

# Cloning and Expression Analysis of the Soybean *CO*-Like Gene *GmCOL9*

Guowen Huang · Jinghua Ma · Yuzhen Han ·  
Xinjian Chen · Yong-Fu Fu

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**Abstract** Photoperiod is one of the important environmental triggers of flowering, and the *CONSTANS (CO)* gene is a key regulator of this pathway in the model plant *Arabidopsis thaliana*. *CO*-like genes are present in many plant species. We describe here the isolation of the *CO* homolog *Glycine max CONSTANS-like 9 (GmCOL9)* from the soybean cultivar Kennong18. Sequence comparisons showed that the closest *A. thaliana* gene to *GmCOL9* is *COL13*. The expression of *GmCOL9* was investigated using quantitative real-time reverse transcriptase polymerase chain reaction, which showed that *GmCOL9* was not regulated by a circadian mechanism, since its expression oscillated at a cycle of 12 h instead of 24 h. *GmCOL9* expression was influenced by the developmental stage of the plant and varied between tissue/organs. The expression of *GmCOL9* was concentrated in the unifoliolate leaves during the vegetative phase of growth and in the stem during the reproductive phase. However, traces of expression were also present throughout the plant, indicating that

*GmCOL9* may be a multifunctional gene in the context of the development of the soybean plant.

**Keywords** *CONSTANS-Like* · Soybean · Gene expression

## Introduction

The successful completion of a plant's life cycle depends heavily on the environmental conditions it experiences. Of the many distinct components of the growing environment, photoperiod is especially important, as it represents the trigger for the switch from vegetative to reproductive growth in many plant species. Plants that respond to a lengthening day length are referred to as long-day plants and those to a shortening one as short-day ones. The sensing of photoperiod and the subsequent molecular events which respond to this stimulus forms the so-called photoperiod pathway. A key component in this pathway is the gene *CONSTANS (CO)*; Putterill et al. 1995; Mouradov et al. 2002), which has orthologs in a wide range of plant species (Yano et al. 2000; Liu et al. 2001; Griffiths et al. 2003; Miller et al. 2008; Holefors et al. 2009; Serrano et al. 2009). *CO* is a transcription factor containing two B boxes and one CCT (CO, CO-like, TOC1) domain (Robson et al. 2001). The *CO* gene family has been classified into three recognizable groups on the basis of B box content. Group I members have both a B1 and a B2 box, group II members a B1 and a variant B2 box, while group III members possess only one B box; all, however, retain the CCT domain (Robson et al. 2001; Griffiths et al. 2003). The expression profiles of some *CO* orthologs are also well conserved, and these *CO* orthologs can complement the *Arabidopsis thaliana co* mutant. For example, when the *Pharbitis nil* (short-day species) gene *PnCO* was over-expressed in the background

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Guowen Huang and Jinghua Ma contributed equally to the work.

G. Huang · Y. Han  
College of Biological Sciences, China Agricultural University,  
Beijing 100094, China

J. Ma · X. Chen  
College of Life Sciences, Henan Agricultural University,  
Zhengzhou 450002, China

Y.-F. Fu (✉)  
Institute of Crop Sciences, National Key Facility of Crop Gene  
Resource and Genetic Improvement,  
Chinese Academy of Agricultural Sciences,  
12 Zhongguancun Nandajie, Haidian District,  
Beijing 100081, China  
e-mail: fuyf@caas.net.cn

of the *A. thaliana co* mutant, flowering was accelerated independently of photoperiod (Liu et al. 2001; Hayama et al. 2007); similarly, *BvCOL1* (isolated from the long-day species *Beta vulgaris*) complements the late flowering phenotype of the *A. thaliana co-2* mutant under various photoperiodic conditions (Chia et al. 2008). Thus, *CO* function seems to be well conserved across the plant kingdom.

However, *CO* orthologs have different functions in different species, and *CO*-like genes have differently functions compared with *CO* flowering activity. Whereas *AtCOL3* is active during root development (Datta et al. 2006), *AtCOL9* is involved in the regulation of flowering time via the suppression of *CO* (Cheng and Wang 2005). Under non-inductive long-day conditions, *Hdl1* (a rice ortholog of *CO*) delays flowering by inhibiting the action of *Hd3a* but accelerates it under inductive short-day conditions (Hayama et al. 2003). The *CO/FT* regulatory module in *Populus trichocarpa* controls both flowering and growth cessation and bud set in the fall (Bohlenius et al. 2006). The over-expression of *CO* impairs tuber formation in transgenic potato grown under short-day conditions (Martinez-Garcia et al. 2002). Thus, the *CO* gene family clearly has a wide-ranging influence over plant development.

Here, we report the isolation of a *CO*-like gene from soybean (*Glycine max* L.), named *GmCOL9* (*CONSTANS-like 9*), and present an analysis of its structure and expression profiles in circadian, developmental, and tissue-/organ-specific patterns. Our results suggested that *GmCOL9* may be involved in several aspects of the development of the soybean plant.

## Materials and Methods

### Plant Materials

The soybean cultivar Kennong18 (*G. max* L. KN18) was employed for all experiments. Plants were grown in a growth room under either short-day conditions (8:16 h light/dark) or long-day conditions (16:8 h light/dark) at 28°C under a light fluency of 100–150  $\mu\text{mol m}^{-2} \text{S}^{-1}$ . Seedlings were harvested before the expansion of the unifoliolate leaves. Various tissues/organs including roots, hypocotyls, epicotyls, cotyledons, unifoliolates, shoot apex (including the apical meristem and immature leaves), stems, leaves on lateral branches, and the whole aerial organs of plants were individually sampled when the unifoliolate leaves, the first trifoliolates, the second trifoliolates, the third trifoliolates, or the fourth trifoliolates had become fully expanded or when plants flowering. Seeds and pods without seeds were sampled at 7, 14, and 21 days after flowering, as well as at maturity. To determine the influence on gene expression of photoperiod, fully expanded unifoliolate leaves were sampled at ZTL0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22,

24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, and 96, which spanned two successive 24-h light/dark cycles followed by two successive 24-h periods of continuous light or darkness. All samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required.

### RNA Isolation and cDNA Synthesis

Total RNA was extracted by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA from the stems was isolated by the cetyltrimethylammonium bromide method (Suzuki et al. 2008). All RNA preparations taken forward had an  $A_{260}/A_{280}$  ratio of 1.8–2.0 and an  $A_{260}/A_{230}$  ratio of  $>2.0$ . The integrity of the RNA was checked electrophoretically. Prior to cDNA synthesis, the RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), and first-strand cDNA synthesis was carried out using 4  $\mu\text{g}$  RNA with the help of a RevertAid first-strand cDNA synthesis kit (Fermentas, St. Leon-Roth, Germany) provided with oligo-dT primers.

### Gene Isolation and In Silico Analysis

The Glyma19g05170 sequence, which included both the untranslated and the coding (cds) regions, was first cloned using reverse transcriptase polymerase chain reaction (RT-PCR), based on the primers GmCOL9F-F and GmCOL9F-R (Table 1). Three independent fragments were sequenced (SunBio, China), and the resulting consensus sequence was used as the template to amplify the cds using the primer pair GmCOL9C-F and GmCOL9C-R (Table 1). This

**Table 1** Primer sequences used in qPCR and PCR gene cloning studies

Name of primers	Sequence (5' to 3')
GmCOL9F-F	ACACCCAAAACAAAAGA
GmCOL9F-R	GATCCTAGACCCAGTAAA
GmCOL9C-F	ATGAGTGGTGCTGAAGCG
GmCOL9C-R	TCAATGTTCATGTCCCATCTT
qGmCOL9-F	GTCTCCGAACCTTGTCCATC
qGmCOL9-R	CACACGAACATATCCGAAAGC
GmACTIN11-F	ATCTTGACTGAGCGTGTTATTCC
GmACTIN11-R	GCTGGTCCTGGCTGTCTCC
GmTUA5-F	TGCCACCATCAAGACTAAGAGG
GmTUA5-R	ACCACCAGGAACAACAGAAGG
GmSKIP16-F	GAGCCCAAGACATTGCGAGAG
GmSKIP16-R	CGGAAGCGGAAGAACTGAACC
GmUKN1-F	TGGTGCTGCCGCTATTTACTG
GmUKN1-R	GGTGAAGGAAGTCTAACAATC

amplicon was inserted into the pGWC vector (Chen et al. 2006), resulting in the construct pGWC-GmCOL9, which was verified by sequencing. The alignment was performed using Molecular Evolutionary Genetics Analysis (MEGA) v4.0. Phylogenetic analyses were conducted using v4 of MEGA software (Tamura et al. 2007). To generate a phylogenetic tree, predicted full-length proteins were aligned by ClustalW software using default parameters. A neighbor-joining method was used to align and construct phylogenetic trees. Bootstraps with 1,000 replicates were performed to provide probabilistic support to tree nodes.

#### Quantitative Real-Time RT-PCR

Expression profiles were evaluated by quantitative real-time RT-PCR, carried out on an ABI StepOne Detection System (Applied Biosystems, USA), based on SYBR Premix Ex Taq polymerase (TaKaRa, Tokyo, Japan). Each 15- $\mu$ l reaction consisted 4  $\mu$ l template, 7.5  $\mu$ l 2 $\times$  SYBR Premix, 200 nM of each primer (qGmCOL9-F and qGmCOL9-R, see Table 1), and 0.3  $\mu$ l ROX. Two independent reference genes were included to improve the level of reliability (Hu et al. 2009)—for the developmental samples, these were *SKIP16* and *UKN1*; for tissues/organs, these were *ACT11* and *UKN1*; and for photoperiod comparisons, these were *ACT11* and *TUA5*. The relevant primer sequences are listed in Table 1. The data were analyzed by StepOne software v2.0. Each experiment was repeated at least three times.

#### Sub-cellular Localization of GmCOL9 Proteins

The coding sequence of *GmCOL9* was introduced in pGWC (Chen et al. 2006) and subsequently cloned into destination vector pENSG-YFP (provided by Dr. Jane Parker, Max Planck Institute, Cologne, Germany) by LR Gateway recombination (Invitrogen). The resulting construct pENSG-YFP-GmCOL9, which includes the CaMV 35S promoter, was biolistically co-transformed into onion epidermis cells with the vector pENSG-CFP-AHL22, which served as a maker of nuclear proteins (Xiao et al. 2009). Transformation was achieved with the PDS 1000/He device (Bio-Rad), utilizing the following parameters: target distance 6 cm, vacuum 25" Hg, and rupture disk pressure 1,100 psi. Yellow fluorescent protein (YFP) was monitored by confocal microscopy (LEICA TCS SP2, Germany).

## Results

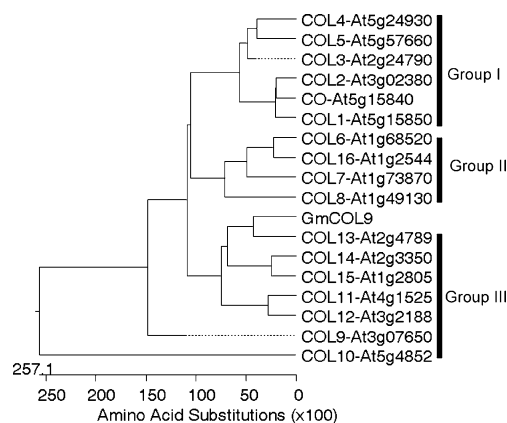
#### Cloning the Gene of *GmCOL9*

A search of the soybean genome sequence (<http://www.phytozome.net/>), based on the CO peptide sequence,

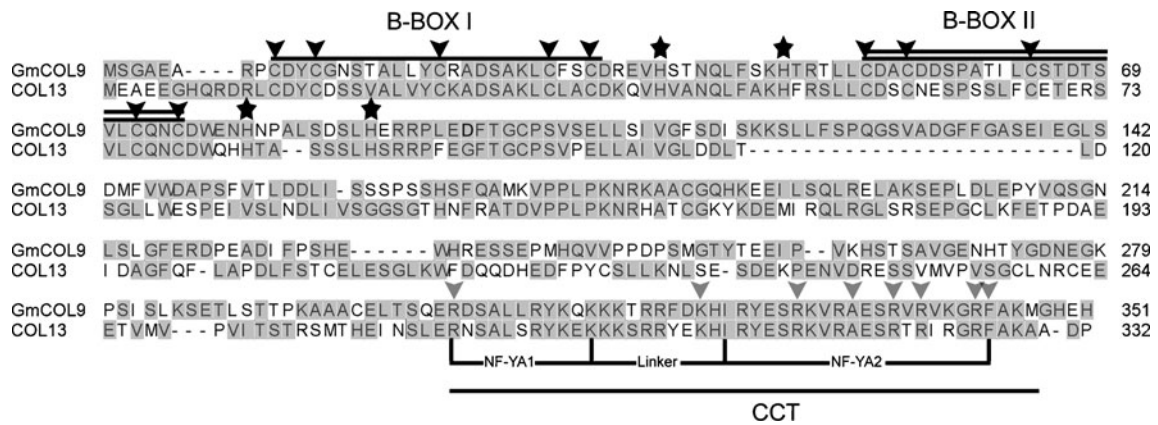
produced 23 hits. The sequence of the locus Glyma19g05170 was related more closely to *AtCOL13* than to any other *AtCO* family member (Fig. 1). The Glyma19g05170 locus was re-sequenced from cv. Kennong18 to produce full-length cds of 1,056 bp, which translated into a 352 residue polypeptide. Its sequence differed from that of Glyma19g05170 with respect to three base positions: G<sup>287</sup> to A, A<sup>882</sup> to G, and T<sup>921</sup> to C, but only the first of these variants resulted in an amino acid change (glycine to aspartic acid; such an amino acid change did not fall in B boxes or CCT domain). Such single-nucleotide polymorphisms may result from the variation of different cultivars (KN18 in this study and Williams 82 in Phytozome). As do all *CO* group III members (Robson et al. 2001), the GmCOL9 protein contained two B boxes and a CCT domain (Fig. 2). The GmCOL9 B box sequences included both the conserved CX<sub>2</sub>CX<sub>8</sub>CX<sub>7</sub>CX<sub>2</sub>C motif, the critical C and H residues, and the consensus spacing defining B box function (Robson et al. 2001; Griffiths et al. 2003). The C-terminus of the GmCOL9 protein contained a conserved CCT domain NF-YA1/linker/NF-YA2 structure, and all the key residues were present (Fig. 2). A 24 residue stretch followed the B2 box, but this lacked any functional motif except for an SVAD phosphorylation site.

#### Circadian Expression Pattern

The expression profile of *GmCOL9* in response to variation in the photoperiod was obtained from measurements of mRNA abundance in unifoliolates over the course of two successive 24-h cycles of either long or short days, followed by 48 h of continuous light or darkness. Under the short-day conditions, expression followed an oscillation period of 12 h, peaking first at dawn (ZTL0) and then again 4 h after the beginning of the dark period (ZTL12; Fig. 3a, b). Such a



**Fig. 1** A sequence-based phylogeny of *GmCOL9* and the *A. thaliana* *CO* family. *GmCOL9* is related to the *A. thaliana* *CO* family group II, and its most closely related member is *AtCOL13*. The classification of *CO* family in *Arabidopsis* is according to Robson et al. 2001



**Fig. 2** Conserved domains alignment of GmCOL9 and COL13 from *Arabidopsis*. Shown are the conserved cysteine (black arrowheads) and histidine (black stars) residues, along with the consensus spacing (Xn) defining the two B box domains (B box1 shown by a single line,

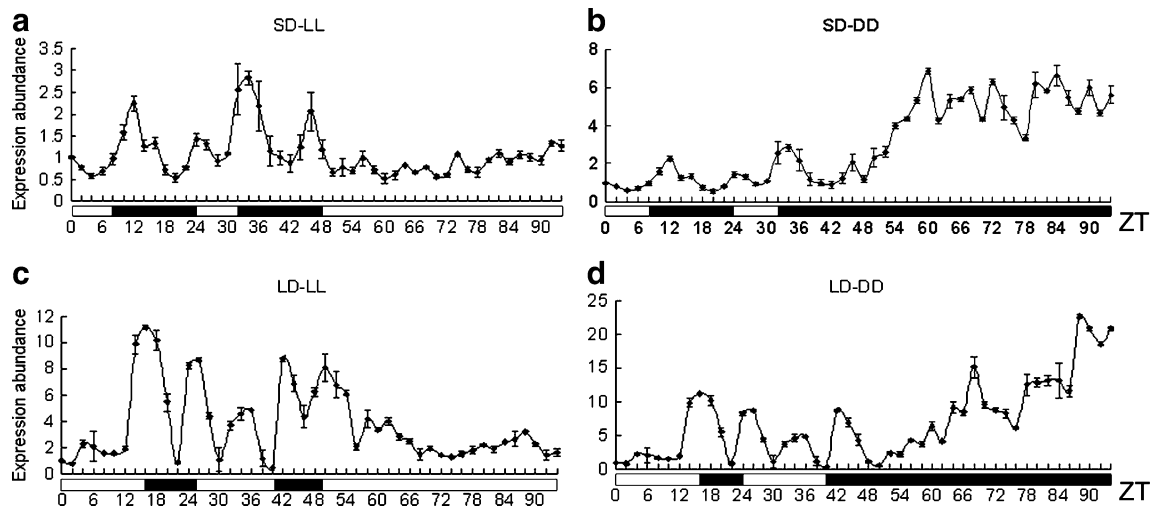
B box2 by a double line). The CCT sub-domains NF-YA1 and NF-YA2 and their linker, as well as the key residues within it, are marked by gray arrowheads

period was damped in subsequent continuous light (LL) but increased the amplitude in subsequent continuous dark (DD). And GmCOL9 expression in LL (Fig. 3a) and DD (Fig. 3b) was random. The expression pattern of *GmCOL9* in long-day conditions was similar to that in short-day conditions, except that the peaks appeared 4 h later. And this pattern was also not maintained once the plants were transferred into continuous light or continuous darkness. In fact, mRNA abundance was generally greater in the dark (Fig. 3d) than in the light (Fig. 3c). The results suggested that *GmCOL9* did not show a circadian expression pattern.

**Developmental Expression Pattern**

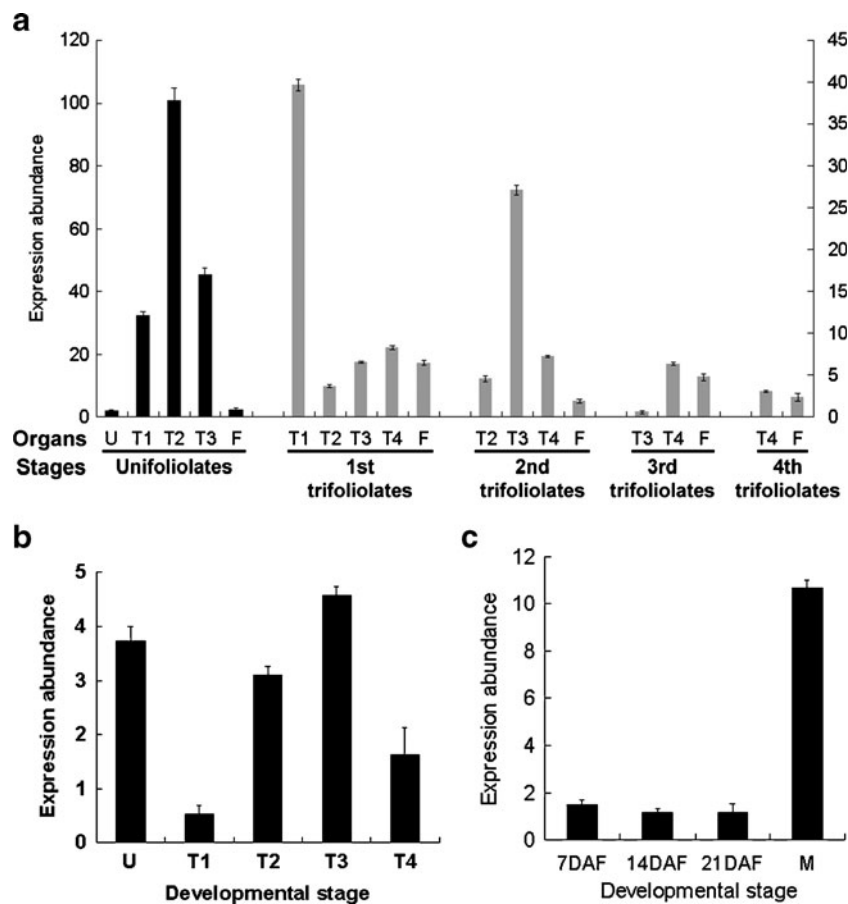
The effect of developmental stage on *GmCOL9* expression was followed from comparisons between leaves harvested

from plants at several range of developmental stages (Fig. 4). *GmCOL9* transcript accumulated at higher levels the younger the plants (Fig. 4a, b). Comparing the expression levels in leaves at distinct developmental stages, higher level was observed in the unifoliolates, while the fourth trifoliolates displayed lowest levels of *GmCOL9* mRNA. At the stage when the first trifoliolates were fully opened, *GmCOL9* was mainly expressed in the unifoliolates and the first trifoliolates. Once the second trifoliolates were fully opened, most of the transcript was present in the unifoliolates, and by the time that the third trifoliolates were fully opened, most of the transcript was present in the unifoliolates and the second trifoliolates. In the developing seed, *GmCOL9* expression was at a low level, but the level of expression rose as the seeds approached maturity (Fig. 4c).



**Fig. 3** The expression of *GmCOL9* in response to either short days (SD; 8:16 h light/darkness. a and b) or long days (LD; 18:6 h light/darkness. c and d) for 48 h, followed by 48 h of either continuous light (LL. a and c) or continuous darkness (DD. b and d). Error bars denote SD

**Fig. 4** The expression profile of *GmCOL9* in leaves (a), aerial parts of the plant (b), and seeds (c). a The left-hand Y-axis shows unifoliolates (black column), and the right-hand Y-axis various trifoliolates (gray columns). U and T1–T4 indicated different leaves (organs) or the developmental stages when leaves opened fully (stages). U fully opened unifoliolates, T1 fully opened first trifoliolates, T2 fully opened second trifoliolates, T3 fully opened third trifoliolates, T4 fully opened fourth trifoliolates, F flowering. 7DAF, 14DAF, and 21DAF indicate, respectively, 7, 14, and 21 days after flowering. M seed maturation. Error bars denote SD



### Tissue/Organ-Specific Expression Pattern

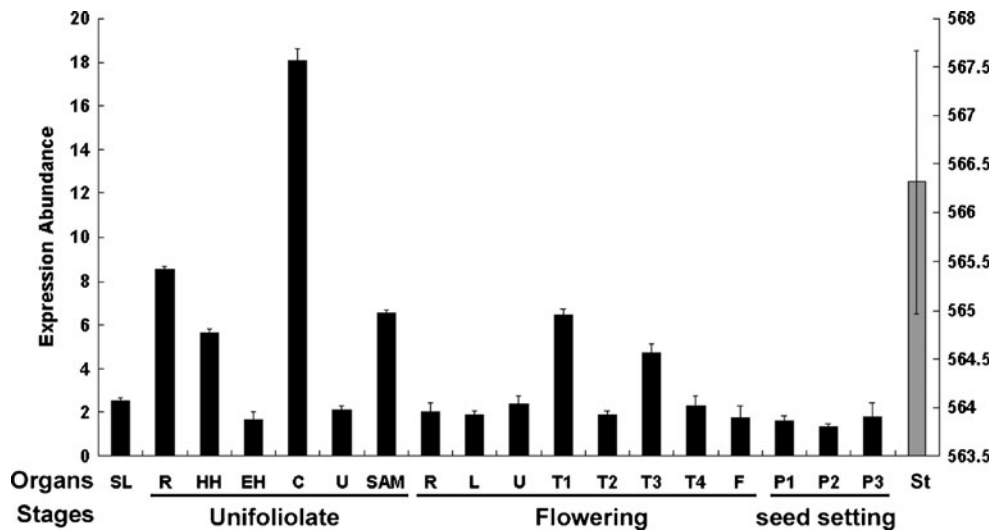
Although *GmCOL9* transcript was detectable throughout the plant, the level of expression varied among tissues/organs (Fig. 5). The level in the stem was up to 100-fold that in other parts of the plant. Transcript abundance was also substantial in the unifoliolates. The expression of *GmCOL9* was detected in roots although in lower levels than that in leaves and cotyledons. Lateral leaves, flower buds, pods excluding the seeds, shoot meristems, epicotyls, and hypocotyls also had different level of *GmCOL9*.

### Sub-cellular Localization of GmCOL9 Protein

To study the sub-cellular localization of GmCOL9 protein, an expression vector containing yellow fluorescence protein-*GmCOL9* (*YFP-GmCOL9*) fusion gene driven by 35S promoter was constructed and introduced into onion epidermis cells by biolistic transformation. As shown in Fig. 6, YFP-GmCOL9 was co-localized with the nuclear protein AHL22 (Xiao et al. 2009) in nuclei, not in the cytoplasm.

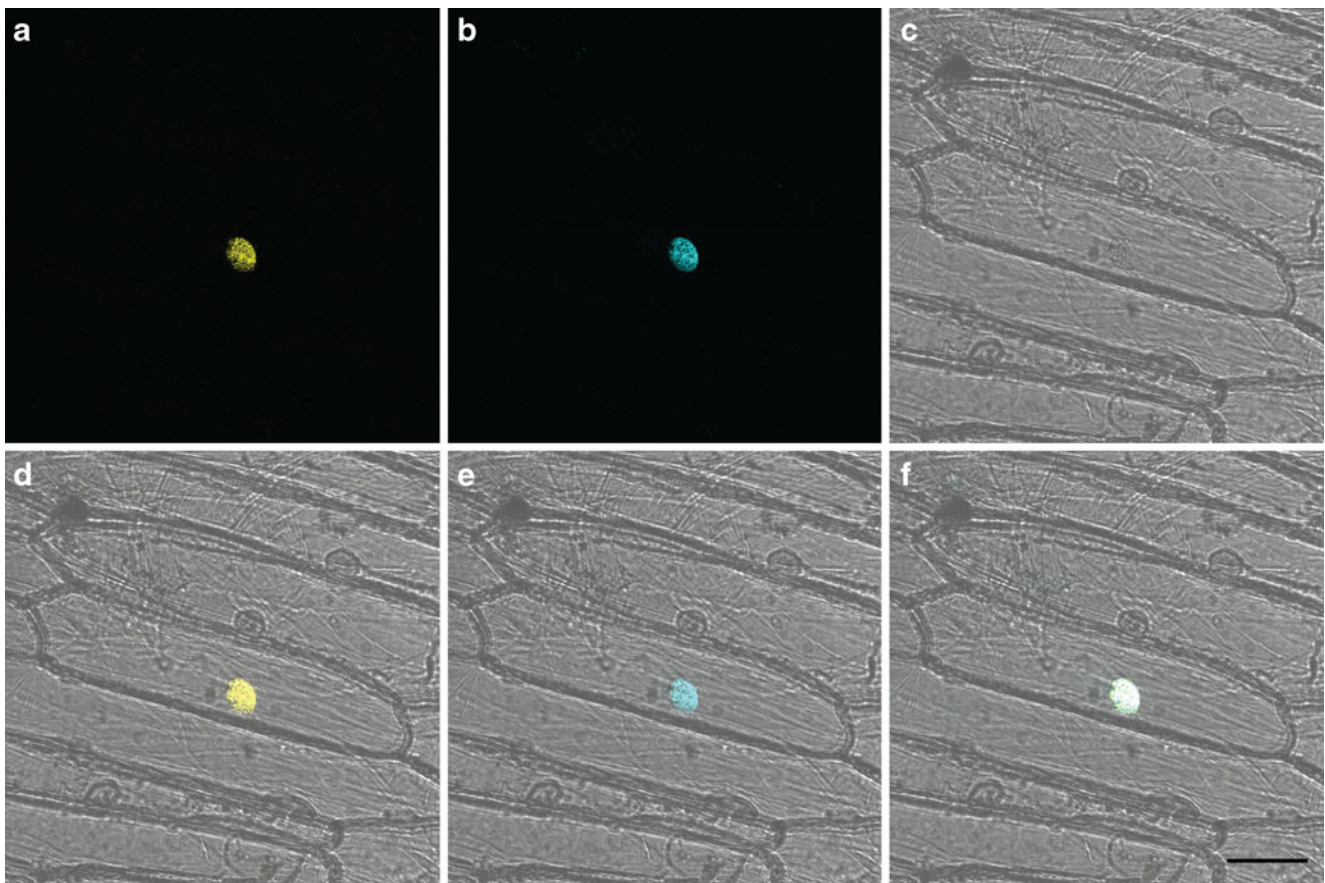
### Discussion

*CO* is a key gene in photoperiod pathway of flowering regulation, and its orthologs were found in various plants (review by Khanna et al. 2009) and green alga (Serrano et al. 2009). These orthologs exhibit high sequence and functional conservation. But the members in *CO* family in various plants may have different variations in sequences and functions. In *Arabidopsis*, there are 17 members in *CO* family, which can be further classed into three subgroups. Group I members contain two B boxes, group II has only one B box, and group III members have a conserved B box and a variant of B box. The proteins in all of three groups contain a CCT (CO, CO-like, TOC) domain at C-terminus (Robson et al. 2001). Based on the characters of all domains, *GmCOL9* belongs to group III and is the closest to *COL13* in *Arabidopsis*, indicating that they may have similar functions. The NF-YA1 sub-domain forms a helix proposed to interact with the HAP3/HAP5 dimer, whereas NF-YA2 is interacted with the DNA of the CCAAT box (Romier et al. 2003; Wenkel et al. 2006). *GmCOL9* shares key residues with *AtCOL13* in this sub-domain, as well as having a similar B box content. Transient expression



**Fig. 5** Tissue/organ expression profiles. *SL* seedling, *R* root, *HH* hypocotyl, *EH* epicotyl, *C* cotyledon, *U* unifoliolate leaf, *SAM* shoot apex (including the apical meristem and immature leaves), *St* stem, *L* lateral leaf, *T1* to *T4* first to fourth trifoliolates, *F* flower buds, *P1* to *P3* pods (excluding seed) at 7, 14, and 21 days after flowering. Stages

defined as follows: *unifoliolates* unifoliolates fully opened, *flowering* onset of flowering, *seed set* initiation of seed growth. The right-hand Y-axis refers only to *St* at flowering (gray column), while the left-hand Y-axis applies to the other samples (black columns). Error bars denote SD



**Fig. 6** GmCOL9 protein co-localized with a nuclear protein AHL22 in nucleus of onion cells. *YFP* was fused to the N-terminus of *GmCOL9*, and *CFP* was also at the N-terminus of AHL22. Both the gene fusions were driven by a constitutive promoter. Two constructs

were co-bombarded into onion epidermis cells and fluorescence visualized by confocal microscopy. **a** Yellow fluorescent signal, **b** cyan fluorescent signal, **c** bright field, **d** merged of **a** and **c**, **e** merged of **b** and **c**, **f** merged of **d** and **e**. Bar, 50  $\mu$ m

analyses in onion epidermal cells have demonstrated the nuclear localization of GmCOL9 protein, in agreement with its role as a transcriptional regulator.

*GmCOL9* is expressed in a non-circadian manner of 24 h, but an oscillation period of 12 h regardless of photoperiod conditions. However, the pattern was largely disrupted when plants were exposed to either continuous light or continuous darkness. And such a pattern was damped in subsequent continuous light conditions or the amplitude was increased in continuous dark. As Aschoff's rule says, in animals, light enhances activity of diurnal model and suppresses the activity of the nocturnal model (Carpenter and Grossberg 1984). Thus, different from *CO* in *Arabidopsis* which oscillates following a typical 24-h circadian pattern (Putterill et al. 1995), the regulation of *GmCOL9* expression appears to be regulated by a non-circadian mechanism, but the light/dark cycle could be an effector for *GmCOL9* expression. A nocturnal model may be involved in the control of *GmCOL9* expression.

*CO* function is largely dependent on its regulation on *FT* expression (An et al. 2004); thus, the CO protein accumulation in cells is more important than its transcript abundance (Valverde et al. 2004; Michaels 2009; Fornara et al. 2010). However, CO protein also shows a circadian pattern similar to its transcripts (Valverde et al. 2004). Therefore, *CO* mRNA change indicates its function as in *Arabidopsis* (Valverde et al. 2004) and other plants such as *P. nil* (Hayama et al. 2007). For lack of specific antibody, we tried to analyze the function of *GmCOL9* by its transcript profiles. As *GmCOL9* was constitutively expressed throughout the plant, it is possible that it participates in a range of functions concerned with plant development. It is of interest that transcript abundance varied substantially between different parts of the plant, with the majority of the message being concentrated in the unifoliolates and stems during vegetative and reproductive stages, respectively. It is possible that *GmCOL9* functions mainly in the unifoliolates during the vegetative phase of growth and shifts its activity to the stem when the plants enter their reproductive phase. Its expression in the hypocotyl, epicotyl, and shoot meristem indicates a possible role in morphogenesis. *GmCOL9* may affect root development due to its expression in both before and after flowering. Its function on seed maturation other than in seed developmental progress may be another important role for *GmCOL9*. In summary, *GmCOL9* may have its functions in multiple aspects.

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