Gou *et al. BMC Plant Biology* (2025) 25:418
https://doi.org/10.1186/s12870-025-06451-8

BMC Plant Biology

RESEARCH Open Access

Genome-wide identification and functional analysis of the longan *CONSTANS (CO)* family

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Abstract

Longans are among the most economically important subtropical fruits. Its flowering is sensitive to the photoperiod, and flowering time has a significant influence on yield and quality. *CONSTANS-like* (*COL*) gene plays a key role in regulating induced flowering in longans. However, the specific role of the *COL* gene family in the regulation of flowering remains unknown. In this study, 10 *DlCOL* genes were identified in longans using comprehensive bioinformatics analysis and named based on their physical chromosomal locations. Phylogenetic tree analysis showed that *DlCOL* genes were divided into three subfamilies, each with a conserved domain. When combined with collinearity analysis, we found *DlCOL* genes were more closely related to *COL* genes of dicotyledons. *DlCOL* family genes are differentially expressed in various longan organs, with *DlCOL1*, *DlCOL3*, and *DlCOL9* expressed in all organs, with the highest expression levels in floral buds. In the differential expression at different flowering induction stages of 'Sijimi' ('SJ') or 'Shixia' longan ('SX'), *DlCOL4* expression was upregulated by 3-fold at the "T1-T2" flowering induction stage in 'SJ', but there was no expression during the three flowering induction stages in 'SX'. Subcellular localization analysis indicated that DlCOL4 is localized in the nucleus. Heterologous transformation of *Arabidopsis* indicated that *DlCOL4* can negatively regulate flowering in transgenic plants. The qRT-PCR (Quantitative real-time PCR) results related to flowering genes indicated that *DlCOL4* may inhibit flowering by interacting with *AtTFL* and *AtCOL*. This study demonstrates the potential functional role of the *DlCOL* gene and the key role of *DlCOL4* in regulating longan flowering.

Clinical trial number

Not applicable.

Keywords Longan, CONSTANS family, Flower induction, Functional analysis

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Introduction

Flowering is a physiological developmental process in which plants change from vegetative growth to reproductive growth. It is induced by a variety of factors [1], and the photoperiod plays an important role in regulating the flowering time of plants [2]. Plants can be divided into long day (LD) and short day (SD) plants according to their different response mechanisms to light [3]. In *Arabidopsis*, the key gene for flowering is *CONSTANS* (*CO*), which can activate *FLOWERING LOCUS T* (*FT*) and its homologous gene *TWIN SISTER OF FT* (*TSF*) when synthesizing a stable coprotein under appropriate LD light [4, 5], thereby inducing flowering primordium germination and promoting flowering [6, 7]. However, rice has two different photoperiod pathways: heading stage 1 (HEADING-DATE1) and heading stage 3a (HEADING DATE 3a) [8], These two pathways are conserved in the *FLOWERING LOCUS T* (*FT*) module of *Arabidopsis* CO flowering base [9]. In addition, the *Hd3a* and *FT* genes are highly similar in rice [10], and *Hd3a* participates in the photoperiod response and promotes flowering under SD conditions [11].

The *CONSTANS-like* (*COL*) gene family is a group of transcription factors that are ubiquitous in plants and play important roles in plant growth, development, and response to environmental changes [12]. *COL* genes affect flowering time by regulating plant responses to photoperiod and are key factors controlling plant growth and development [13]. *CO* encodes a zinc finger transcription factor belonging to the B-box protein (BBX) family of proteins, with specific domains such as the N-terminal B-box domain and the C-terminal CCT (CO, CO-like) and TOC1 (TIMING OF CAB EXPRESSION1) domain [14, 15]. The number of gene members of *CO/COL* varies in different dicots and monocots. For instance in dicots, *Arabidopsis thaliana* [16] had 17 members, tomato [17] had 13 members, mango [18] had 31 members, and in monocots, rice (*Oryza sativa*) had 16 members and Barley had 9 members [2]. In these plants, COL members are subdivided into three groups: Group I contains two B-box domains, a CCT domain; Group II contains a B-box and CCT domain; and Group III contains an intact B-box, an emanating zinc finger structure, and a CCT domain. *COL* gene is conserved in the regulation of plant flowering, but its regulatory mechanisms differ among plant species. In *Arabidopsis*, flowering is promoted by direct activation of the expression of the *FT* gene [19]. Among the members of rice *COL* gene, *OsCOL10* was found to be associated with the regulation of rice flowering. Studies have shown that overexpression of *OsCOL10* can lead to late flowering in rice under both long- and short-day conditions, accompanied by increases in plant height, panicle size, and yield [20]. *OsCOL16* negatively regulates downstream flowering genes by inhibiting *Ghd7*, a factor that regulates flowering in rice, resulting in the downregulation of *Ehd1*, *Hd3a* and *RFT1* expression, thereby negatively regulating flowering [8]. The expression of *MiCOL* genes in most mango leaves and stems is significant, especially in the leaves at the flowering induction and flower organ differentiation stages [21]. Drought and salt stress promoted the expression of the *CO/COL* genes in mangoes. Notably, overexpression of the *MiCOL* gene has been shown to inhibit flowering under long-day conditions in transgenic *Arabidopsis thaliana* [18].

The *CO* gene has multiple functions in plant biology, not only regulating flowering, but also participating in the plant response to salt stress, and studies have shown that salinity treatment affects the *CO* gene [22]. Compared to wild-type *Arabidopsis*, mutants with missing *CO* gene function exhibited stronger salt tolerance; However, *OsCOL10* in *Arabidopsis thaliana* with high *CO* gene expression showed lower salt tolerance. CO and ABFs (ABF1, ABF2, ABF3, and ABF4), key transcription factors in ABA signaling pathways, interact to form protein complexes and, by binding to the promoter regions of downstream response genes in certain salt stress signaling pathways, such as *RD29A* and *RD20*, inhibit the plant's ability to tolerate salt stress, while opposing the transcriptional function of ABF3 [23].

However, *AtCOL4* is involved in ABA and salt stress responses and is associated with ABA-dependent signaling pathways [24]. In *Arabidopsis*, *AtCOL3* is a positively regulates photomorphological development in *Arabidopsis* It has an effect downstream of *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*), while also promoting lateral root development independently of *COP1* and playing a role in the day-length-sensitive regulation of shoot branches [25]. In addition, *CO/COL* plays a very important role in the regulation of leaf senescence and drought resistance in rice, fruit ripening mechanisms and stress response in bananas, and height regulation of flowering time and stem length in *Medicago sativa* [26–28].

Longan (*Dimocarpus longan* Lour) belongs to the *Sapindaceae* family and is an important subtropical fruit tree [29] that is widely cultivated and consumed because of its unique flavor and nutritional value. In the model plant *Arabidopsis thaliana* and in some fruit trees, *COL* gene family members have been shown to affect flowering time by regulating the photoperiod response of plants [30], which is important for controlling crop production cycles and also improving yields. However, The flowering process of longan is highly susceptible to many environmental factors, especially frost in spring and high temperature and humidity in winter, which often lead to irregular flowering of longan [29]. and the stability of flowering time directly affects the yield of longan; therefore, it is very important to study the flowering regulation

of longan for yield development [31]. Existing literature has not elucidated the genes involved in the regulation of flowering in the *COL* gene family of longans. Therefore, in this study, bioinformatics methods were used in order to identify and analyze the *COL* gene of longan, including physicochemical properties, structural characteristics, phylogenetic analysis, and expression process analysis of different organs and different flowering stages, and the differential gene (*DlCOL4*) was selected for subcellular localization and gene function verification analysis. Thus, we elucidated the potential function of *DlCOL* in the regulation of longan growth, development, and flowering. In addition, this study provides an important molecular basis for improving the quality and yield of longans.

Methods

Identification of *DlCOL* gene in longan genome

All of the *DlCOL* genes (http://www.sapindaceae.com/Download.html) in the longan genome database were identified using the hidden Markov model (HMM) program and the related Pfam germplasm (B-box and CCT domains corresponding to PF00643.19 and PF06203.9), and 14 candidate genes were obtained. Pfam (http://pfam.xfam.org/) [32] and Blastp in NCBI (https://www.ncbi.nlm.nih.gov/) are further identifying candidate genes to ensure that all *COL* genes contain both B-box and CCT domains. Finally, 10 *DlCOL* genes were obtained. we named them according to their physical ocation on the chromosomes of the longan genome. The physicochemical properties of the CO/COL proteins were analyzed using the ProtParam tool (http://web.expasy.org/protparam/), including basic information such as the amino acid number, molecular weight, theoretical isoelectric point (pI), and instability index (value < 40 is stable). Subcellular localization was predicted using the online tool WoLF PSORT (https://www.genscript.com/wolf-psort.html).

Analysis of chromosomes and replication events of the *DlCOL* gene

The chromosome position information of longan DlCOL was obtained from the Longan Genomics Database, and each gene was mapped to the corresponding chromosome using TBtools v1.09876 software [33]. The TBtools software was used to analyze and visualize homologous replication events in the *DlCOL* gene family.

Construction of the phylogenetic tree of the *DlCOL* gene

CO/COL homologous genes of a series of species were used for phylogenetic analysis, and the longan genomics database (http://www.sapindaceae.com/Download.html), *Arabidopsis thaliana* information resource database (http://cucurbitgenomics.org/), Rice Information Resource Database (http://plants.ensembl.org/Oryza_sativa/Info/Index) and Maize Information Resource Database (http://plants.ensembl.org/Zea_mays/Info/Index) download the protein information data of *COL*. The phylogenetic tree was constructed using the adjacency method (NJ) by MEGA software, with 1000 bootstrappy repeats. Visualization was performed using the online tool Interactive Tree of Life (iTOL) v5 (https://itol.embl.de/).

DlCOL gene structure and protein sequence analysis

Basic information of *DlCOL* gene of longan was obtained from the genomics database of longan, such as amino acid sequence, nucleotide sequence, and physical location, Conserved motifs were identified using the MEME website (https://meme-suite.org/meme/tools/meme) with the following parameters: maximum number of motifs 3, minimum width, 6; and maximum width, 50. The NCBI BLAST search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the query coverage and identity of each gene. The basic information on the motifs is listed in Table S1. The three conserved domains of the DlCOL protein (B-box1, B-box2, and the CCT motif) were compared by the Web logo3 online system (http://weblogo.threeplusone.com/) using the default data.

DlCOL genomic collinearity analysis

Collinearity analysis between longan and two monocotyledonous model plants (rice and maize) and one dicot model plant (*Arabidopsis thaliana*) was performed using the TBtools software.

Analysis of the cis-acting element of DlCOL

The 2000 bp upstream sequence of *DlCOL* gene family members was found from the Longan genome database as the promoter sequence of *DlCOL* gene, the cis element was predicted using plantCAPE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), and the predicted data were visualized using TBtools software.

Expression profiles of *DlCOL* genes in different organs and at different flowering stages

RNA-seq data from the NCBI Sequence Reading Archive (GSE84467) were obtained to analyze the expression patterns of *DlCOl* gene in the roots, stems, leaves, seeds, young fruits, pulp, pericarp, flowers, and flower buds of Longan, and TBtools were used to generate heat maps. According to our previous study, the expression profiles of *DlCOL* genes at three different flowering stages, including dormant stage (T1), flower primordium stage (T2) and flower organ-forming stage (T3) of 'Sijimi' ('SJ') and 'Shixia' longan ('SX'), was performed by using the RNA-seq data (SRS2241241, SRS2241242, SRS2241243, SRS2241244, SRS2241245, SRS2241246, SRS2241247, SRS2241248, SRS2241249, SRS2241250, SRS2241251,

SRS2241252, SRS2241253, SRS2241254, SRS2241255, SRS2241256, SRS2241257, and SRS2241258) [34].

RNA extraction, cDNA synthesis, and qRT-PCR analysis

Using a Plant Total RNA Extraction Kit (BL1301A; Biosharp), RNA was extracted from the sample by grinding it into a powder. Then the Script III RT Kit gDNA Eraser kit was used to synthesize 20μL cDNA with 1μL RNA according to the instructions. Quantitative real-time PCR was performed using a PCR instrument (Bio-Gener RePure-A). Three biological replicates were used for each treatment group. The total reaction system was 20 μL, including 1 μL cDNA, 0.5 μL of upstream and downstream primers, 10 μL of SYBRGreenMasterMix, and the rest was supplemented with ddH_2O. Reaction program: pre-denaturation at 94 °C for 2 min, 94 °C 30s, 60 °C 30s, 72 °C 30s, after 35 cycles, the melting curve was used for calculation and analysis by the $2^{-\Delta\Delta Ct}$ method [35], Excel software was used for mean statistics, SPSS software was used for one-way ANOVA analysis of the difference significance of the changes of the target gene in different tissues and materials ($P < 0.05$), and then the data were sorted out and the heat map was plotted using TBtools software.

Overexpression vector construction and *Arabidopsis thaliana* transformation

The full-length CDS of *DlCOL4* was cloned and inserted into the BamHI and SacI sites in pBI121 under the control of the CaMV35S promoter to construct the overexpression vector. Then, pBI121-*DlCOL4* was transferred to *Agrobacterium* strain GV3101 using the freeze-thaw method, and *DlCOL4* was overexpressed *Arabidopsis thaliana* lines using the floral dip method [36]. The positive *Arabidopsis thaliana* transgenic lines were screened on MS solid medium containing 30 μg/mL hygromycin under 16 h light/8 h dark conditions at 24 °C.

Subcellular localization analysis

The stop codon-free *DlCOL4* coding sequence was inserted into the pBI121 vector, and *DlCOL4* and GFP were used to form a vector for the fusion expression of 35 S::DlCOL4-GFP. Then, the correct constructs were transferred into *Arabidopsis* protoplasts using the PEG-mediated method in *Agrobacterium* strain and cultured in the dark for 24–48 h. Subsequently, the expression of DlCOL4-GFP in *Arabidopsis* protoplasts was observed using a laser scanning confocal microscope (TCS-SP8MP; Leica, Germany). At the same time, the pBMA(V)-HS-osgfp empty vector is used as a control.

Phenotypic analysis of Transgenic plants

The T3 generation transgenic plants were cultured in the same environment as the wild type, The photoperiod setting for cultivation is configured to 16 h of light and 8 h of darkness, with 3000 Ix light intensity and 24 °C. The flowering time and number of rosette leaves of transgenic and wild-type plants were measured and recorded. RNA was extracted from transgenic and wild-type plant leaves for qRT-PCR analysis, the flowering-related genes *AtTFL*, *AtCOL* and *AtLFY* were used for qRT-PCR analysis. *AtTUB* was used as an internal control. Finally, $2^{-\Delta\Delta Ct}$ was used to calculate and analyze, and SigmaPlot 12.0 was used for Bar chart plotting. (The primers used in this study are listed in Supplementary Table 1)

Results

Identification of *DlCOL* gene

The longan genome database was retrieved using the HMM program, and 10 putative *DlCOL* genes were identified and verified using the Pfam and blastp databases (Table 1). The results showed that all *DlCOL* genes contained B-box and CCT domains. In order to distinguish these 10 genes more clearly, these genes are named *DlCOL1* to *DlCOL10* according to their physical location on the chromosome (see supplementary Table 2 for details). As shown in Table 1, the results of the

Table 1 Analysis of basic information of *DlCOL* family

Name	Chr	Number of Amino Acid	Molecular Weight (KDa)	Theoretical pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity	Localization predicted
DlCOL1	1	346	37.55	5.75	45.5	60.38	-0.522	cytosol
DlCOL2	1	375	40.70	5.79	41.56	62.03	-0.327	chloroplast
DlCOL3	4	368	41.78	5.69	48.91	65.73	-0.77	nucleus
DlCOL4	10	499	56.43	6.03	61.37	59.8	-0.725	nucleus
DlCOL5	10	426	47.45	5.44	47.35	59.15	-0.883	chloroplast
DlCOL6	10	513	55.60	5.53	36.67	61.97	-0.609	nucleus
DlCOL7	12	384	43.41	8.54	48.37	76.43	-0.422	nucleus
DlCOL8	14	349	38.95	5.96	39.94	65.19	-0.623	nucleus
DlCOL9	14	348	38.73	6.19	36.83	67.04	-0.6	chloroplast
DlCOL10	15	436	48.72	5.34	37.98	63.28	-0.692	nucleus

bioinformatics analysis showed that the number of amino groups in the DlCOL family members ranged from 346 to 513aa, among which the number of amino acids in the DlCOL1 (346aa) protein was the lowest, and the number of amino acids in DlCOL6 (513aa) was the highest. The number of amino acids in the DlCOL family proteins was relatively similar, thus indicating that the replication and differentiation time of the DlCOL protein during the development of longan was relatively short, the function was relatively simple, and the differentiation of *COL* genes was relatively independent compared with that of other species. The molecular weight of the DlCOL family protein members is between 37.55–56.43kD, and the theoretical isoelectric point (pI) of the protein is between 5.34 and 8.54, among which DlCOL9 is a alkaline protein and the other 9 DlCOL proteins are acidic proteins. The instability index of the DlCOL members was between 36.67 and 61.37, only DlCOL6 and DlCOL9 had instability coefficients less than 40, which were stable proteins. However, the instability coefficients of the other 8 DlCOL proteins were all greater than 40. The fat coefficient of DlCOL was between 59.15 and 76.43, and the hydrophilic value of 9 DlCOL proteins ranged from − 0.33 to -0.88, all of which were hydrophilic proteins. The WoLF PSORT online software was used to analyze the subcellular localization of DlCOL proteins. The results showed that DlCOL2, DlCOL5, and DlCOL14 proteins were localized and expressed in chloroplasts, and DlCOL1 may be localized to the cytosol, whereas other DlCOL protein members are localized and expressed in the nucleus.

In addition, we analyzed the chromosomal localization of *DlCOL* gene. As shown in Fig. 1A, these 10 genes are distributed on six chromosomes of the longan genome, of which chromosomes 4 (*DlCOL3*), 12 (*DlCOL7)* and 15 (*DlCOL10*) each have one gene; and chromosome 1 (*DlCOL1*, *DlCOL2*) and chromosome 14 (*DlCOL8*, *DlCOL9*) each have 2 genes; and chromosome 10 (*DlCOL4*, *DlDCL5*, *DlCOL6*) has three genes. Among them, there was a collinearity relationship between seven *DlCOL* gene pairs (Figure. 1B), and the gene duplication events were mainly concentrated on chromosomes Chr10 and Chr15, which were all tandem replication events. Only *DlCOL1*, *DlCOL2* and *DlCOL3* had no collinear relationship with other genes in the family.

Phylogenetic analysis of the DlCOL proteins

In order to understand the evolutionary relationships between the gene homologs of the different *COL* families, a phylogenetic tree was constructed using 57 COL proteins from longan (10), *Arabidopsis thaliana* (16), rice (15), and maize (16) (Fig. 2). All of the COL proteins were validated using the Pfam and BLASTP databases, and all 57 COL proteins contained B-box and CCT domains (Table S1). These COL proteins were divided into three groups, each containing at least four different plant COL proteins. In the phylogenetic tree, four longan *DlCOL* family genes were in group I, four in group II, and two in group III. Phylogenetic tree analysis showed that the COL proteins in groups I and II contained two B-boxes and one CCT domain, whereas the COL proteins in group III contained only one B-box and one CCT domain. Dicotyledons (*Arabidopsis thaliana*) had the highest number of genes in group II (7 out of 16), group I had five AtCOL proteins, and group III had four AtCOL proteins. As shown in Fig. 2, the AtCOL proteins, except AtCOL7 and AtCOL8 were always found to be clustered near the DlCOL proteins and showed high sequence similarity. In addition, monocots (rice and maize) have a tight grouping of COL proteins, such as ZmaCOL4 and OsCOL10, ZmaCOL13, OsCOL4, ZmaCOL9, OsCOL6, ZmaCOL3, and OsCOL1, suggesting a common origin.

Sequence structure analysis of DlCOL members

To analyze the conserved peptide motifs and conserved domains of the *COL* gene family of longans, the structure was analyzed using MEME online software and CDD in NCBI, and visualized using TBtools software (Fig. 3). The results showed that each *DlCOL* subfamily had the same motif structure, and the DlCOL protein family contained motif1 and motif2, indicating that motif1 and motif2 are highly conserved in the DlCOL protein family (Fig. 3 A-3B). Similarly, the DlCOL protein family has conserved Bbox1, Bbox2, and CCT domains, indicating that the *DlCOL* gene has been highly conserved during evolution (Fig. 3 C). Analysis of the gene structure of longan revealed that the *DlCOL* genes were generally similar, and the introns of *DlCOL4*, *DlCOL3*, *DlCOL6* and *DlCOL7* genes were longer. However, the untranslated region UTR and CDS (protein coding region) were similar to those of other members of the *DlCOL* gene family (Fig. 3D). These results indicate that the *DlCOL* gene is highly conserved during evolutionary processes.

Collinearity analysis of the *DlCOL* gene

We performed a collinearity analysis between longan and the dicot model plant *Arabidopsis thaliana*, monocot model plant rice, and maize (Fig. 4). The results showed that the *COLs* of longan and *Arabidopsis thaliana* had a high degree of homology, 11 pairs of orthologous genes were identified between longan and *Arabidopsis*, while relatively fewer were found in rice (4 pairs) and maize (2 pairs). The results indicated that longans were most closely related to the dicot model plants. However, relatively few collinear gene pairs have been detected between longans and rice or maize. The results showed differences in the structure and function of *COL* genes between longan, rice, and maize. It could also be inferred that there were different evolutionary directions between

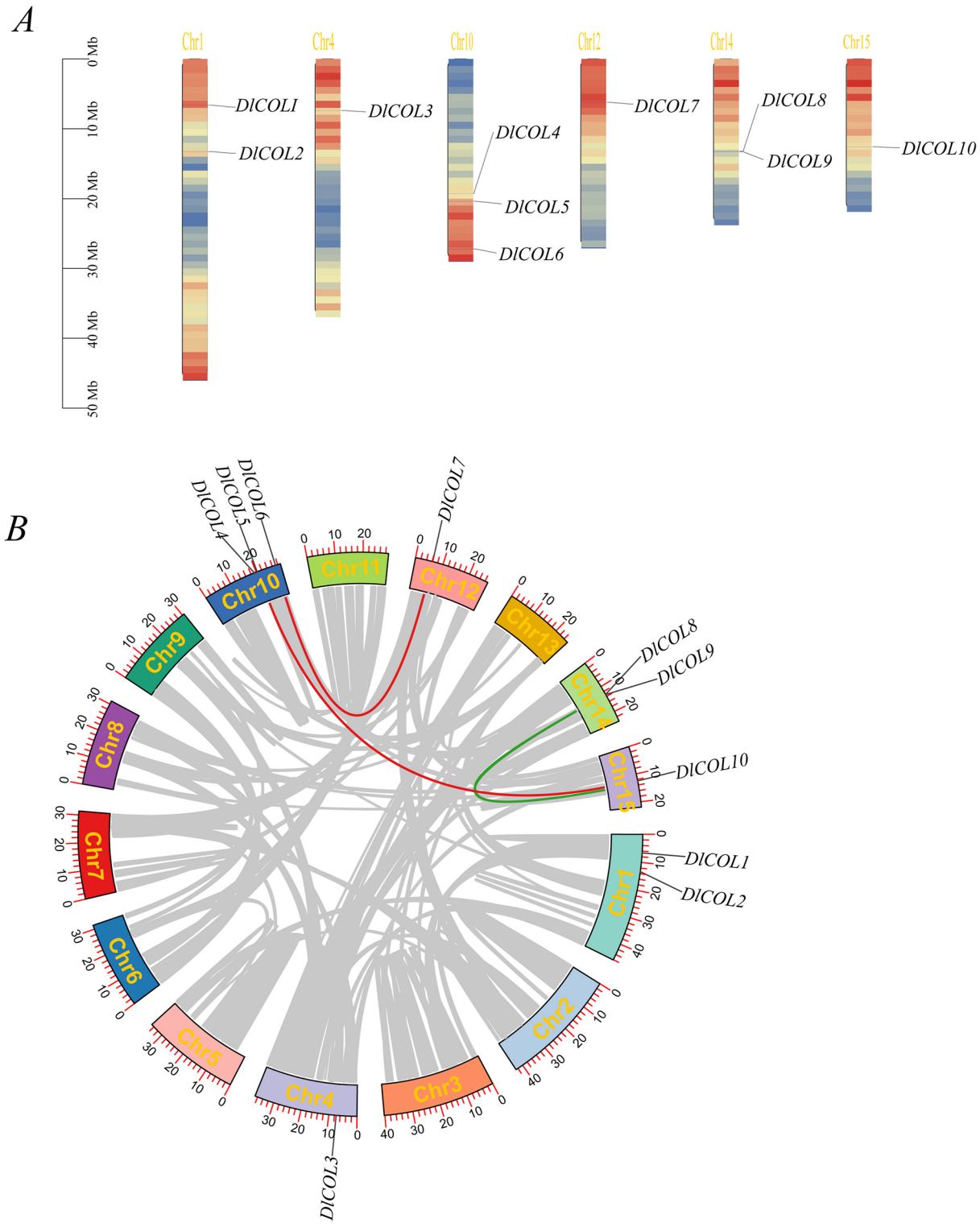

Fig. 1 Chromosomal and collinearity analysis of *DlCOL* gene, (**A**) chromosomal analysis of the *DlCOL* genes; (**B**) Collinear analysis of the *DlCOL* genes, the colored line represents the collinear gene pair, and the gray line represents the homolog block in the longan genome

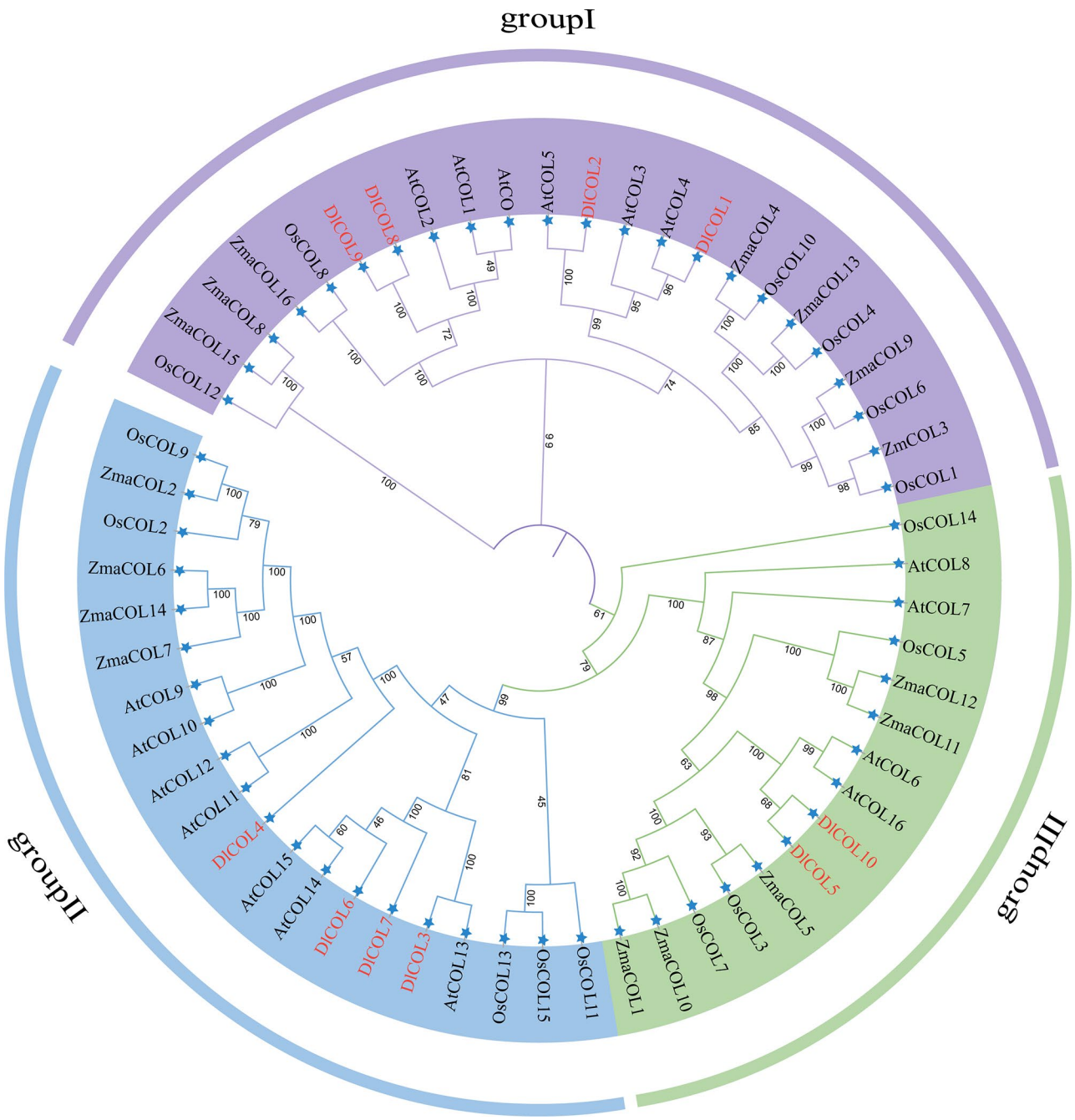

Fig. 2 Phylogenetic tree of COL members from longan, tomato and *Arabidopsis*, with longan COL family members in red font

the longan, rice, and maize *COL* gene families during the evolutionary process.

Analysis of cis-element elements of the *DlCOL* gene family promoter

The function and expression pattern of the *COL* gene may be related to the composition of cis-acting elements. The results showed that nine unique cis-acting elements were identified in the *DlCOL* gene family. The cis-acting elements of the *DlCOL* starter were highly similar, and the occurrence frequency of light-responsive elements was the highest in each *DlCOL* promoter (Fig. 5). In addition, the *DlCOL* gene can be induced by a variety of hormones, such as methyl jasmonate, abscisic acid, gibberellin, salicylic acid, and auxins. However, most promoter regions of the longan *COL* gene contain a variety of stress-responsive elements, including drought-induced elements and low-temperature-responsive elements. These results indicate that the expression of *DlCOL* is strongly related to light and stress conditions. *DlCOL*

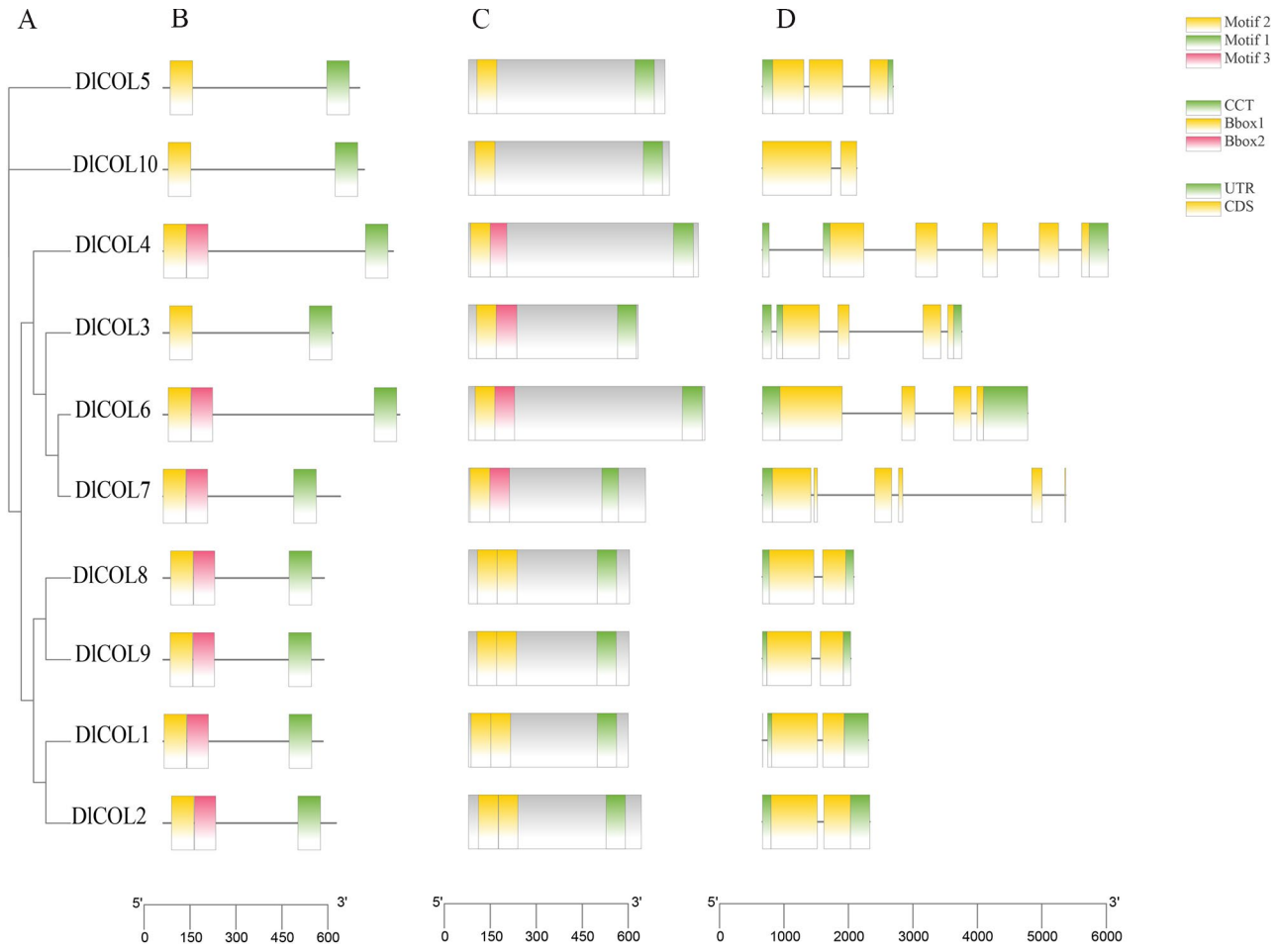

Fig. 3 *DlCOL* gene and protein structure. (**A**) Phylogenetic analysis of 10 *DlCOL* genes; (**B**, **C**) Distribution characteristics of the conserved motifs of the DlCOL protein; (**D**) Intron-exon analysis of the *DlCOL* gene

gene family members play key roles in photoperiod, circadian rhythm, hormones, and stress pathways, demonstrating the diversity of *DlCOL* functions.

Expression profiles of *DlCOL* genes in different organs and at different flowering times

In order to study the expression patterns of *DlCOL* gene in different organs and at different flowering stages, we analyzed the expression of 'SX' longan and 'SJ' in different tissues and at different stages using the downloaded transcriptome data, and the results showed that the expression levels of the *DlCOL* gene in different organs were significantly different (Fig. 6 A). Most genes were expressed in the leaves and flower buds, including *DlCOL1*, *DlCOL3* and *DlCOL9*, which were expressed in all organs, and the highest levels were expressed in flower buds. The expression levels of *DlCOL10* and *DlCOL5* were significantly higher in the leaves but low or no expression in other organs.

It was found that most of *DlCOL* genes, except for *DlCOL1*, *DlCOL3* and *DlCOL5*, were significantly expressed in 'SX' and 'SJ' based of our previous transcription data (Fig. 6B). In the three stages of 'SX' flower induction, the expressions of *DlCOL2*, *DlCOL8* and *DlCOL9* were down-regulated in the late flower formation stage (SXT2-SXT3), and the expressions of *DlCOL7* were up-regulated in the late flower formation stage (SXT2-SXT3). While, *DlCOL4* was up-regulated in the early flower induction stage (SJT1-SJT2), and *DlCOL7* was down-regulated in the early flower induction stage (SJT1-SJT2) of 'SJ'. The expression of *DlCOL10* was down-regulated in the early floral induction stage (SJT1-SJT2) and upregulated in the late floral induction stage (SJT2-SJT3). The expression of *DlCOL4* was up-regulated 3 times in the early flowering induction stage (SJT1-SJT2) of 'SJ'. whereas, there was no expression change of this gene in during flower induction process of 'SX' these results suggested that *DlCOL4* plays a role in regulating flowering. Therefore, *DlCOL4* was selected for further functional studies.

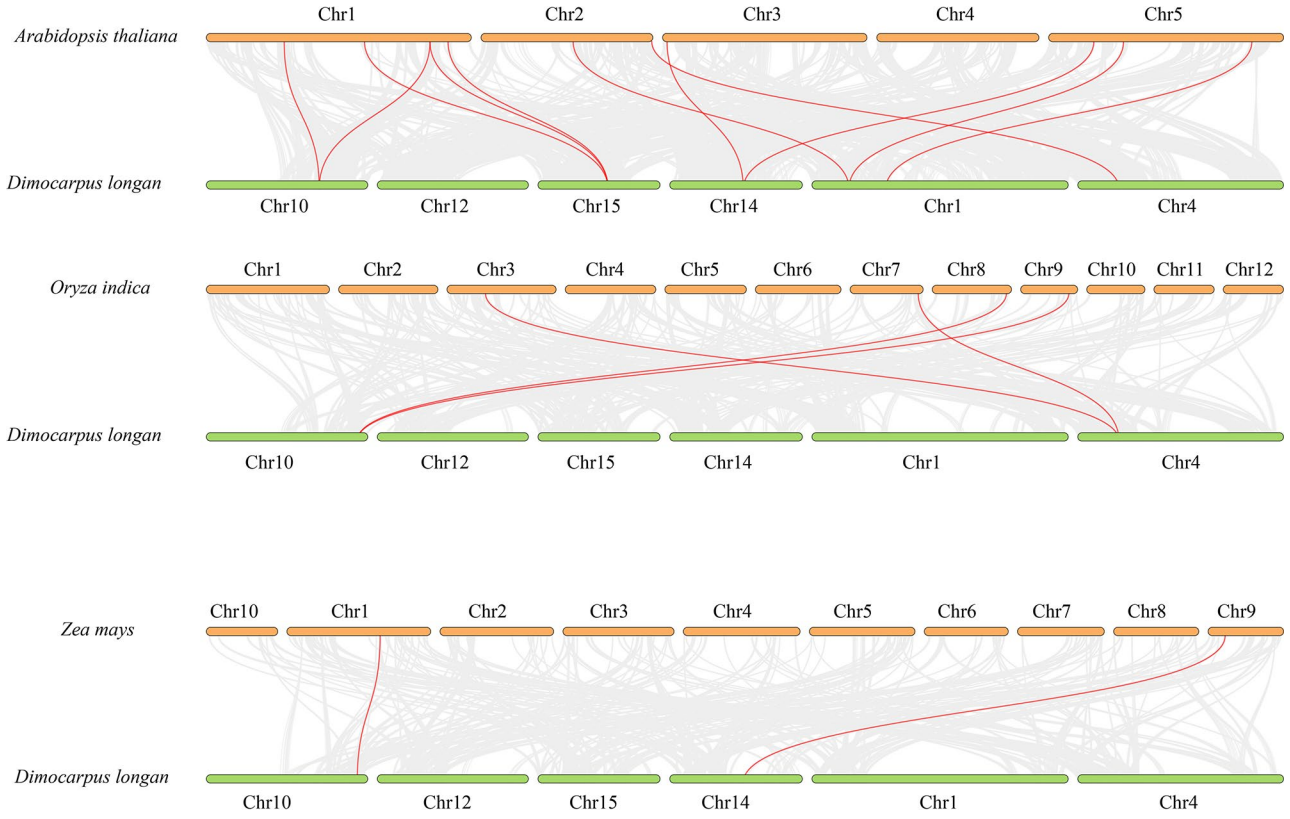

Fig. 4 Collinearity analysis of the *COL* genes between longan and *Arabidopsis*, rice and maize. The red lines highlight collinear gene pairs, while the gray lines represent homologous blocks in the longan and other plant genomes

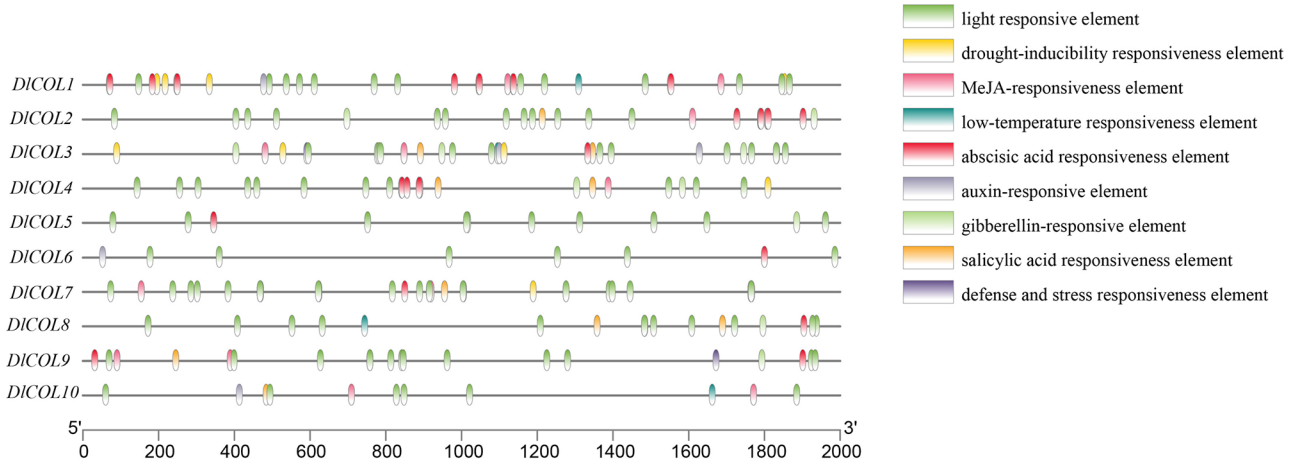

Fig. 5 10 distribution of cis-acting elements in the promoter sequences of the *DlCOL* genes. Different cis-acting elements are represented by different color blocks

Subcellular localization analysis of the DlCOL4 protein

To analyze the location of the DlCOL4 protein in cells, we constructed a fusion protein expression vector (35 S::DlCOL4-GFP) and introduced it into *Arabidopsis* mesophyll protoplasts using the PEG-mediated method. The results are shown in Fig. 7, under the action of the 480 mm wavelength laser, the fluorescence signal of 35 S::DlCOL4-GFP can only be detected in the nucleus, with no GFP signal presented in the cytoplasm and cell membrane. In contrast, the 35 S::GFP control exhibited a GFP signal throughout the cell. The results showed that *DlCOL4* is a nuclear-localized protein.

Overexpression of *DlCOL4* inhibits *Arabidopsis* flowering

To better investigate the potential flowering time regulatory mechanism of *DlCOL*, two positive T3 *DlCOL4*

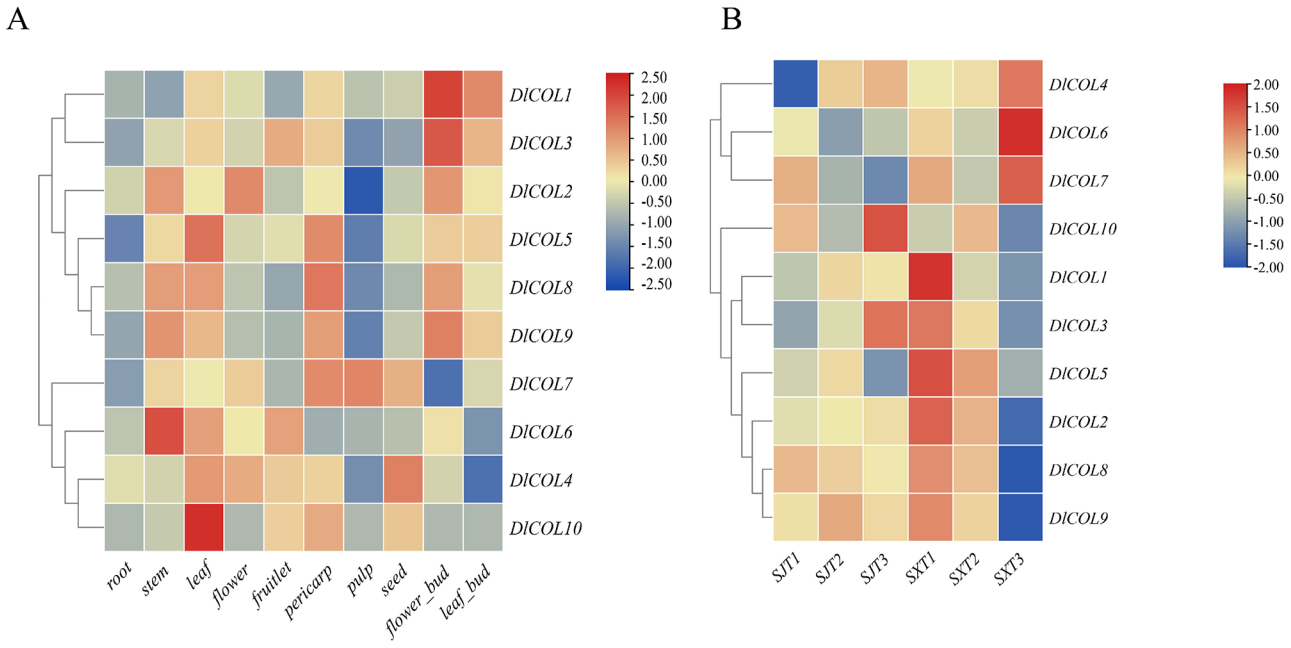

Fig. 6 Expression patterns of *DlCOL* gene in different organs and different flowering times. (**A**) Expression of the *DlCOL* genes in different longan organs. (**B**) Expression levels of the *DlCOL* genes in different flowering periods. T1 represents the dormant stage (before the emergence of the flower primordium), T2 represents the flower primordium stage, and T3 represents the flower organ-forming stage. The color gradient (red/yellow/blue) indicates the level of expression (from high to low)

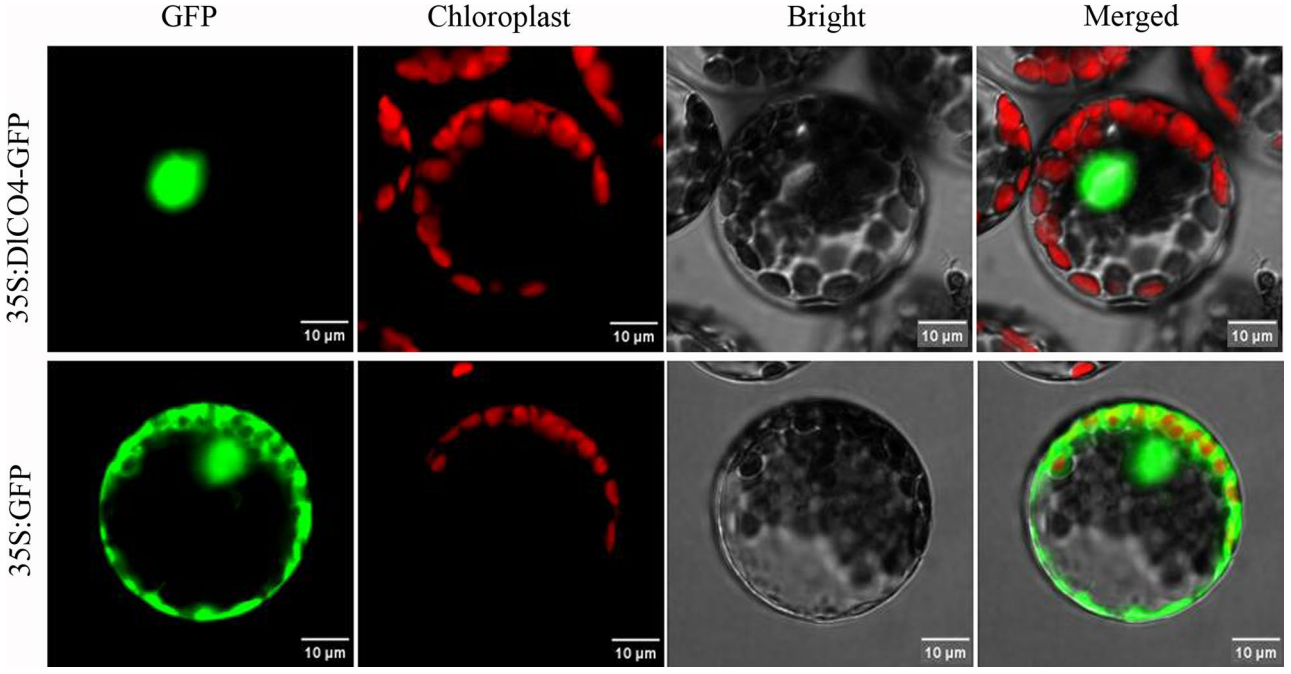

Fig. 7 Subcellular localization of DlCOL4 protein

overexpression transgenic plants (OE4 and OE5) were randomly selected. The qRT-PCR results showed that *DlCOL4* was only highly expressed in transgenic lines, and under LD conditions, the flowering time of *DlCOL4* transgenic *Arabidopsis thaliana* was later than that of wild-type (WT) plants (Fig. 8 A and 8E). The transgenic flowering time ranged from 42.66d to 44.57d (OE4 flowering time 42.66d, OE5 flowering time 44.57d), and the WT flowering time ranged from 29.53d to 32.66d (WT1 flowering time 29.53d, WT2 need 32.66d). WT *Arabidopsis thaliana* had an average of 14.5 rosette leaves, whereas transgenic lines had an average of 12.3 (OE4)

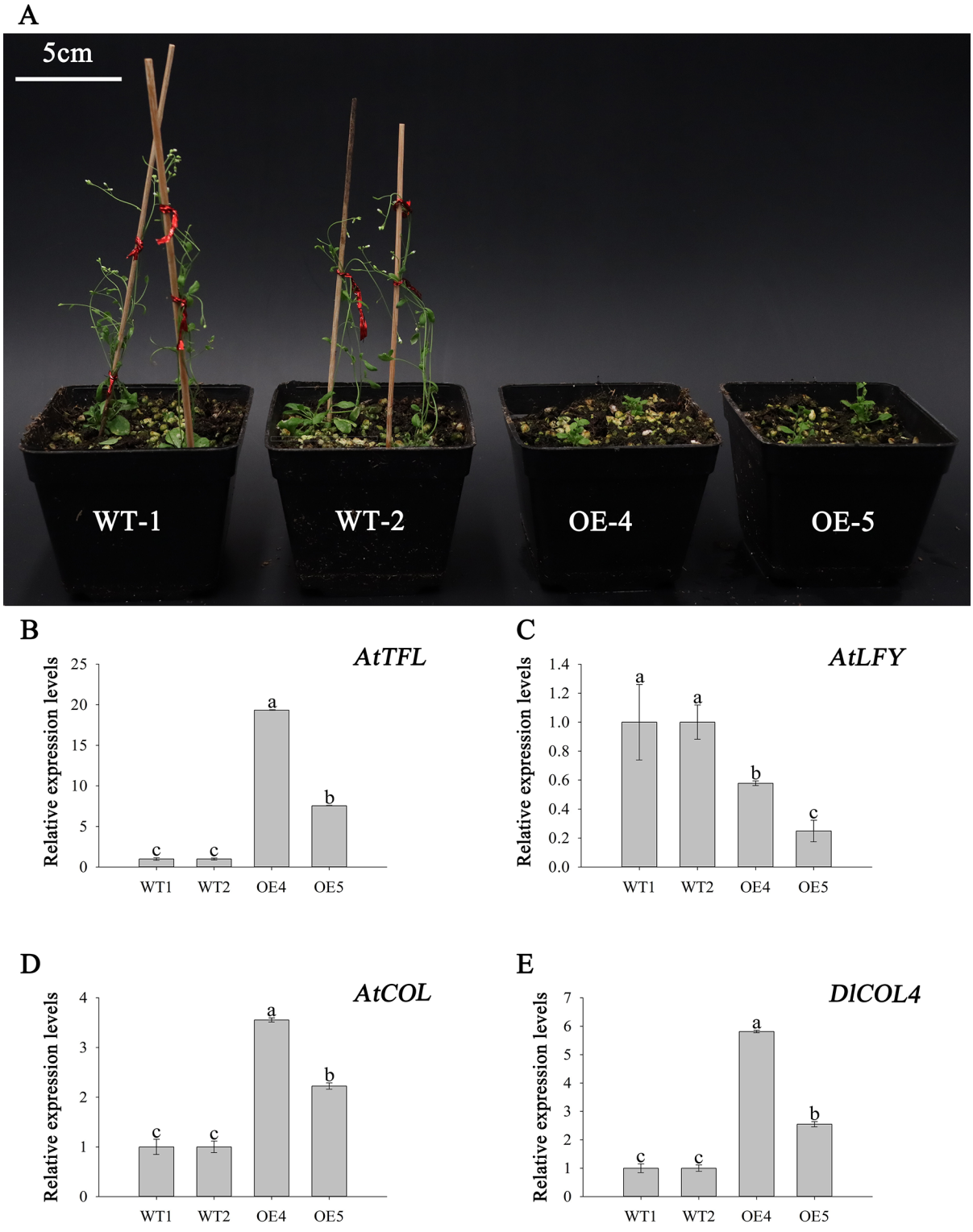

Fig. 8 *DlCOL4* transgene overexpression analysis. (**A**) Comparison of the flowering time between WT and transgenic lines. (**B**-**E**) The expression levels of *AtTFL*, *AtLFY*, *AtCOL* and *DlCOL4* genes in transgenic and WT lines were detected by qRT-PCR

and 11 (OE5) rosette leaves, significantly lower than WT (Table S5). To further analyze the flowering regulatory mechanism of *DlCOL4* in plants, we performed qRT-PCR on flowering-related genes (Fig. 8B and E). The results showed that, compared with wild-type plants, the expression levels of *AtTFL* and *AtCOL* genes in the OE4 and OE5 lines were significantly increased, whereas the expression levels of *AtLFY* flowering-promoting genes were not significantly different. These results suggest that *DlCOL4* inhibits flowering by interacting with *AtTFL* and *AtCOL*.

Discussion

Flowering is an important phenological mechanism in the transition of plants from vegetative to reproductive growth [37]. Longan is a fruit with high nutritional and economic value and is mainly distributed in subtropical areas. Its economic value is greatly affected by its relationship with flowering time [36]. Therefore, it is important to effectively regulate flowering time during longan production. *COL* gene family members can affect flowering time by regulating the plant response to photoperiod, which is a key factor in controlling plant growth and development [21]. In this study, 10 *COL* gene family members were identified in the longan genome using bioinformatic methods. According to the phylogenetic tree results (Fig. 2), these members can be divided into three subfamilies, and genes in the same subfamily have similar domains, similar to the grouping of *Arabidopsis* [2] and tomato [17]. According to previous reports, the number of *COL* genes identified in *Fragaria vesca* (10 *COL* genes; genome size 230 Mb) [38], *Setaria italica(*11; 490 Mb) [39], cannabis (13; 808 Mb) [40], and *Capsicum annuum(*10; 2700 Mb) [41] were similar to those of longan *COL* genes (10; 455.5 Mb) [42]. The differences in the gene numbers and genome sizes of the five plants indicated that the number of *COL* gene families was relatively stable and did not change with genome size.

The *COL* gene is a universal gene family in the plant kingdom that controls flowering time. *CONSTANS-like* gene family members all contain B-box and CCT domains. Phylogenetic analysis of *DlCOL* (Fig. 2) and sequence structure analysis of *DlCOL* (Fig. 3B) showed that four *DlCOL* genes were found in longan subfamilies I and II, and two *DlCOL* genes were found in subfamily III. Members of the *DlCOL* gene in subfamilies I and II contain two B-box domains, whereas subfamily III contains only one. When compared with *Arabidopsis*, the *DlCOL* gene structure of longan subfamilies I and II is relatively conserved, whereas the structural differentiation of *DlCOL* gene in subfamily III is relatively large [2]. The results showed that expansion of the *COL* family mainly occurred in subfamily III.

The structural framework of proteins plays an important role in predicting protein function, and gene function and classification can be performed by analyzing gene structure and conserved motifs [43]. Analysis of gene structure has shown that most *DlCOL* within the same subfamily have the same exon-intron structure, and their location information in the genome provides important evidence for their evolutionary relationships [44, 45]. Analysis of conserved motifs revealed that almost every member contained both Motif1 and motif2, and that genes with the same motif arrangement clustered in the same subgroup, consistent with their evolutionary classification. Therefore, this unique set of genetic architectures plays an important role in the regulation of protein function. Sequence structure analysis of *DlCOL* members revealed that *DlCOL5* and *DlCOL10* have highly similar motif1 and motif2 and conserved domains, B-box1 and CCT. In the phylogenetic tree of *DlCOL*, two genes, *DlCOL5* and *DlCOL10*, are located in a common evolutionary clade (Group II). In *Arabidopsis thaliana*, *AtCOL6*, *AtCOL7*, *AtCOL8*, and *AtCOL16* are also in Group II. *AtCOL7* and *AtCOL8* play the role of transcriptional suppressors in the regulation of flowering, and the overexpression of *AtCOL7* and *AtCOL8* will cause the flowering delay of transgenic *Arabidopsis thaliana* [21, 40, 46]. Therefore, the *DlCOL* gene may be involved in the regulation of flowering in longans.

Plant flowering regulation is determined by various cis-acting response elements and environmental factors, and hormone response elements play an important regulatory role in flowering changes [47, 48]. For example, gibberellin can inhibit apple flowering by inhibiting CK response and signaling [49]. Auxin-related genes and signaling pathways play key roles in flower development. For example, the growth hormone regulator ARF2 mutant in *Arabidopsis* shows delayed flowering times [50]. According to this analysis, cis-acting elements in the *DlCOL* promoter are closely related to stress, hormones, growth, and development. Photoresponsive and hormone-related cis-acting elements were particularly abundant in the promoter family. The results showed that *DlCOL* family genes are involved in regulating the flowering and development processes of longans and have important functional roles.

Studies have shown that *CO/COL* gene is widely expressed in different plant organs, and its expression level in leaves is relatively high. For example, in mangoes, *MiCOL16A* and *MiCOL16B* are highly expressed in leaves [51]. In rice, *Ghd2*, a *COL* gene, is mainly expressed in the leaves and is involved in the regulation of leaf senescence and drought resistance [52]. In this study, we examined the transcript levels of 10 *DlCOL* genes in flowers, stems, leaves, roots, and other organs. It was found that *DlCOL* gene was significantly expressed

in leaves. Analysis of the expression patterns at different flowering stages revealed that The *COL* gene expression patterns of the two longan species were distributed at different flowering stages. For example, most *COL* gene expression patterns in 'SX' are distributed in the late flowering stage (SXT2-SXT3). The *COL* gene expression pattern in 'SJ' tended to be distributed during the early flowering stage (SJT1-SJT2). However, the expression level of *DlCOL4* gene in the "SJT1-SJT2" stage of 'SJ' was up-regulated 3 times, while there was no expression in the three stages of 'SX'flower induction, indicating that *DlCOL4* gene may be involved in flowering induction.

CO/COL is an important gene in plant photoperiodic pathways that regulates plant flowering under different light conditions [53]. For example, under LD conditions, overexpression of *AtCOL9* and *AtCOL4* genes delayed flowering in transgenic *Arabidopsis* plants [30, 54]. In rice, the *OsCOL9* gene inhibits flowering of transgenic rice under SD conditions [55]. Consistent with these studies, in the present study, over-expressed *DlCOL4* gene, belonged to the same subfamily as *AtCOL9* and *OsCOL9*, in *Arabidopsis*, the transgenic lines also showed a delayed flowering phenotype. As a transcription factor, *CO* regulates the flowering time of plants, mainly by acting on downstream functional genes related to flower formation. For example, *CO* can induce *FT* expression in *Arabidopsis thaliana* to control flowering [56, 57]. It has been found that *CO* can up-regulate the expression of *FT* in *Arabidopsis thaliana* under LD conditions to control the flowering time of *Arabidopsis thaliana* [58]. *AtCOL8* inhibits *FT* expression and flowering under LD conditions [59]. In mango, *MiCOL16A* and *MiCOL16B* genes can inhibit mango flowering by reducing the expression of *AtSOC1* and *AtFT* under SD and LD conditions [37] in orchids with *CsCOL8* gene under LD conditions, the expression levels of *AtCO* and *AtFT* in *CsCOL8* transgenic *Arabidopsis thaliana* significantly increased, delaying the flowering of *Arabidopsis thaliana* [60]. In this study, overexpression of *DlCOL4* under LD conditions may inhibit flowering by regulating the expression of *AtTFL* and *AtCOL*; however, the specific regulatory mechanisms require further study.

Conclusions

In this study, we have identified 10 *DlCOL* genes, all of which were highly conserved according to bioinformatics analysis. Eleven pairs of genes directly homologous to the *DlCOL* gene and *Arabidopsis COL* gene were identified, indicating that the longan and *Arabidopsis COL* genes were highly correlated. According to the analysis of expression patterns, most members of *DlCOL* gene family are mainly expressed in leaves and during the flower bud stage. *DlCOL4* is transcriptionally active in the nucleus, and the tissue-specific features of *DlCOL* indicate the function and differentiation of *DlCOL* genes in different tissues. The promoter of *DlCOL* gene contains many light-response elements, plant hormone-response elements, and various stress-response elements. Functional analysis of *DlCOL4* showed that *DlCOL4* may inhibit flowering by interacting with *AtTFL* and *AtCOL*. The results of this study can better analyze the function of *DlCOL* gene, further reveal the regulation of *DlCOL4* gene in the flowering mechanism of longans, and provide a theoretical basis for the genetic breeding of longans.

Abbreviations

COL	CONSTANS-like
LD	Long day
SD	Short day
FT	FLOWERING LOCUS T
TSF	TWIN SISTER OF FT
BBX	B-box protein
COP1	Constitutive Photomorphogenic 1
HMM	Hidden Markov model
qRT-PCR	Real-time reverse transcription PCR
WT	Wild-type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06451-8.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements

Not applicable.

Author contributions

JG and XS and performed the experiments, conducted the formal analysis, and wrote the manuscript. DJ and SS designed the experiments, acquired funding, revised the manuscript. JC, YL, CR and ZZ analyzed the data. LL revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the Natural Science Foundation of China (33272674), the Science and Technology Research Program of the Chongqing Education Commission (KJQN202401302, KJZD-K202201301 and KJQN202301301), the Guangxi Science and Technology Plan Project (Guinongke AB241484020), the Project of National Key Laboratory for Tropical Crop Breeding (No. NKLTCBCXTD11), the Guangdong province Litchi and Longan Industry Technology System (2024CXTD19), the Scientific Research Projects of Chongqing University of Arts and Sciences (R2020FYL01).

Data availability

The "SJ" and "SX" data were uploaded to the NCBI database (http://www.ncbi.nlm.nih.gov/sra) with accession number SRS2241241–SRS2241258.

Declarations

Ethics approval and consent to participate

No specific permits were needed, and material collection and molecular experiments were carried out following current Chinese regulations.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 January 2025 / Accepted: 24 March 2025
Published online: 02 April 2025

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