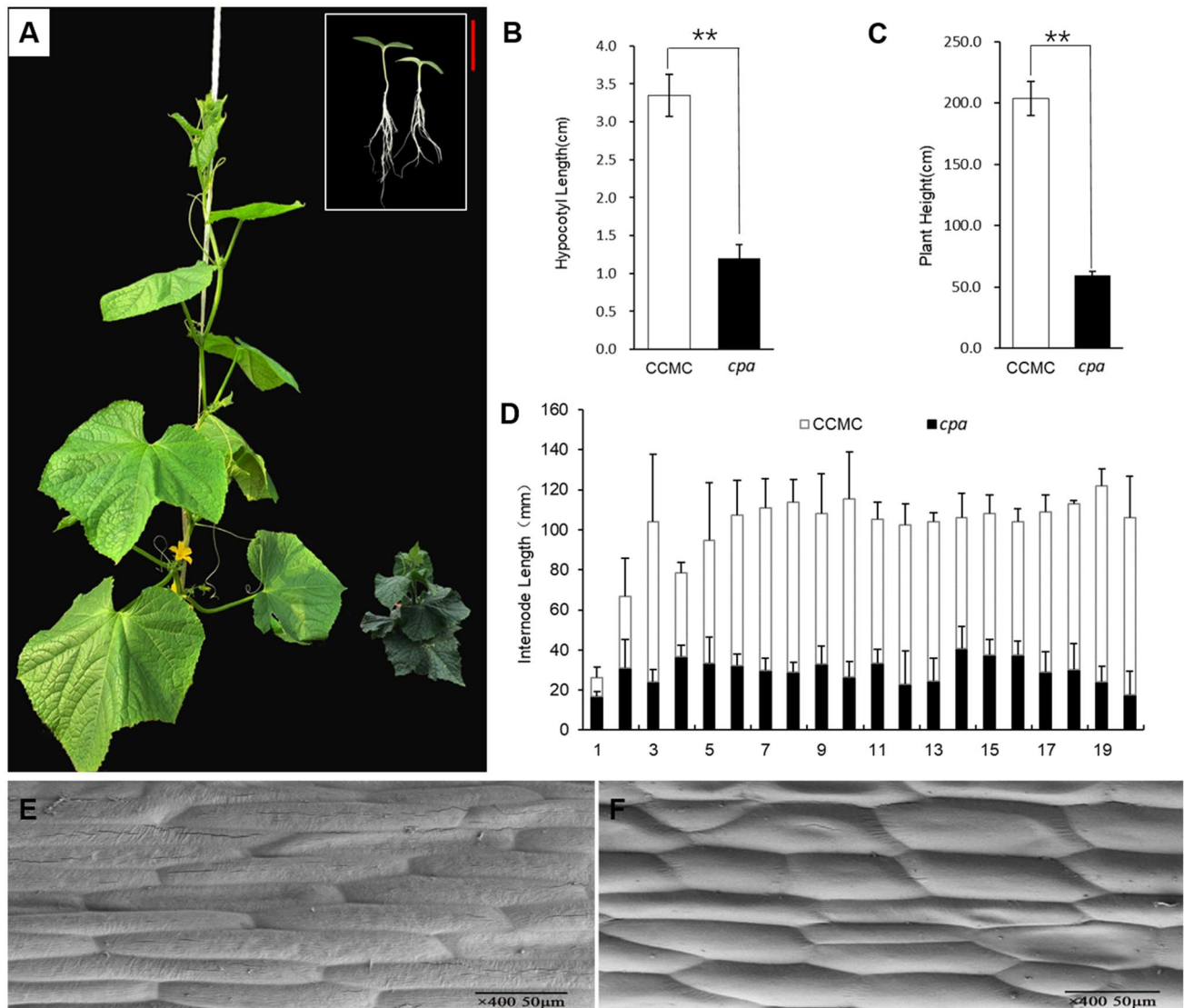


Fig. 1 Phenotypic characterization of cucumber CCMC (WT) and *cpa* mutant. **a** Phenotype comparison between WT (left) and *cpa* (right) at cotyledon stage and flowering stage. Bar = 10 cm. Statistics comparison of hypocotyl length at cotyledon stage **b** and plant height at mature stage **c** between WT and *cpa*. **d** Statistics on the internode

lengths of WT and *cpa* at the mature stage, the digits on the abscissa means the internode numbers. Stem longitudinal sections of CCMC **e** and *cpa* **f**. Values are the mean \pm SD, $**P < 0.01$ (**b**, **c**). Bar = 50 μ m (**e**, **f**)

Identification of the candidate gene *CsDWF5* responsible for *cpa*

To determine the inheritance pattern of the *cpa* trait, we constructed two F_2 populations from the crosses of *cpa* with CCMC and Hazerd. All the F_1 plants displayed normal phenotype. Among the 107 CCMC \times *cpa* F_2 plants, there were 82 wild types and 25 *cpa* types which were consistent with the 3:1 Mendelian inheritance law ($\chi^2 = 0.078 < \chi^2_{0.05, 1}$). Of 967 Hazerd \times *cpa* F_2 population, 733 had normal phenotype, and 234 exhibited the *cpa* phenotype ($\chi^2 = 0.331 < \chi^2_{0.05, 1}$). All the above results indicated that the *cpa* trait was caused by a single recessive gene.

BSA-seq result showed that there are 9294 million filtered reads from the WT pool and 8961 million from the mutant pool. Then, the clean data were aligned to the 9930 reference genome (V2.0) (Table S3). The genomic region near 15 M with a cluster of SNPs harboring high Δ SNP index on chromosome 7 was identified as the candidate region of *CsDWF5* locus (Fig. S2). In order to identify the causal SNP, we calculated the indices of all SNPs on the seven chromosomes of cucumber and plotted them into scatter plots. To eliminate background noise, we calculated the Δ SNP index. Considering the preference of EMS-induced mutagenesis, we selected a subset of SNPs with the following criteria for further research: (1) base mutation with transitions of G–A or C–T; (2) SNP index (aa) of mutant pool = 1; (3) the SNP with the non-synonymous mutation. This screening resulted in only one SNP which was located in the splicing site in the sixth intron of the *Csa7G447780* gene, and it was considered as the candidate SNP which is responsible for the *cpa* in cucumber (Table S4).

Genetic mapping was used to verify the reliability of the result with the *cpa* locus. Fifty-two pairs of SSR and 9 out of 110 pairs of Indel makers distributed on 7 chromosomes were detected to be polymorphism between CCMC and Hazerd. The detection of polymorphism between WT pool and *cpa*-pool identified that only two markers, Zindel113 and Zindel116, were linked to the *cpa*, and both of them were located on chromosome 7. An initial physical map was constructed with nine polymorphic markers on chromosome 7 by genotyping 96 individuals of F_2 population of from Hazerd \times *cpa* (Fig. 2a). Based on these results, the target interval was narrowed down to about 1.4 Mb by the flanking markers Zindel116 and Zindel113. In order to further narrow the candidate interval, a larger population including 907 individuals were used to screen more recombinants. A total of 44 SNP markers in this interval were developed, and five polymorphic markers (11.4%) were filtered out. After the genotypes of the recombinant individuals were identified, the *cpa* locus was delimited to a 260 kb genomic interval flanked with ZSNP72 and ZSNP116. Then, ZSNP52 among this region was identified to be co-segregation with

cpa-locus by linkage analysis (Fig. 2b), and this marker was consistent with the candidate SNP which was screened by MutMap. This result verified the accuracy of the causal mutation in *cpa*.

Gene annotation of the *Csa7G447780* displayed that it encodes a 7-dehydrocholesterol reductase protein and has 13 exons and 12 introns (Fig. 2c). This gene was homologous of Arabidopsis *DWF5* gene, so it was named as *CsDWF5*. The coding sequence of this gene was cloned from WT and *cpa*, and the full length of the gene was 1308 bp. The sequence alignment revealed that a single nucleotide mutation (G to A) which caused abnormal splicing and 3 bp insertion (TAG) at the first base of the sixth intron of the *CsDWF5* in *cpa* were identified. These changes resulted in the frameshift mutation and got a premature stop codon (Fig. 2c and d).

Homology and expression analysis of *CsDWF5*

We examined the protein sequence of *CsDWF5* in cucumber and other 10 species. The protein sequence of *CsDWF5* was used for blasting homology sequences in NCBI, and the multiple sequences alignment was conducted by DNAMAN. The amino acids in cucumber showed high similarity with that of other crops including Legumes, Solanaceae, Cruciferae and so on (Fig. S3). Among them, melon held the highest similarity with 98.16% and the identities of amino acid of the remaining homologous ranged from 81.15–90.11%. We constructed a phylogenetic tree with the amino acid sequence using MEGA to understand the phylogenetic relationship of the *DWF5* gene (Fig. 3). The cucumber was clustered with zucchini, sweet potato, melon and had the closest relationship with melon. The relative mutant concerning *DWF5* gene in Arabidopsis has been published; its lesion in BR biosynthesis is account for the abnormalities coding of 7-dehydrocholesterol reductase in complex sterol synthesis network (Choe et al. 2000). The identities of amino acid sequence between cucumber and Arabidopsis are 84.92%, and this similarity may lead to analogous function between them.

The results of qRT-PCR showed the expression level of *CsDWF5* in different tissues all was higher in *cpa* in comparison with WT. It is worth nothing that root and leave hold the high level of expression among all tissues (Fig. 4a). These results indicated that *CsDWF5* had a great influence on the growth and development of various tissues in cucumber.

To explore the expression location of *CsDWF5* in cells, subcellular localization was conducted with tobacco leaves and protoplasts. The fluorescence results of infected tobacco leaves displayed that the *CsDWF5*-GFP fusion protein was located in the cell membrane, while free-GFP was observed in the nucleus and cell membrane (Fig. 4b). The results of subcellular localization in protoplasts evidenced that *CsDWF5*-GFP mainly localized in plasma membrane

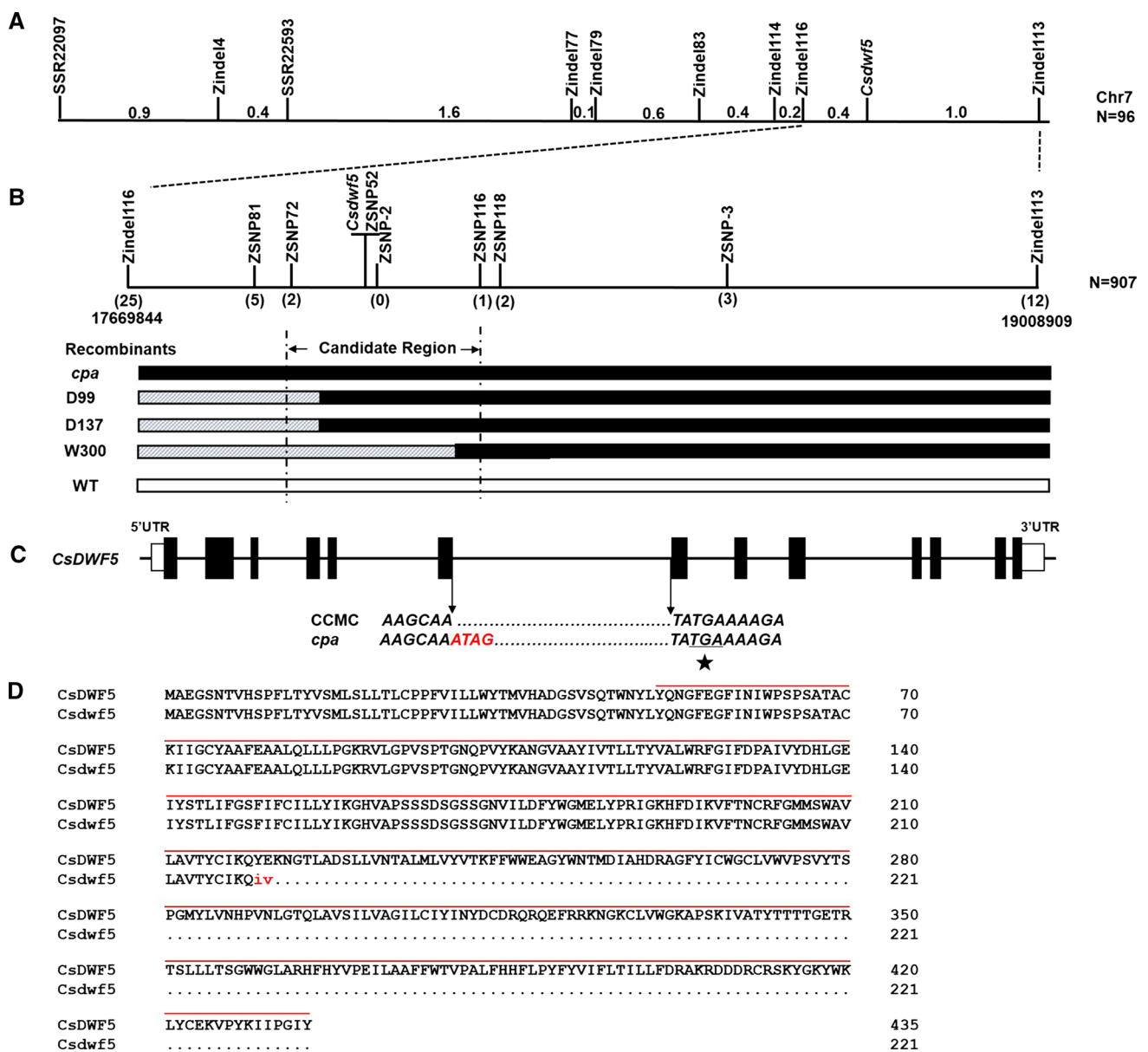


Fig. 2 Identification of candidate gene for *compact plant architecture* (*cpa*) gene in cucumber. **a** Initial mapping with BSA based on 96 plants from the F_2 population placed *Csdwf5* locus on chromosome 7 at a 1.4 cM interval flanked by markers Zindel 116 and Zindel 113. The numbers on chromosomes are genetic distance (cM). **b** Further genetic position analysis with 907 F_2 individuals delimited *Csdwf5* locus into a region between ZSNP72 and ZSNP116, and then, co-segregation detection was conducted by ZSNP52 with 4 recombinants. The digits in parentheses indicate the number of recombinants of

each marker. 17,669,844 and 19,008,909 means the physical position in chromosome 7. The different colors of rectangles were used for genotypes, black for *cpa*, white for WT, and stripe for heterozygous. **c** The structure of predicted *CsDWF5* and the coding sequence alignment of *CsDWF5* in CCMC and *cpa*. White boxes, black box and solid line represent 5' and 3'UTR, exons and introns respectively, asterisk indicated the stop codon. **d** The protein sequence alignment of *CsDWF5* gene in CCMC and *cpa*. And red line indicated the conserved domain

excluding the nucleus, which was consistent with that of leaves (Fig. S4).

The response mechanism of BR

We conducted the BR treatment on the *cpa* mutant by spraying the seedling with EBR. The color, shape and petiole

length treated with EBR had a clear tendency to be restored to the wild type phenotype (Figs. 5 and S5). But there is no obvious recovery in the plant height of *cpa* mutants (Fig. S5). This result indicated that BR can partially restore the mutant to the wild type phenotype. The endogenous BR contents of hypocotyl, stem and male flower in *cpa* were significantly reduced compared with those in wild type (Fig. S6).

Fig. 3 Phylogenetic analysis of CsDWF5 in cucumber and its homologs in other species. The phylogenetic tree was constructed by the neighbor-joining method built in MEGA X, and the inferred phylogeny was tested by bootstrap analysis with 1000 replicate datasets. Numbers at the tree forks indicated bootstrap values

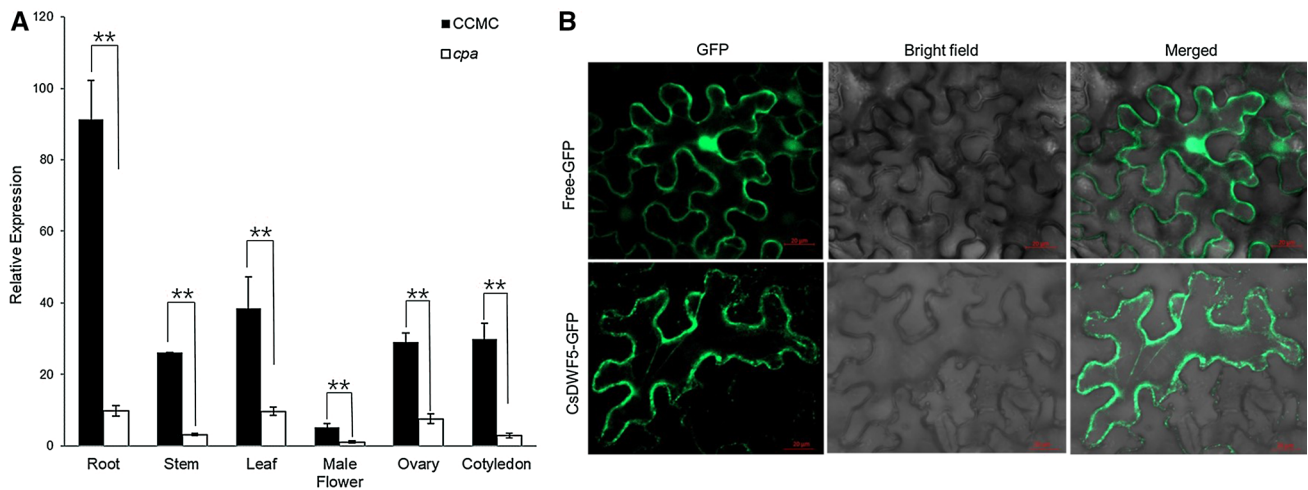
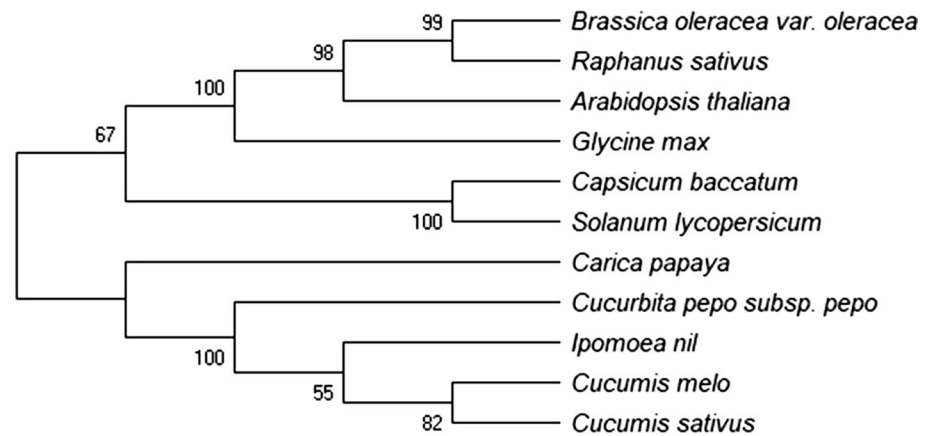
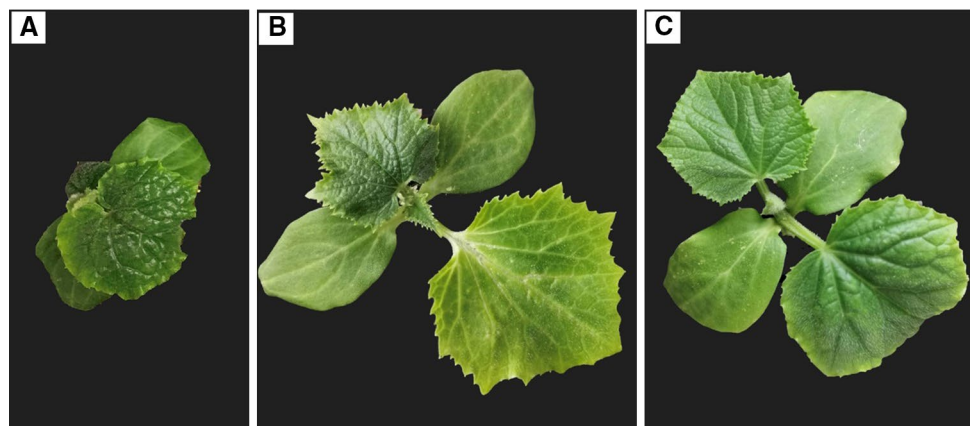


Fig. 4 Expression pattern analysis of *CsDWF5* **a** Relative expression of *CsDWF5* in different tissues of WT and *cpa* plants measured by qRT-PCR. Each experiment with three replications, and the error bars represent SE, asterisks indicate significant difference between *cpa*

and WT (t test, $P < 0.01$). **b** Subcellular localization of *CsDWF5*. A *CsDWF5*-GFP fusion protein was transiently expressed in tobacco cell, and GFP signal was observed by confocal fluorescence microscopy. Bar = 20 μ m

Fig. 5 Recovery phenotypes of *cpa* mutant by exogenous application of BR. **a** The top view of *cpa* seedling in the two-leaf stage. **b** The top view of *cpa* mutant seedling at the two-leaf stage with 0.2 μ M EBR application. **c** The top view of WT seedling in the two-leaf stage



Comparative transcriptome analysis of CCMC and *cpa*

In order to investigate the regulation mechanism referring to the formation of the dwarf in mutant, RNA-seq analysis was performed with leaves of WT and mutant. Pearson correlation and PCA analysis were firstly performed to value the reliability of all samples (Fig. S7). Compared with WT, 1096 genes exhibited differential expression in the *cpa* mutant, of which 527 and 588 DEGs were downregulated and upregulated, respectively. GO enrichment analysis showed that DEGs were mainly enriched in the biological process involving the movement of cells or subcellular components, microtubule-based movement and the molecular function which includes microtubule binding, microtubule motor binding, microtubule motor activity and tubule binding. Most of the GO items enriched by these DEGs were related to the vigorous growth of the seedling stage. KEGG enrichment analysis indicated that DEGs were significantly enriched in BR biosynthesis and plant–pathogen interaction (Figs. S8 and 9).

Based on RNA-seq results, we further analyzed the expression patterns of several key genes related to hormone synthesis and signal transduction pathways (Fig. S10). Compared with those in WT, 7 dehydrocholesterol reductase in sterol synthesis pathway was downregulated, and auxin response regulator, GH3 auxin-responsive promoter and AUX/IAA family genes were downregulated, while other genes are upregulated in *cpa* mutants. Besides, 12 cucumber genes were randomly selected for qRT-PCR to verify the reliability of RNA-seq results. The qRT-PCR results held high consistency with the transcriptome data, which evidenced the credibility of RNA-seq results (Fig. S11).

Discussion

Cucumis sativus L., as one of the most popular vegetables, is cultivated widely around the world. The plant height is not only the important trait of plant architecture but also the vital agronomic trait referring to the crop yield. Recently, several genes that are responsible for the dwarf phenotype have been cloned in cucumber, including *cp*, *si*, *scp-1*, *scp-2*, *Csdw* (Li et al. 2011; Lin et al. 2016b; Wang et al. 2017a; Hou et al. 2017a; Xu et al. 2018). Among them, *scp-1*, *scp-2* and *Csdw* were confirmed to be connected with the biosynthesis of BR. In this study, a novel dwarf mutant similar to the above two mutants was identified, which displayed the typical phenotype of BR-deficient mutants including dwarf architecture plant, shrunk and dark-green leaves, and female sterility.

In this paper, we used BSA-seq combined with linkage analysis to identify the locus of the candidate gene. Direct sequencing which is regarded as the most effective method in

SNP detection was used here for SNP genotyping. BSA-seq result revealed that only one splicing locus SNP7G18016936 was passed by filtering criteria and this locus was consistent with result of the linkage analysis with a large population. Gene cloning showed that this SNP caused confused splicing leading to a frameshift mutation and premature stop codon. qPCR results showed that the expression of *CsDWF5* in *cpa* mutant is significantly lower than that in wild type plant, which also was proof for being the candidate gene. Besides, the similar dwarf phenotype in a homologous mutant that has been reported in Arabidopsis is favorable evidence. Collectively, all the above evidence favorably demonstrated that the *CsDWF5* acts as the target gene for controlling the formation of the dwarf mutant phenotype.

Functional annotation and structural analysis revealed that *CsDWF5* encodes sterol Δ^7 reductase (S7R), which contains a superfamily domain of the PEMT (phospholipid methyltransferase), and is highly conserved in other species. Steroid 5- α -reductase, as another member of PEMT, was reported to be involved in the biosynthesis of brassinolide in plants (Li et al. 1996; Hou et al. 2017). Homology analysis indicated that *CsDWF5* shares high similarity with the homologous protein in humans. Sterol Δ^7 reductase (S7R) was studied deeply on the growth of humans. Children who are short of this enzyme will be troubled by Smith–Lemli–Opitz syndrome (SLOS). Symptoms of this disease manifest congenital anomalies including mental retardation and malformations in many different organs (Smith et al. 1964). And SLOS patients were reported to have the malfunction proteins that are homologous to *CsDWF5* (Fitzky et al. 1998). Similar to that of humans, the mutation of the corresponding *DWF5* gene in Arabidopsis also showed defects in growth and development. *dwf5* plants exhibit typical characteristics of BR-deficient mutants such as small, round and dark-green leaves, and short stems, pedicels and petioles (Choe et al. 2000). It has been reported that some specific moiety including a 7-reductase signature and MCC (mixed charge cluster) domain located on the C-terminal half of the protein has the proper function on sterol reductase (Lecain et al. 1996; Choe et al. 2000). *CsDWF5* here holds the truncation protein at the C-terminal half in the perspective of molecular structure that accounts for the abnormal development of *cpa*, which was consistent with the above reports.

According to the transcriptome sequencing result, more than 1000 differentially expressed genes were filtered out between the wild type and *cpa* mutant. Those genes are significantly enriched in the biosynthesis of brassinosteroids, movement of cell or subcellular component, microtubule binding, tubulin binding and some other pathways that connected with the growth and development in plants. BR has a great impact on the vital processes underlying the plant growth and development, including the dormancy and

germination of seed, organ differentiation, vascular tissue development, flowering and senescence, tropism. The block of cell elongation and differentiation are the direct reasons for shorter internodes. Microtubules reorientation was closely related to the cell elongation during the plant growth stage (Shibaoka 1994). BRs could affect the cell elongation by influencing the formation and stability of MTs based on the research of BR-deficient mutants (Catterou et al. 2001). Our RNA-seq results showed that DEGs were enriched in microtubule activity, which offered certain guiding significance for us to explore the relationship between sterol and cell morphogenesis and microtubule movement. Brassinosteroids (BRs) participated in the regulation of physiological and molecular processes to ameliorate various biotic and abiotic stresses (Nawaz et al. 2017). So we speculated that enrichment of DEGs in plant–pathogen interaction in KEGG analysis might be related to the aberrant BR synthesis.

By analyzing the DEGs, we found that the candidate gene encoding 7-dehydrocholesterol reductase was down regulated in *cpa* which is consistent with qRT-PCR. Sterol acts as the synthetic precursor of brassinolide, and its abnormal coding affects the synthesis of BRs. DEGs results also revealed several genes referring to the synthesis of BRs, which are involved in coding P450 family proteins. Cytochrome P450 monooxygenase were reported to be participated in some key catalytic steps of BR biosynthesis (Wang et al. 2017a). A large number of mutants which are blocked in sterol or BR biosynthesis were reported in dwarf or compact phenotypes. All these point out the importance of sterols and BRs roles in the regulation of plant architecture and provide another evidence for candidate gene. Surprisingly, most of the differentially expressed P450 genes were upregulated in *cpa* (Fig. S8), so we can conclude that sterols influence the pathway of BR synthesis, but the specific regulatory network still needs further study. Furthermore, some genes related to plant hormone signal transduction pathway including auxin response factor (ARF), GH3-auxin-responsive promoter, jasmonate ZIM domain protein, gibberellin receptor (GID1), ethylene insensitive 3 and other hormone signal transduction pathway genes were differently expressed. Auxins play critical roles in the major growth responses during plant development. BRs were reported interacted synergistically with auxin in hypocotyl elongation in several plant species. Previous studies have revealed that IAA and BRs exhibit cross talk with other plant hormones. IAA and GH3 genes are important cross talk points in BR, auxin, light and other signaling pathways (Goda et al. 2004). We speculate that all these differential expression might be related to the aberrant coding of the candidate gene in *cpa*. These findings are helpful to revealing complex phytohormone cross talk and plant signaling networks.

All above results highlight the necessity and importance of brassinolides and sterols in the growth and development

of plants. As yet, there has been no report on the genes involved in the biosynthesis pathway of sterol in cucumber. Our study herein revealed that the aberrant of *CsDWF5* gene located on chromosome 7 led to the defection in the biosynthesis pathway of steroid, which acts as the precursor of BR biosynthesis. The increase of chlorophyll content in *cpa* explains the deep green color of leaves. The supplement of exogenous BR revealed that the *cpa* mutant can be partially rescued. The partial recovery with BR in *cpa* mutant might be explained by that *cpa* mutant is not due to the lesion at BR-specific but sterols synthesis pathway. Sterols's work on plant development is not completely BR dependent. By changing the content and proportion of cell structural substances, sterols disrupted the properties of cell membranes and interfered the function of membrane-binding proteins (Schaller 2003). In addition, sitosterol can also affect plant growth and development by participating in the biosynthesis of cellulose (Peng et al. 2002). But the specific roles that sterols and BRs play in the formation of morphology in cucumber still need more in-depth research based on our study. Moreover, since *cpa* is also accompanied by other phenotypic abnormalities such as poor fertility of female flowers, the regulation of sterols for the growth and development in cucumber, especially the development of the female flower, still needs to be further studied.

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Author contribution statement QL and JC conceived the research and designed the experiments. MZ performed research, analyzed the data and wrote the manuscript; FC and ZY involved in the phenotypic selection, DNA extraction. MS participated in genome re-sequencing analysis. QL and DM revised the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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