Genome-Wide Identification and Expression Analyses of *CONSTANS-Like* Family Genes in Cucumber (*Cucumis sativus* L.)

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Received: 14 June 2020 / Accepted: 5 June 2021 / Published online: 15 June 2021
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Abstract

The *CONSTANS-like* (*COL*) gene family is one of the plant-specific transcription factor families that play important roles in plant growth and development. However, the knowledge of *COLs* related in cucumber is limited, and their biological functions, especially in the photoperiod-dependent flowering process, are still unclear. In this study, twelve *CsaCOL* genes were identified in the cucumber genome. Phylogenetic and conserved motif analyses provided insights into the evolutionary relationship between the *CsaCOLs*. Further, the comparative genome analysis revealed that *COL* genes are conserved in different plant species, especially collinearity gene pairs related to *CsaCOL5*. Ten kinds of cis-acting elements were vividly detected in *CsaCOLs* promoter regions, including five light-responsive elements, which echo the diurnal rhythm expression patterns of seven *CsaCOL* genes under SD and LD photoperiod regimes. Combined with the expression data of developmental stage, three *CsaCOL* genes are involved in the flowering network and play pivotal roles for the floral induction process. Our results provide useful information for further elucidating the structural characteristics, expression patterns, and biological functions of *COL* family genes in many plants

Keywords *COL* genes · Cucumber · Expression pattern · Photoperiod-dependent flowering process

Abbreviations

CO	CONSTANS
COL	CONSTANS-like
DAS	Days after sowing
FT	FLOWERING LOCUS T
qRT-PCR	Quantitative real-time PCR
SAM	Shoot apical meristem

Introduction

Successful transition from vegetative to reproductive growth is important in a plant life cycle (Srikanth and Schmid 2011). It is affected by both external and internal factors, and among them, photoperiod (day length) is a pivotal environmental signal associated with inception and process of flowering. The regulation of flowering by photoperiod has been reported in many plants, such as *Arabidopsis*, rice and maize (Putterill et al. 1995; Yano et al. 2000; Jin et al. 2018). Despite the different responses to the day length in plant species, the specific molecular components are conserved in the photoperiod-dependent flowering pathway (Fu et al. 2015). For example, *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) genes were considered as the main regulators (Putterill et al. 1995; Corbesier et al. 2007), and the *CO/FT* module is conserved in many plants (Song et al. 2010). It is apparent that *CO* acts as a hub gene of the photoperiodic flowering network (Shim et al. 2017). At the transcriptional level, the expression abundance of *CO* gene is mainly regulated by *FKF1-GI* complex degrading *CDFs* gene (Imaizumi et al. 2005; Sawa et al. 2007). Later, multi-photoreceptor-mediated mechanisms have been revealed to be involved in the post-translational regulation level. The blue-light

Handling Editor: T. Casey Barickman.

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photoreceptors cry1 and cry2, and phytochromes phyA and phyB, jointly regulate the stability of *CO* protein (Valverde et al. 2004; Shim et al. 2017). Through a complex regulatory network, *CO* proteins are accumulated and modulate the transcription of the downstream *FT* (Corbesier et al. 2007; Tamaki et al. 2007). The mobile florigen gene *FT* synthesized in the leaves is transmitted to the shoot apical meristem to initiate floral transition (Abe et al. 2005).

The *CO* and *CONSTANS-like (COL)* genes belong to plant-specific transcription factors, and they play diverse roles during the plant life cycle (Almada et al. 2009). Notably, many *COL* members participate in the photoperiodic flowering process; however, significant variation of the function has been reported. In *Arabidopsis*, *CO* plays a positive role and promotes flowering under LD condition (Putterill et al. 1995); overexpression of *COL5* can induce flowering in SD grown *Arabidopsis* (Hassidim et al. 2009). The study of *COL1* and *COL2* revealed that they have little effects on the flowering time (Ledger et al. 2001). Meanwhile, overexpression *COL8* and *COL9* result in late-flowering phenotype in *Arabidopsis* (Cheng and Wang 2005; Takase et al. 2011). The homologs of *CO* in other plant species are also thought to be involved in the photoperiod-associated flowering pathway. In rice, the Heading date 1 (*Hd1*) gene, the homologous of *Arabidopsis CO* gene, induces flowering under SD condition and exhibits the opposite response under LD treatment (Yano et al. 2000). Another homologous *CO* gene, *HvCO9*, contributes to delay flowering in barley (Kikuchi et al. 2012). In *Lilium × formolongi*, three *COL* genes (*LfCOL5*, *LfCOL6*, and *LfCOL9*) are involved in initiating flowering induction under LD treatment (Li et al. 2018). In addition, the functional *COL* genes with flowering-inducing effects tend to belong to the group I members (Zhang et al. 2015; Chaurasia et al. 2016).

Cucumber is one of the most popular vegetables, and its fruits are rich in health-promoting properties and are consumed worldwide. It is generally considered to be a day-neutral flowering plant. However, the special Xishuangbanna cucumber (XIS, *Cucumis sativus* L. var. *xishuangbannensis* Qi et Yuan) is a typical SD flowering plant, and their flowering time is delayed in the temperate regions (Qi et al. 1983). Previous studies by Bo et al. (2015) and Pan et al. (2017) have identified QTLs related to photoperiod-dependent flowering time in XIS cucumber. Wang et al. (2020) suggested that the florigen gene *CsaFT* was presumably an important genetic determinant of flowering time variation when measured in four cucumber sub-groups. The key genes involved in the photoperiod-mediated flowering network are worth exploring in the photoperiod-sensitive XIS cucumber.

As the core gene of photoperiodic flowering pathway, *CO* and *COL* family genes need further investigation. In this study, we identified the *CsaCOL* family genes in the cucumber genome. The phylogenetic tree and conserved domain

analysis were used to show the system classification and structure information of *CsaCOL* members. Collinearity analysis was performed to investigate the evolutionary relationship. Related cis-acting elements were found to predict the possible expression network of *COL* genes. The expression analysis and interaction network prediction were performed to understand the specific function of the *CsaCOL* genes. All these analyses provided insight into the regulation network of *COL* genes in the photoperiod-dependent flowering pathway.

Materials and Methods

Identification of *CsaCOL* Genes in the Cucumber Genome

All *COL* proteins were defined as genes containing both B-box and CCT domains (Robson et al. 2001). The hidden Markov model (HMM) program and related Pfam accession (B-box and CCT domains corresponding to PF00643.19 and PF06203.9) were used to find out all the *CsaCOL* genes in the cucumber genome database (<http://cucurbitgenomics.org/organism/2>, Cucumber genome sequence, Chinese Long, Version 2). All the selected *CsaCOL* proteins were further identified by Pfam database (<http://pfam.xfam.org/>) and Blastp in NCBI (<https://www.ncbi.nlm.nih.gov/>) to confirm the conserved domains B-box and CCT. In order to distinguish the *CsaCOL* genes, we named them based on the physical location on chromosomes in the cucumber genome. The ProtParam tool (<http://web.expasy.org/protparam/>) was used to provide basic information of the number of amino acids, molecular weight, theoretical iso-electric point (pI), and instability index (with a value < 40 considered as stable). Then the online tool PSORT (<http://www.genscript.com/psort.html>) was performed to reveal the subcellular location information. All the basic information of *CsaCOL* genes is shown in Table 1.

Phylogenetic Tree Construction

The homologs of *COL* genes were obtained from *Arabidopsis*, watermelon, rice, tomato, and maize, and the corresponding websites are as follows: TAIR database (<http://www.arabidopsis.org/>), Cucurbit Genomics database (<http://cucurbitgenomics.org/>), Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>), Sol Genomics Network (<https://solgenomics.net/>), and Maize Genetics and Genomics Database (<https://maizegdb.org/>). The *COL* proteins from six plant species (Table S1) were used to construct the phylogenetic tree. All the amino acid sequences were aligned by Clustal W program and constructed by MEGA6 software,

Table 1 Basic characterizations of *CONSTANS -Like (COL)* genes in cucumber

Name	Gene ID	Chr	No. of AA	MW(kDa)	pI	Instability index	Localization predicted
<i>CsaCOL1</i>	<i>Csa1G023050</i>	1	360	41.63	5.25	51.60	Nuclear mitochondrial
<i>CsaCOL2</i>	<i>Csa1G420310</i>	1	368	40.46	5.77	40.26	Nuclear
<i>CsaCOL3</i>	<i>Csa1G420320</i>	1	375	40.99	5.79	42.50	Nuclear cytoplasmic
<i>CsaCOL4</i>	<i>Csa2G057080</i>	2	319	35.16	8.20	47.38	Nuclear mitochondrial
<i>CsaCOL5</i>	<i>Csa2G383330</i>	2	337	36.93	6.08	45.36	Extracellular(including cell wall) nuclear mitochondrial
<i>CsaCOL6</i>	<i>Csa2G423550</i>	2	396	43.90	5.43	52.33	Nuclear
<i>CsaCOL7</i>	<i>Csa4G011750</i>	4	491	54.36	5.50	41.47	Nuclear
<i>CsaCOL8</i>	<i>Csa4G124910</i>	4	344	38.47	5.24	44.78	Nuclear
<i>CsaCOL9</i>	<i>Csa5G609670</i>	5	403	45.47	5.55	47.61	Nuclear mitochondrial
<i>CsaCOL10</i>	<i>Csa6G039540</i>	6	334	38.40	8.33	51.69	Nuclear
<i>CsaCOL11</i>	<i>Csa6G113560</i>	6	542	60.05	5.52	58.56	Nuclear mitochondrial
<i>CsaCOL12</i>	<i>Csa7G031530</i>	7	407	44.56	5.42	58.02	Nuclear

Chr. chromosome, No. of AA the number of amino acids, MW molecular weight, pI iso-electric point

with neighbor-joining method and 1000 times bootstrap replications.

Conserved Motifs of *CsaCOL* Genes

The basic information of *CsaCOL* genes in cucumber was obtained from Cucurbit Genomics database, such as physical location, sequences of amino acid, and nucleotide. The MEME online website (<http://meme-suite.org/tools/meme>) was used to identify the conserved motifs, with parameters' arrangements as follows: maximum number of motifs, 10; minimum and maximum width, 6 and 200. Basic sequence information of motifs is listed in Table S2. Three conserved domains (B-box1, B-box2, and CCT motif) of *CsaCOL* proteins were aligned and presented by the WebLogo 3 online system (<http://weblogo.threeplusone.com/>) with default parameters (Crooks et al. 2004).

Comparative Genome Collinearity Analysis

To exhibit the collinearity relationship of the *COL* genes in cucumber and other five plant species (melon, watermelon, *Arabidopsis*, maize and rice), corresponding *COL* genes were mapped to chromosomes based on physical location from the database of Cucurbit Genomics Database, TAIR database, Maize Genetics and Genomics Database and Rice Genome Annotation Project. The collinearity analysis was realized through Perl and Python language in linux system.

Analysis of the Cis-Acting Elements

The upstream sequences (1500 bp) of *CsaCOL* genes' were collected for analysis of cis-acting elements in their

promoter region. Corresponding analysis was performed by PlantCARE program (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>; Lescot et al. 2002) and then exports the results with online tools Gene Structure Display Server program (GSDS2.0, <http://gsds.cbi.pku.edu.cn/index.php>).

Growth Conditions of Cucumber Plants and Sample Collection

The cucumber inbred line 'SWCC8,' which belongs to XIS cucumber with property of short-day flowering, was used for the expression analysis. All seeds were sowed in the matrix (peat: vermiculite, 3:1) and then put in the incubator with 12 h/12 h (day/night) photoperiod regime, 28/18 °C temperature (day/night), a relative humidity of 80%, and 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photo flux density at Nanjing Agricultural University.

For studying the diurnal expression patterns of *CsaCOL* genes, the cucumber seedlings were transferred to two photoperiod conditions 8 h/16 h and 16 h/8 h (day/night) regimes when the first true leaf just appeared. One week later, the first true leaves were sampled every 4 h in 2 days, with three biological replicates at each time point. All samples were frozen in liquid nitrogen immediately and stored at $-80\text{ }^{\circ}\text{C}$ for RNA extraction.

To study the expression changes of *CsaCOL* members at different developmental stages, the second leaves counting from top were sampled from three biological replicates every 10 days within 80 days after sowing (DAS). Here, the photoperiod regime 8 h/16 h (day/night), which was verified by Bo et al. (2010) for the proper flowering of XIS cucumber 'SWCC8,' was used and other conditions were the same as

stated above. The slices of shoot apical meristem (SAM) at key developmental phases were selected and presented as the phase markers.

RNA Extraction, cDNA Synthesis and qRT-PCR Analysis

The above leave samples were ground into powder. Total RNA of these samples was extracted using Trizol Reagent (Invitrogen), and then 1 µg RNA was used to synthesize a 20 µL cDNA system following the instructions in PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA). Quantitative real-time PCR (qRT-PCR) was carried out on Bio-Rad iCycler Real-Time PCR Detection System (USA) by TaKaRa SYBR Premix Ex Taq™ (Tli RNaseH Plus) with three biological replications. Total reaction system is 20 µL, containing 10 µL SYBR Premix (2×), 1 µL cDNA, 1 µL sense and anti-sense primer separately (10 µM), and 7 µL ddH₂O. The qRT-PCR program was listed below, pre-denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s. Primer pairs were designed using Primer Premier 5.0, and NCBI blast program was used to identify the specificity of all primers (Table S3). The β-actin gene (*Csa2G301530*) in cucumber was used as internal reference, and the relative expression levels were calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Prediction of Protein–Protein Interaction Network

Based on the interolog in *Arabidopsis*, the interactions between *CsaCOL* members and photoperiodic flowering-related genes were predicted by STRING database (<http://string-db.org>). Then the interaction network was presented by Cytoscape_v3.7.2 software (National Institute of General Medical Sciences, MD, USA). Combined with the expression results, the schematic diagram, including *CsaCOL*, and other critical genes and elements were presented.

Results

Basic Characterization of *CsaCOL* Genes in Cucumber

Twelve putative *CsaCOL* genes were finally identified in cucumber by the HMM program and then verified by Pfam and blastp database, with all the *CsaCOL* genes both harboring B-box and CCT domains. In order to distinguish the twelve genes, we named them *CsaCOL1* to *CsaCOL12* according to their physical location on chromosomes. Detailed information is presented in Table 1. The *CsaCOL* genes were distributed on six chromosomes of cucumber genome, with three in chromosome 1 (*CsaCOL1*–*CsaCOL3*)

and 2 (*CsaCOL4*–*CsaCOL6*), two in chromosome 4 (*CsaCOL7* and *CsaCOL8*) and 6 (*CsaCOL10* and *CsaCOL11*), and one in chromosome 5 (*CsaCOL9*) and 7 (*CsaCOL12*), respectively. The amino acid sequences of *CsaCOL* proteins are between 319 (*CsaCOL4*) and 542 (*CsaCOL11*) in length, and the molecular weight is ranging from 35.16 kDa (*CsaCOL4*) to 60.05 kDa (*CsaCOL11*). The iso-electric points are various between *CsaCOL* genes, and the minimum and maximum iso-electric points are 5.24 (*CsaCOL8*) and 8.33 (*CsaCOL10*) separately. Protein instability index analysis showed that all *CsaCOL* members belong to instable proteins (instability index > 40). The predicted results of sub-cellular location presented that *CsaCOL* genes localized on nuclear, mitochondrial, and multiple other locations.

Phylogenetic Analysis of *COL* Genes

To understand the evolutionary relationship of the *COL* family genes, we constructed an unrooted neighbor-joining tree (Fig. 1) using 84 *COL* proteins from cucumber (12), watermelon (11), *Arabidopsis* (17), tomato (13), maize (16), and rice (15). All *COL* proteins have been verified by Pfam and Blastp database to contain both B-box and CCT domains (Table S1). In *Arabidopsis*, through sequence alignment, the *COL* members can be divided into three groups according to the divergence of B-box domain, with group I *COLs* containing two B-boxes; one normal B-box and another diverged B-box in group II; and group III *COL* members only including one B-box domain (Robson et al. 2001; Griffiths et al. 2003). Phylogenetic analysis showed that 84 *COL* proteins were indeed classified into three groups, with each group containing at least one *COL* protein from six different plant species. The distribution of cucumber *CsaCOL* proteins was five (*CsaCOL2*–*CsaCOL5*, *CsaCOL8*) in group I, four (*CsaCOL6*, *CsaCOL7*, *CsaCOL11*, *CsaCOL12*) in group II, and three (*CsaCOL1*, *CsaCOL9*, *CsaCOL10*) in group III. However, not all the *COL* proteins clustered into group I have two B-box domains. For example, *CsaCOL8* was classified in group I but only contains one B-box domain. In this study, classification results based on phylogenetic tree are not exactly the same when compared with that in *Arabidopsis* (Robson et al. 2001; Griffiths et al. 2003).

Sequence Structure Analysis of *CsaCOL* Members

MEME program was used to predict the sequence structure information. Ten kinds of conserved motifs were presented by numbers from 1 to 10 (Fig. 2a; Table S2). All the *COL* members in cucumber include two conserved domains, one or two B-boxes (motif 1) at the N-terminus and one CCT domain (motif 2) near the C-terminus. In addition to the representative B-box and CCT domains, each group *CsaCOLs* has their specific motif composition. For example,

Fig. 1 Neighbor-joining phylogenetic tree of *COL* proteins from six plant species. The group I, group II, and group III sub-families are indicated by light purple, blue, and green colors, respectively. The *COL* genes in cucumber are marked in red. The prefixes of *Csa*, *Cla*, *Ath*, *Sly*, *Zma*, and *Osa* indicate *COL* genes are found in cucumber, watermelon, *Arabidopsis*, tomato, maize, and rice, respectively (Color figure online)

three *CsaCOL* proteins in group III, *CsaCOL1*, *CsaCOL9*, and *CsaCOL10*, share the same composition of motifs, and the specific motif is motif 4. All the *CsaCOL* members in group I contain a valine-proline motif (VP motif, motif 3) near the CCT domain in the C termini, which is important for the interaction with *COPI* gene (Gangappa and Botto 2014). The conserved motifs of *CsaCOL* proteins show a certain similarity within groups.

The alignment of amino acid sequences of *CsaCOLs* identified three key domains of *COL* family genes, namely B-box1, B-box2, and CCT domains (Fig. S1). Then, the representative domains of *COL* proteins were shown separately (Fig. 2b). The CCT domain among the *CsaCOLs* is highly conserved, and the conservation is 45.24%. The amino acid sequences of B-box1 domain in the three groups are not identical, but the five cysteine residues (C in green) are fully conserved among twelve *CsaCOL* members, with the consensus sequence unified as C-X₂-C-X₈-C-X₂-D-X-A-X-L-C-X₂-C-D-X₃-H-X₈-H. The CCT and B-box1 are relatively conserved domains of *COLs*. Group III *COL* members have no B-box2 domain. In the group I and II, the B-box2 domains also contain five cysteine residues, even though the

interval sequences are different, indicating that the cysteine residues are conserved in the B-box domains of the *COL* proteins. The sequence conservation in normal B-box2 is up to 78.95% (group I *CsaCOLs*) and only 34.48% in the divergent B-box2 (group II *CsaCOLs*). The representative differences of *CsaCOL* proteins are in the sequence of the B-box2.

Collinearity Analysis of *COL* Genes Between Cucumber and other Plant Species

The collinearity analysis was carried out between cucumber and five plant species (Fig. 3), including three dicots (melon, watermelon, and *Arabidopsis*) and two monocots (maize and rice). The number of *COL* collinearity gene pairs between cucumber and melon, watermelon, *Arabidopsis*, maize, and rice are 11, 10, 7, 1, and 0 in order (Table S4). Cucumber, melon, and watermelon belong to the cucurbit crops, and the comparative genomic analysis yields more conserved collinear blocks, along with more collinearity gene pairs of the *COL* genes (one on one). However, relatively few collinearity gene pairs were detected between cucumber and maize

Fig. 2 Sequence structures of *CsaCOL* proteins in cucumber. **a** Conserved motifs of *CsaCOL* proteins. In total, 10 motifs were identified and indicated by increasing numbers from 1 to 10. Different motifs are indicated by different colors. **b** Sequence logo for B-box and CCT

domains of the *COL* proteins in cucumber. In the Fig. 2a, Motif 1 stands for B-box domain (B-box1, Motif 1 on the left; B-box2, Motif 1 on the right), and Motif 2 represents the CCT domain (Color figure online)

or rice. Multiple-to-one and one-to-many phenomena were detected in the collinearity analysis with the model plant *Arabidopsis*. For example, both *CsaCOL3* and *CsaCOL4* have a collinearity relationship with *AthCOL5*. In addition, *CsaCOL9* has a collinearity relationship with both *AthCOL6* and *AthCOL16*. The collinearity analysis between cucumber and cucurbit crops showed that *COL* genes in group I, II, and III participate in the formation of collinearity gene pairs; however, only group I and III *COL* members in cucumber produced collinearity with those in *Arabidopsis* and maize, implying that the *COL* sequences in group I and III are relatively conserved in the evolutionary history. In addition to the comparison of cucumber and rice, *CsaCOL5* has a collinearity relationship in the other four comparisons, which

indicates that *CsaCOL5* is highly conserved among different plant species (Table S4).

Cis-Acting Elements in the Promoter Regions of *CsaCOL* Genes

The composition of cis-acting elements was detected in the *CsaCOL* genes' promoter regions (Fig. 4; Table S5). Five elements evolved in light-responsive were found, such as G-Box, GT1-motif, GATA-motif, ACE, and 3-AF1-binding site, indicating that *COL* genes can be used as a light sensor in flowering plants (Simon et al., 2015). Among them, GT1-motif and G-box cis-acting elements were detected in seven and six promoter regions, respectively. Plant hormones-responsive

Fig. 3 Comparative genome collinearity analyses of *COL* genes between cucumber and other plant species. Gray lines indicate the collinearity blocks within cucumber and other plant genomes, while the red lines highlight the collinearity *COL* gene pairs. The collin-

earity analysis between cucumber (*Cucumis sativus* L.) and melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus*), *Arabidopsis* (*Arabidopsis thaliana*), maize (*Zea mays* L.), and rice (*Oryza sativa* L.) were presented in that order (Color figure online)

elements were also detected in the promoter regions, mainly correlated to GA (GARE-motif), MeJA (CGTCA-motif), and Auxin (AuxRR-core). In addition, development-related element CAT-box was found in five promoter regions, and its specific function is related to meristem expression. The circadian element was detected in *CsaCOL3*, which is corresponding to the circadian expression pattern of *COL* genes (Campoli et al. 2012; Kikuchi et al. 2012). The compositions of cis-acting

elements are related to the possible functions and expression patterns of the *COL* genes.

Diurnal Rhythm Expression Patterns of *CsaCOL* Genes Under SD and LD Photoperiod Regimes

Previous study suggested that the expression of *COL* genes has been shown to be affected by the circadian clock and

Fig. 4 The prediction of cis-acting elements in the promoter regions of *CsaCOL* genes

light-dependent diurnal oscillations (Campoli et al. 2012; Kikuchi et al. 2012). In order to check the daily oscillation rhythm, we detected the expression levels of cucumber *COL* genes under SD (8 h/16 h) and LD (16 h/8 h) regimes every 4 h in 2 days.

Under SD treatment (Fig. 5; Table S6), eight *CsaCOL* genes showed a diurnal expression pattern. Six of them, *CsaCOL1*, *CsaCOL2*, *CsaCOL3*, *CsaCOL5*, *CsaCOL6*, and *CsaCOL8*, showed a significant diurnal rhythm, peaking at dawn, and descending to different troughs. In addition, the expression peak of *CsaCOL9* and *CsaCOL11* appeared after dusk, then reaching their troughs during the day. And both of *CsaCOL9* and *CsaCOL11* hold a relative high expression level when compared with above six rhythm expression genes.

Different diurnal expression profiles were presented under LD regime (Fig. 6; Table S7), and the number of genes with daily oscillation rhythm is also eight. *CsaCOL1*, *CsaCOL2*, *CsaCOL3*, *CsaCOL5*, and *CsaCOL8* reached their peaks at the same time that was 4 h after dawn. *CsaCOL9*, *CsaCOL11*, and *CsaCOL12* presented their expression peak at dusk or 4 h after dusk.

The diurnal rhythm expression patterns of *CsaCOL* genes showed different peaks and troughs under SD and LD photoperiod regimes. Summarizing the rhythm expression results, seven *COLs* in cucumber, *CsaCOL1*, *CsaCOL2*, *CsaCOL3*, *CsaCOL5*, *CsaCOL8*, *CsaCOL9*, and *CsaCOL11*, presented the diurnal expression patterns under both SD and LD photoperiod regimes.

Expression Profiles of *CsaCOL* Genes at Different Developmental Stages

In order to better understanding the functions of *CsaCOL* genes in the flowering pathway, their expression patterns at different developmental stages were analyzed. The short-day treatment 8 h/16 h (day/night) was considered to be a relatively suitable photoperiod regime for the flowering of XIS cucumber ‘SWCC8’ (Bo et al. 2010). The expression analysis was carried out under 8 h/16 h (day/night) regime.

Previous study showed that *COL* family genes play critical roles in the flowering induction process (Suárez-López et al. 2001; Li et al. 2018). In the pre-experiment, the average flowering time of ‘SWCC8’ was 80 DAS. Then the expression profiles were detected every 10 days until 80 DAS. Comparative analysis of the slices at 10 DAS (Fig. 7a) and 30 DAS (Fig. 7b) showed that the floral primordia appeared at 30 DAS, indicating that the cucumber plants changed from vegetative to reproductive growth at 30 DAS. The expression profiles of *CsaCOL* genes were detected, and their expression patterns could be classified into two types (Fig. 8; Table S8). In the first type, which showed their highest expression level before or after the floral induction stage, comprised *CsaCOL2*, *CsaCOL3*, *CsaCOL5*, and *CsaCOL11*. In the second type, the highest expression profile was presented close to the flowering induction phase (30 DAS), including *CsaCOL1*, *CsaCOL8*, and *CsaCOL9*.

Previous study showed that *COL* genes have been correlated with the flowering induction by affecting the

Fig. 5 Diurnal expression patterns of *CsaCOL* genes under 8 h/16 h day/night photoperiod treatment

expression of florigen gene *FT* (Kobayashi et al. 1999). The expression level of *CsaFT* was also detected in this study (Fig. 8). The florigen gene *CsaFT* had an up-regulated expression trend around 30 DAS. The consistent higher transcripts' accumulation of *CsaFT* and *CsaCOL1*, *CsaCOL8*, and *CsaCOL9* indicated that the three *CsaCOL* genes may play positive roles in the flowering induction process. In addition, the expression level of *CsaCOL8* is significantly higher when compared with other *CsaCOL* genes. And the opposite expression pattern of other four *CsaCOL* members

(*CsaCOL2*, *CsaCOL3*, *CsaCOL5*, and *CsaCOL11*) and *CsaFT* suggested that they may inhibit the expression of *CsaFT*, thereby delaying the flowering process.

Prediction the Interaction Network Between *CsaCOL* and Photoperiodic Flowering-Related Genes

Previous study showed that *CO* was the hub gene of the photoperiod-mediated flowering pathways (Shim et al. 2017). To further elucidate the function of *CsaCOL* genes,

Fig. 6 Diurnal expression profiles of *CsaCOL* genes under 16 h/8 h (day/night) photoperiod regime

the interaction relationships between *CsaCOL* and photoperiodic flowering-related genes were predicted according to the network of *Arabidopsis*. Ultimately, three *COL* genes in cucumber, *CsaCOL3*, *CsaCOL5*, and *CsaCOL8*, participated in the photoperiodic flowering network. Genes with more interaction gene pairs are generally thought to be more important. Based on this principle, *CsaCOL8* had more interaction partners (9) compared with *CsaCOL3* (6) and *CsaCOL5* (3), indicating that *CsaCOL8* may be more

active in the photoperiod-associated flowering network (Fig. S2; Table S9).

The predicted network was complex for containing multiple interaction gene pairs, so we selected the critical elements to draw the schematic diagram (Fig. 9). Light is perceived by the specialized photoreceptors, such as *CsaPHYA*, *CsaPHYB*, and *CsaCRY1*. The cucumber plants measure the light signal by the internal oscillators, i.e., the genes (*CsaLHY*, *CsaTOC1*, *CsaGI*, and *CsaFKF1*)

Fig. 7 The comparative analysis of shoot apical meristem (SAM) sections at the key time points. **a** The SAM section at 10 days after sowing (DAS). The section presented the vegetative growing parts (IM and LP) at 10 DAS. **b** The SAM section at 30 DAS. When the floral primordia appear, it indicates the transition from vegetative to reproductive growth at 30 DAS. IM, inflorescence meristem; FP, floral primordia; LP, leaf primordia. Bars = 100 μ m

Fig. 8 Expression patterns of *CsaCOL* and *CsaFT* genes at different developmental stages

regulated by the circadian clock, all of which have direct or indirect effects on *CsaCOLs*' expression. *COL* genes affect the flowering process by regulating the expression of florigen gene *FT* (Kobayashi et al. 1999). Combined

with the expression results (Fig. 8), *CsaCOL8* directly promotes the expression of *CsaFT*, while *CsaCOL3* and *CsaCOL5* suppress it.

Fig. 9 The schematic diagram of *CsaCOL* and other related genes. Arrow represents activation while T-bars represent repression

Discussion

CO has the function of measuring day length, and meanwhile *CO* plays a central role during the photoperiod-regulated flowering process (Putterill et al. 1995; Suárez-López et al. 2001). Benefiting from the genome sequencing technology, *CO* and *COL* family genes have been found and characterized in different plants, for example, seventeen *COL* genes in *Arabidopsis* (Robson et al. 2001), sixteen and nine in rice and barley (Griffiths et al. 2003), thirteen in sugar beet (Chia et al. 2008), twelve in soybean (Wu et al. 2014), eleven in *Chrysanthemum lavandulifolium* (Fu et al. 2015), twenty five in Chinese cabbage (Song et al. 2015), and twenty in radish (Hu et al. 2018). The numbers of *COL* genes are not proportional to the size of corresponding plant genome (Table S10). In our study, ignoring the exceptions, most of the *CsaCOL* genes are structurally and evolutionarily conserved (Figs. 2, 3), and they have diverse expression patterns (Figs. 5, 6, 8).

Structural and Evolutionary Characteristics of the *COL* Genes

According to the definition of *COL* genes in *Arabidopsis*, all the *COL* genes have one CCT domain (C-terminus), and the differences come from the numbers of B-box domain (N-terminus), with group I *COL* members containing two B-box, group II one normal B-box, one diverged B-box and group III only including one B-box (Robson et al. 2001; Griffiths et al. 2003). Twelve *COL* genes in cucumber are also classified into three groups (Fig. 1), but not every *CsaCOL* gene fits the grouping principle in *Arabidopsis*. For example, *CsaCOL8* just contains one B-box domain (Table S1), while *CsaCOL8* is classified into group I in the phylogenetic analysis (Fig. 1). According to the previous analysis, the similar phenomenon occurs in multiple plants, such as *Brachypodium distachyon*, *Brassica rapa*, and *Citrus clementina* (Song et al. 2015).

Gene's structure affects its function. Therefore a comprehensive understanding of its structural features is necessary. The study of *COL* genes in *Lilium formolongi* showed that *LfCOL13*, *LfCOL14* and *LfCOL15*, which only contain one

B-box domain, were classified into group II (Li et al. 2018). In this study, the exception *CsaCOL8* just includes one B-box domain and belongs to a member of group I (Fig. 1; Table S1). Since it might be insufficient to comprehend the features of a gene just based on the number of B-box or phylogenetic analysis. The VP motif was detected in all of the group I *COL* members in cucumber (Fig. 2a, motif 3), which also has been verified in the group I *COL* genes in *Arabidopsis* (Gangappa and Botto 2014) and Banana (Chaurasia et al. 2016). The VP motif may be one of the criteria for judging the structural characteristics of group I *COL* genes. In addition to VP motif, the nuclear localization signals (NLSs) and other novel motifs also play important roles in the function of *COL* genes (Crocco and Botto 2013). It has been suggested that the B-box and CCT domains are necessary structures of *COL* members, and the radiated variation into other motifs and phylogenetic analyses also should be taken into consideration.

The evolution and origin of *COL* genes in plants are well worth exploring. According to the sequence logo results (Fig. 2), the B-box1 and CCT domains, namely the structural features of group III *COL* genes, are highly conserved when compared with others. Previous study shows that early *COL* proteins only contain B-box1 and CCT domains in green plants (Crocco and Botto 2013), implying that group III members may be the origin of *COL* proteins. The high similarity between B-box2 and B-box1 in group I *COLs* suggested that B-box2 may originate from the replication event of B-box1. Because of the differences of amino acid sequence and protein length (Fig. 2b), the B-box2 in group II *COL* members may derived from the mutation of B-box2 in group I. In summary, we proposed that group III members are the early *COL* proteins; thereafter, through the replication event, the group I *COLs* are generated; finally, group II *COL* members appeared to be initiated by gene mutation. The origin and differentiation of *COLs* need further exploration.

Multiple Expression Patterns of *COL* Genes

Previous studies have shown that some of the *COL* genes exhibit a distinct diurnal rhythm expression pattern under

different photoperiod regimes (Suárez-López et al. 2001; Li et al. 2018). For example, in *Arabidopsis*, *CO* showed distinct diurnal expression patterns under LD and SD light regimes, with the expression peak occurring at dusk and night under LD condition; however, *CO* reached its peak only at night under SD treatment (Turck et al. 2008). In the LD plant *Lilium × formolongi*, the rhythm expression patterns of *LfCOL* genes were also different (Li et al. 2018). In this study, under 8 h/16 h and 16 h/8 h regimes, the diurnal expression patterns of *CsaCOL* genes were varied in the time points and the expression level of the peak (Figs. 5, 6). These results suggested that *CsaCOL* genes are sensitive to photoperiod, thus, intriguing different responses according to the external photoperiod conditions. The discovery of light-responsive cis-acting elements also demonstrates that the *CsaCOL* genes typically respond to photoperiod changes (Fig. 4).

The *COL* genes play critical roles in the photoperiodic flowering induction process (Suárez-López et al. 2001); however, the functions of *COL* genes are diverse among plant species (Chaurasia et al. 2016; Li et al. 2018). Some of the studies highlighted the importance of group I *COL* members. For example, in wild and domesticated cotton, eight *COL* genes in group I were demonstrated to be involved in the photoperiod-regulated flowering process (Zhang et al. 2015). Previous study of banana showed that the group I type *COL* genes were also regulated by the photoperiod regime (Chaurasia et al. 2016). However, not all the *COL* genes, participating in the flowering network, belong to group I members. In *Lilium × formolongi*, *COL* genes in group I (*LfCOL5*), II (*LfCOL9*), and III (*LfCOL6*) play positive roles in the flowering induction process. In this study, group I (*CsaCOL2*, *CsaCOL3*, *CsaCOL5*, *CsaCOL8*), II (*CsaCOL11*), and III (*CsaCOL1*, *CsaCOL9*) *COL* genes were involved in the flowering induction network, and the functions of *CsaCOL* members were varied (Fig. 8). These results demonstrated that *CsaCOL* genes have different roles, and even the same group members may have diverse functions.

Conclusions

In conclusion, this study systematically analyzed the *COL* family genes in cucumber. The twelve *CsaCOL* genes are distributed on six chromosomes of the cucumber genome and can be divided into three groups through phylogenetic analysis. Conserved domain analysis reveals high similarity of *CsaCOLs* within groups, and the *COL* genes are evolutionarily conserved in different plants. Multiple *CsaCOL* members showed light-dependent diurnal rhythm changes, which echo the detection of light-responsive cis-acting elements in their promoter regions. Three *CsaCOL* genes are

involved in the flowering network; notably, *CsaCOL8* plays a positive role at the flowering induction phase. Our study provides a comprehensive understanding of cucumber *COL* genes on structure and expression regulation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00344-021-10420-4>.

Acknowledgements This work was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions. The authors would like to thank Hesbon Ochieng Obel for the revision of language.

Author Contributions JFC, JL, and ZT planned and designed the work. ZT, XDQ, and HW participated in the preparation of plant materials. ZT and HW conducted all experiments. ZT, XDQ, and HW analyzed the data. JFC, JL, and ZT wrote the manuscript. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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