Gene xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Identification of a *CONSTANS* homologous gene with distinct diurnal expression patterns in varied photoperiods in ramie (*Boehmeria nivea* L. Gaud)

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ARTICLE INFO

Article history:
Received 10 August 2014
Received in revised form 6 January 2015
Accepted 21 January 2015
Available online xxxx

Keywords:
CO/COL gene
Ramie
Phylogenetic analysis
Expression pattern
Photoperiod

ABSTRACT

Ramie is an important natural fiber and forage crop in China. Breeding of late- or non-flowering varieties, with higher vegetative yields, is an important goal in ramie improvement. However, the ramie genes involved in flowering regulation have not previously been identified. In model plants, such as rice and *Arabidopsis*, the *CONSTANS* (*CO*) and *CONSTANS-like* (*COL*) genes play key roles in flowering regulation. In the present study, six ramie *COL* genes (*BnCOL1*–*BnCOL6*) with a full-length open reading frame (ORF) were identified. Sequence alignment revealed that all six BnCOL proteins contained conserved CCT (*CO*, *COL*, *TOC1*) and B-box I domains, but that only four of these proteins contained the B-box II domain. Expression pattern analysis showed that *BnCOL1*–*BnCOL6* were mainly expressed in the stem xylem, flowers, and leaves. Phylogenetic analysis classified the six newly identified BnCOL proteins, and also 16 COL proteins with known functions in other species, into three groups. The *BnCOL2*-encoded protein was assigned to the same group as the *CO*- and *Hd1*-encoded proteins, suggesting that this *BnCOL2*-encoded protein showed the highest level of homology with the *CO*/*Hd1*-encoded proteins. Photoperiodic experiments showed that *BnCOL2* exhibited a diurnal expression pattern under long- and short-day conditions. Subcellular localization examination revealed that the BnCOL2 protein fused with YFP was localized in the nucleus. Because the homologous sequence and similar expression pattern between *BnCOL2* and *CO*/*Hd1*, the *BnCOL2* possibly has a role in flowering modulation, and can be used as a candidate gene for research in the flowering regulation of ramie.

1. Introduction

Ramie (*Boehmeria nivea* L. Gaud), popularly known as "China grass," is a perennial short-day (SD) plant belonging to the family Urticaceae. Ramie fibers extracted from the bast layer of the stem have a smooth texture, long strands, and excellent tensile strength and are important natural fibers. In China, ramie is the second most important fiber crop; its growth acreage and quantity of fiber production are second only to those of cotton. In addition, ramie has been used as a livestock forage crop because of its high level of crude protein (Squibb et al., 1954); moreover, the ramie variety Zhongsizhu 1 has been bred for animal feed (Xiong et al., 2005). The production of ramie for fiber and feed use requires a high vegetative yield. Thus, breeding of varieties with a longer period of vegetative growth is a key goal in ramie improvement.

Ramie has poor eco-adaptability and almost 90% of crop production in China occurs in the Yangtze Valley. Investigation of the relationship between prevailing environmental conditions in the ramie cultivation region and fiber yield has shown that high temperature and high rainfall are essential for high fiber yield (Liu et al., 2011). However, low fiber yields occur in some low-latitude region, even when the climate conditions are suitable for ramie growth. Nanning, which is the capital city of the Guangxi Zhuang Autonomous Region in southern China is a subtropical region with an average annual temperature of 22 °C and high annual rainfall; these optimal climate conditions allow ramie to be harvested four times per year. In the Yangtze Valley, ramie is harvested only three times per year. However, the total annual fiber yield per hectare in Nanning is approximately half of that obtained in the Yangtze Valley (Tang et al., 2010). Nanning is located at lower latitude than the Yangtze Valley and therefore has shorter days. Thus, flowering occurs twice a year (during April and July) in Nanning, but only once a year (during August) in the Yangtze Valley. As a consequence of the early flowering in Nanning, the period of vegetative growth (including stem elongation) is inadequate, and this is probably a major reason for the low yield. Thus, flowering trait has an important influence on the eco-adaptability of ramie. In order to improve the eco-adaptability and to

Abbreviations: *CO/COL*, *CONSTANS/CONSTANS-like*; ORF, Open reading frame; CCT, *CO*, *COL*, *TOC1*; SD/LD, Short day/long-day; *FT*, *FLOWERING LOCUS T*; TF, Transcription factor; qRT-PCR, Real-time quantitative PCR.

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http://dx.doi.org/10.1016/j.gene.2015.01.045
0378-1119/

breed late- or non-flowering varieties, the modulating mechanism of flowering trait must be elucidated. However, the genes involved in flowering regulation of ramie have not previously been identified, and this has severely hindered crop improvement programs.

In model plants, such as the short-day (SD) rice and the long-day (LD) *Arabidopsis*, the regulatory network for flowering has been extensively studied, and a large number of molecular components, including activators and repressors, have been identified (Imaizumi, 2010; Tsuji et al., 2011). These molecular components are involved in several regulatory pathways, such as the autonomous, gibberellin, photoperiod, and vernalization pathways. Each independent pathway works in response to different environmental and endogenous signals. *FLOWERING LOCUS T* (*FT*)/*heading date 3a* (*Hd3a*) is commonly regulated expression in all of these pathways, and it ultimately determines the transition to reproductive growth (Parcy, 2005; Tsuji et al., 2011). In the photoperiod pathway of *Arabidopsis*, the *CONSTANS* (*CO*) protein plays a central role by mediating the circadian clock and floral integrators, via positively regulating *FT* expression (Turck et al., 2008; Kovi et al., 2013). The *CO* protein is a zinc-finger transcription factor (TF) containing two conserved domains (i.e., a B-box zinc finger domain and a CCT (*CO*, *CO-like*, *TOC1*) domain), which are located in the region near the amino- and carboxy-terminus, respectively (Robson et al., 2001; Chou et al., 2013). In rice, the *CO* homologous gene, *heading date 1* (*Hd1*), induces the expression of *Hd3a* (the *FT* homologous gene in rice) and promotes flowering in SD plants (Yano et al., 2000; Hayama and Coupland, 2004). These findings indicate that the *CO*/*FT* mechanism is conserved between species that respond to different day lengths, and also between monocot and dicot lineages (Ballerini and Kramer, 2011).

The *CONSTANS-like* (*COL*) family, which is defined by the conserved B-box and CCT domains, has been identified in many species. Seventeen members, including *CO* genes, were reported in *Arabidopsis* (Robson et al., 2001), whereas 16 COL TFs and 9 COL TFs were identified in rice and barley, respectively (Griffiths et al., 2003). Some *COL* members that were identified from *Arabidopsis*, rice, barley, and ryegrass have been characterized by their functions; the results revealed that most *COL* TFs either promoted or delayed flowering under LD or SD conditions (Yano et al., 2000; Suarez-Lopez et al., 2001; Martin et al., 2004; Cheng and Wang, 2005; Kim et al., 2008; Xue et al., 2008; Hassidim et al., 2009; Lee et al., 2010; Takase et al., 2011; Campoli et al., 2012; Kikuchi et al., 2012; Wu et al., 2013). These findings suggest that many *COL* family members have a conserved function in modulating flowering via the photoperiodic response.

The *CO* gene and its homologous genes have been shown to play central roles in flowering regulation in model plants (Yano et al., 2000; Suarez-Lopez et al., 2001; Martin et al., 2004; Miller et al., 2008; Campoli et al., 2012), however, the ortholog of *CO* in ramie has not previously been identified. In this study, we identified six ramie *COL* genes with a full-length open reading frame (ORF), and phylogenetic analysis and expression pattern analysis of these six *BnCOL* genes were further performed. In addition, for the *BnCOL2*, whose encoding protein showed a high level of homology compared to the *CO* protein, the subcellular localization and photoperiodic response were further characterized.

2. Materials and methods

2.1. Assembly of BnCOL genes and ORF analysis

The transcriptome of ramie had previously been sequenced using the variety Zhongzhu 1, based on the Illumina paired-end sequencing technology (Liu et al., 2013a, 2014a). A total of 43,990 and 50,486 unigenes were de novo assembled, respectively, which constituted two pools of unigenes (designated as pool 1 and pool 2, respectively). The keywords "COL," "CCT," or "CONSTANS" were used as queries to search against the annotation of the unigenes. *COL* genes from the two pools were aligned based on nucleotide sequencing by using Clustal X (Thompson et al., 1997). Those genes that overlapped by more than 50 bp were further assembled. If two COL genes identified from two pools overlapped completely, the *COL* gene with the longer nucleotide sequence was reserved. In addition, *COL* genes that appeared in a single pool of unigenes were reserved. Finally, all the assembled and reserved *COL* genes were analyzed for their ORF, by using the findorf program (https://github.com/vsbuffalo/findorf). All the non-redundant putative COL protein sequences were manually checked for the B-box and CCT domain.

2.2. Phylogenetic analysis of COL protein

The 16 COL genes with known functions in other species are shown in Table 1. We conducted phylogenetic analysis of these 16 COL proteins, and also the BnCOL proteins identified in the present study. Multiple sequence alignments of the full-length protein sequences, including the highly conserved N-terminal B-box domain and the C-terminal CCT domain, were determined by using Clustal X (version 1.83) (Thompson et al., 1997). Unrooted phylogenetic trees were constructed by using MEGA 4.0 with the neighbor-joining (NJ) method. Bootstrap tests were conducted with 1000 replicates (Tamura et al., 2007).

2.3. Plant growth, photoperiodic treatment, and tissue sampling

Cuttings of Zhongzhu 1 were transplanted into pots in March 2013. In May 2013, when the plants were 60 days old, we sampled the stem bark, stem xylem, shoots, leaves, and roots of three plants. In September 2013, when the plants had flowered, we sampled the floral organs, including the female and male flowers, from three plants. The sampled tissues were immediately frozen in liquid nitrogen and stored at −80 °C until use. We separately stored three samples of each tissue as three biological replicates.

To determine the circadian regulation of the *BnCOL2* transcripts, the 60-day-old potted plants were transferred into a growth chamber (SPX-250-GB, Shanghai Yuejin, China) and were subjected to different light treatments (16-h dark/8-h light for SD and 8-h dark/16-h light for LD) at a temperature of 30 °C. After 7 days, the aboveground tissues, including the leaves and stem of each individual, were harvested as a single sample; these samples were collected at 4-h intervals, with three biological replicates for each sampling. All of the samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.4. RNA extraction and qRT-PCR analysis

The total RNA of each sample was extracted by using the EZNA Plant RNA Kit (OMEGA Bio-Tek, USA) according to the manufacturer's protocol. For each sample, first-strand cDNA was reverse-transcribed from RNA treated with DNase I (Fermentas, Canada), by using M-MuLV Reverse Transcriptase (Fermentas, Canada) according to the manufacturer's instructions. Reverse transcription quantitative polymerase chain reaction (qRT-PCR) was performed by using an optical 96-well plate with an iQ5 multicolor real time PCR system (Bio-RAD, USA). Each reaction contained 1 μL of cDNA template, 10 nM gene-specific primers, and 10 μL of iTaq™ Universal SYBR Green Supermix (Bio-RAD, USA), in a final volume of 20 μL. To analyze the expression in different tissues, we used three genes, namely, the cellulose synthase gene unigene21178, the 18s ribosomal RNA (18s) gene, and the *actin* gene, as candidate internal controls. To analyze the photoperiodic response, we used four genes, namely, the cellulose synthase gene unigene16487, the cellulose synthase gene unigene21994, the 18s gene, and the *actin* gene, as candidate internal controls. We used the program GeNorm to determine the M value for each gene in each sample and to identify the gene with the highest stability of expression; this gene was designated as an internal control (Vandesompele et al., 2002). We found that the unigene21178 had the smallest M value (0.202), and showed the highest stability of expression in different tissues; on the other hand, the *actin* gene had the smallest M value (0.237), and

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Table 1
The *CONSTANS-like* genes published in other species.

Gene published	Gene ID	Species	Group	Function	Reference
CO	AT5G15840	*Arabidopsis*	I	Accelerating flowering in response to long days	Suarez-Lopez et al. (2001)
COL1	AT5G15850	*Arabidopsis*	I	Accelerating the circadian clock	Ledger et al. (2001)
COL2	AT3G02380	*Arabidopsis*	I	No influence on flowering time in overexpression plant	Ledger et al. (2001)
COL3	AT2G24790	*Arabidopsis*	I	Promoting lateral root development; daylength-sensitive regulator of shoot branching	Datta et al. (2006)
COL5	AT5G57660	*Arabidopsis*	I	Inducing flowering under short days condition	Hassidim et al. (2009)
COL7	AT1G73870	*Arabidopsis*	III	Regulating branching and shade avoidance response	Wang et al. (2013)
COL8	AT1G49130	*Arabidopsis*	III	Delaying flowering under long-day conditions	Takase et al. (2011)
COL9	AT3G07650	*Arabidopsis*	II	Delaying flowering	Cheng and Wang (2005)
Hd1	Os06g0275000	Rice	I	Promotion of flowering under short-day conditions and inhibition under long-day conditions	Yano et al. (2000)
Ghd7	Os07g0261200	Rice	III	Delaying flowering under long day condition	Xue et al. (2008)
OsCO3	AB001887	Rice	III	Delaying flowering under short day condition	Kim et al. (2008)
OsCOL4	Os02g0610500	Rice	I	Delaying flowering	Lee et al. (2010)
DTH2	JX202590	Rice	II	Promoting flowering under long day condition	Wu et al. (2013)
LpCO	AY600919	Ryegrass	I	Accelerating flowering	Martin et al. (2004)
HvCO1	AF490467	Barley	I	Accelerating flowering	Campoli et al. (2012)
HvCO9	AB592332	Barley	III	Delaying flowering	Kikuchi et al. (2012)

displayed the highest stability of expression when ramie was grown for different time periods under different light treatments. Therefore, we selected the unigene21178 as the internal control to analyze the expression of BnCOL genes in different tissues, and the *actin* gene as the internal control to analyze the photoperiodic response of *BnCOL2*. The primer sequences of the *BnCOL* genes, unigene21178, unigene16487, unigene21994, 18s gene, and *actin* gene are listed in Table 2. The thermal cycle used was as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 10 s, and 55 °C for 25 s. Relative expression levels were determined as described previously by Livak and Schmittgen (2001).

2.5. Subcellular localization

The *BnCOL2* cDNAs containing the full-length ORF were amplified by using PCR. Specific 5′ and 3′ primer (the sequence is 5′-GACGGATCCGCC ACCATGGGTATTGGACACGG-3′ and 5′-GACCTCGAGAAACGTCGGAACG ACGCCGTAC-3′, respectively) that contained the BamH I and SalI recognition sites was designed to facilitate cloning of the cDNAs. The amplified PCR fragment was ligated into the constitutive expression vector pCAMBIA1301-YFP, which contained the cauliflower mosaic virus (CaMV) 35S promoter and the YFP reporter gene. The reporter construct was isolated and transformed into leaf cells of 30-day-old tobacco plants by using agrobacterium-mediated transformation. After 5 days, the fluorescence in the transformed cells was observed under a laser-scanning confocal microscope.

3. Results

3.1. Identification of six BnCOL genes with a full-length ORF

By searching against the annotation of unigenes, we screened 15 and 14 genes annotated as *COL* transcription factor in pools 1 and 2, respectively (Table S1). We identified one gene containing a full-length ORF in pool 2, but no *BnCOL* genes containing a full-length ORF in pool 1. In order to eliminate redundant genes and obtain a higher number of genes with a full-length ORF, we aligned genes from the two pools based on the nucleotide sequences. Genes with overlapping sequences were further assembled, and redundant genes were eliminated. Finally, we identified 13 non-redundant BnCOL genes (Table S1). Six of these 13 genes contained a full-length ORF; we designated these genes *BnCOL1*–*BnCOL6* (Table 3). The sequences were deposited in GenBank under accession numbers KF928219–KF928224.

3.2. Sequence analysis of BnCOL proteins

Analysis of the conserved domains of the six BnCOL proteins showed that all of the proteins contained the B-box I type domain; in addition, four proteins (encoded by *BnCOL1*, *BnCOL2*, *BnCOL5*, and *BnCOL6*) contained the B-box II type domain (Fig. 1). Furthermore, all of the proteins contained a conserved CCT domain; however, in the *BnCOL5*-encoded protein, this domain was incomplete (Fig. 1). Sequence homology analysis revealed a similarity of 33–50% between the six BnCOL proteins and homologous COL proteins of other species (Table 3).

To reveal the evolutionary relationship between COL TFs, we conducted phylogenetic analysis of the six newly identified BnCOL proteins and 16 COL proteins with known functions in other species (Table 1). The results showed that the 22 COL proteins could be classified into three groups (groups I–III) (Fig. 2). Proteins encoded by *BnCOL1*, *BnCOL5*, and *BnCOL6* were assigned to group II, whereas proteins encoded by *BnCOL3* and *BnCOL4* were assigned to group III (Fig. 2). Interestingly, the *BnCOL2*-encoded protein was assigned to the same group as *CO*- and *Hd1*-encoded proteins, namely, group I, suggesting that this protein showed the highest level of homology compared to *CO*/*Hd1*-encoded proteins.

Table 2
The primer sequence of internal control and BnCOL genes for qRT-PCR.

Gene	Forward primer	Reverse primer	Reference [a]
actin	GTTGAACCCTAAGGCTAACAGAG	GGAATCCAGCACGATACCAG	Liu et al. (2014b)
Unigene21178	TTCCGAAGGCTACTTGGATG	GGAGGTTTCACCATCACCTG	Liu et al. (2013a)
18s rRNA gene	TGACGGAGAATTAGGGTTCGA	CCGTGTCAGGATTGGGTAATTT	Liu et al. (2014b)
Unigene16487	GAGACGAAGACGTCGATGC	CCTTCTCATGGCTTCTCGAC	Liu et al. (2013a)
Unigene21994	TCAAGGGTACAGCTCCCATC	AGCTTCCCTTCCTTGTAGCC	Liu et al. (2013a)
BnCOL1	GCACGACACTTTGTTCCCTT	TTACAACCCTAAGCTGCCGA	
BnCOL2	CGTTCAGAATCGGTCAACCC	CCGACGAGTCTCTCCAGAAA	
BnCOL3	TGGCCAGACTGCATGGATAT	TTCTTGGCCTCTTCTCTGCA	
BnCOL4	GGAAGAGCAAGACCAACAGC	GAGAGGGTCCAATATCGGCA	
BnCOL5	AGATGGACGGGGAACAAGAG	AGAGGAAGAGGTTGTCGGTG	
BnCOL6	CTCTCGAAGGCTTCAATGGC	ACCCACAGAGCAAGGAACAT	

[a] The references in which the primer sequence had been published.

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Table 3
The information of BnCOL genes.

Gene	GenBank ID	Gene length	Predicted protein	Major tissue	The most similarity genes		Group
		(bp)	(aa)	expressed	Gene	RS [a]	
BnCOL1	KF928219	3121	408	Xylem	*COL9*	47%	II
BnCOL2	KF928220	3007	392	Xylem	*COL5*	50%	I
BnCOL3	KF928221	1973	453	Leaf	*COL7*	35%	III
BnCOL4	KF928222	1942	458	Xylem	*COL7*	36%	III
BnCOL5	KF928223	1929	444	Xylem	*DTH2*	44%	II
BnCOL6	KF928224	1713	371	Xylem	*DTH2*	33%	II

[a] RS indicated the ratio of similarity of the protein sequence.

3.3. Expression patterns of BnCOL genes

In order to characterize the expression patterns of the *BnCOL* genes in different organs, we analyzed the relative expression levels of the six *BnCOL* genes in six different tissues (leaf, stem bark, stem xylem, shoot, root, and flower). Our results showed that the six genes were expressed in all of the investigated tissues. With the exception of *BnCOL3*, which showed the highest expression level in the leaf, all of the genes exhibited the highest expression levels in the stem xylem (Table 3, Fig. 3). In addition, *BnCOL1*, *BnCOL4*, and *BnCOL5* showed relatively high expression levels in the flower (Fig. 3).

3.4. Photoperiodic response of BnCOL2

Among the six BnCOL genes identified, the *BnCOL2*-encoded protein showed the highest levels of homology compared to *CO* and *Hd1*-encoded proteins, namely, 34% and 27% sequence similarity, respectively. Therefore, we further analyzed the circadian response of *BnCOL2* in the ramie variety Zhongzhu 1. We found that *BnCOL2* showed a higher expression level under SD conditions than under LD conditions. In addition, this gene exhibited a diurnal expression pattern under LD and SD conditions (Fig. 4). We observed a higher expression level for *BnCOL2* during the night than during the day, under SD and LD conditions (Fig. 4).

3.5. Subcellular localization of the BnCOL2 protein

The putative function of the BnCOL2 protein is as a transcription factor, and therefore, we predicted that this protein would be localized in

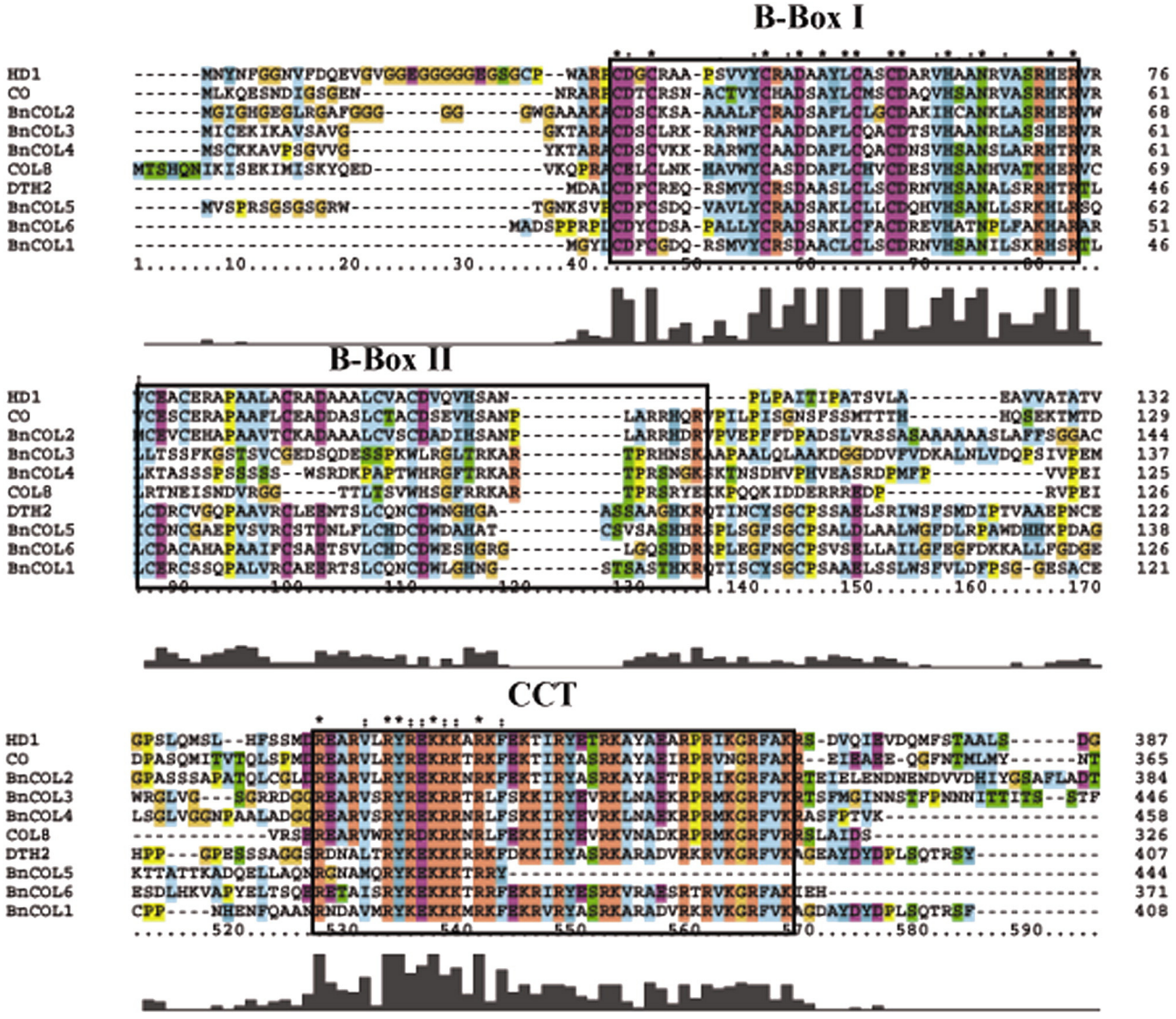

Fig. 1. Sequence alignment of six BnCOL proteins, a CONSTANS protein, and three COL proteins with known functions by using Clustal X. The conserved B-box and CCT domains that are characteristic of the *CONSTANS/CONSTANS-like* gene families are boxed.

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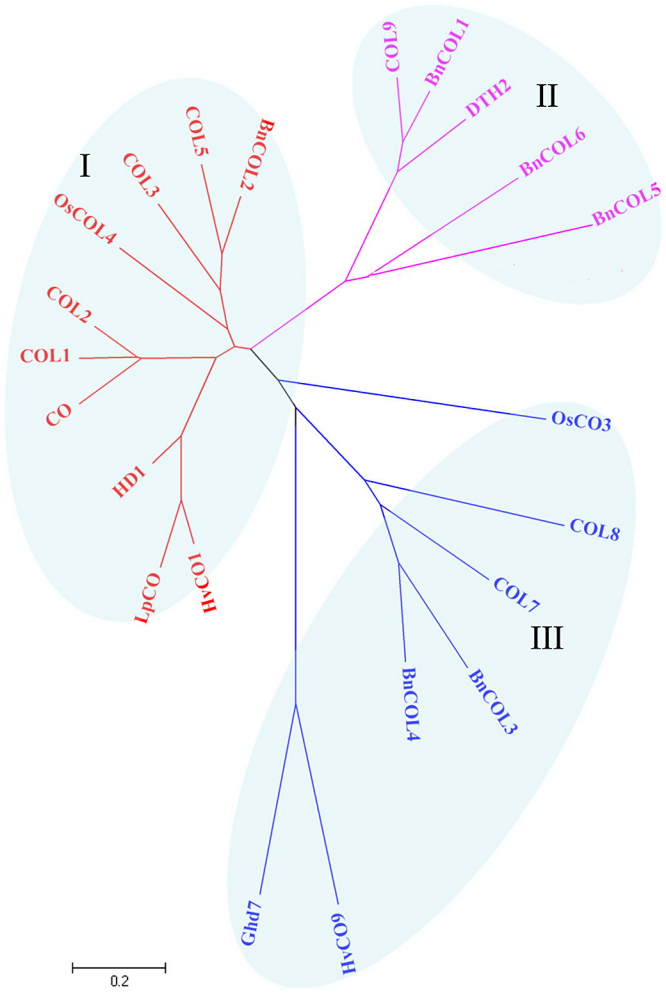

Fig. 2. Phylogenetic tree of six BnCOL proteins and 16 CO/COL proteins with known functions. The unrooted tree was generated by using the MEGA 4.0 program with the neighbor-joining method. Bootstrap values from 1000 replicates are indicated at each node.

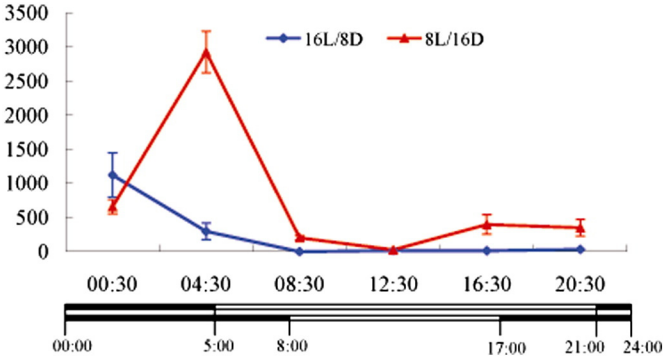

Fig. 4. Diurnal expression pattern of *BnCOL2* in the variety Zhongzhu 1, under long-day (LD) and short-day (SD) conditions. The mean of each point is based on the average of three biological replicates and was calculated by using the relative quantification method. The ramie at 08:30 under LD conditions constituted a control; the expression level of *BnCOL2* in the control was designated as one unit. The *y*-axis represents the expression level of *BnCOL2* at each time point under SD or LD conditions relative to the expression level of the control. The black and white bars indicate the dark and light periods, respectively. The numbers below the bars indicate the hours of the day. Error bars represent the standard error. The blue and red curves represent the expression change of *BnCOL2* under LD (16-h light/8-h dark) and SD (8-h light/16-h dark) conditions, respectively.

the nucleus. In order to investigate the subcellular localization of the BnCOL2 protein, we transformed the plasmid harboring an *YFP* gene fusion with *BnCOL2* driven by the 35S promoter into the leaf cells of tobacco and observed the fluorescence in the transformed cells. The result showed that significant signal of fluorescence can be observed from the nucleus, which suggested that the BnCOL2 protein fused with YFP was localized in the nucleus (Fig. 5), implying that BnCOL2 is a transcription regulator.

4. Discussion

4.1. Identification of six BnCOL TFs with a full-length ORF

In model plants, *CONSTANS* and its homologous genes have been shown to play central roles in regulating flowering (Table 1). These genes have been identified as belonging to a gene family with conserved B-box and CCT domains. To date, 854 *COL* genes from 81 species have been collated in the PlantTFDB database (Jin et al., 2014). However, no *COL* gene has previously been identified in ramie, despite the importance of ramie as a natural fiber crop. In the present study, we identified six *COL* genes in ramie. All of the BnCOL proteins encoded by these genes contained conserved B-box I and CCT domains. However, the B-box II

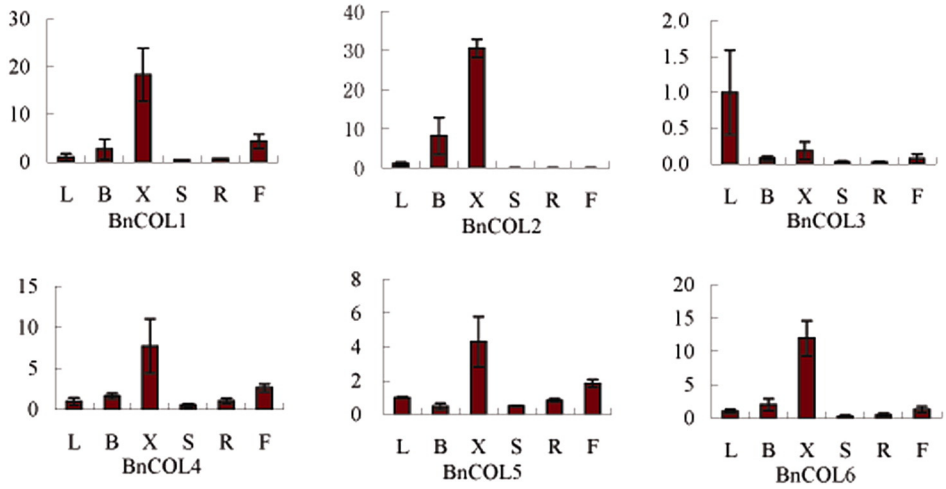

Fig. 3. The expression patterns of *BnCOL* genes in specific tissues. The L, B, X, S, R, and F in the *x*-axis represent the leaf, stem bark, stem xylem, shoot, root, and flower, respectively. The *y*-axis represents the expression levels in the stem bark, stem xylem, shoot, root, and flower relative to the expression level in the leaf, which was designated as one unit. Error bars represent the standard error.

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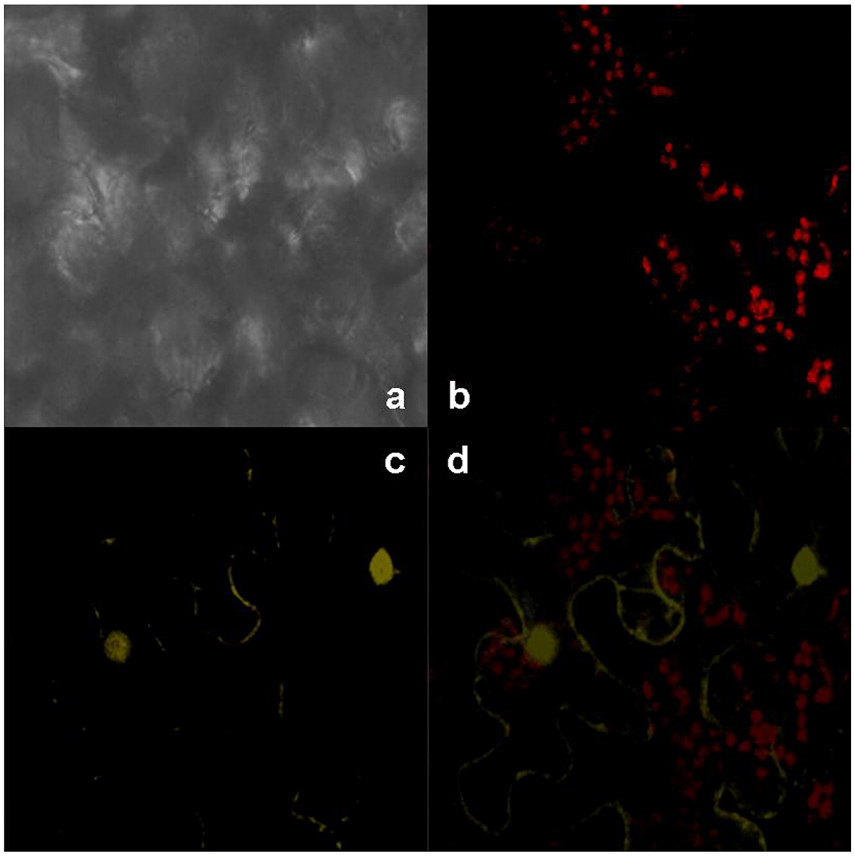

Fig. 5. Subcellular localization of the BnCOL2 protein. Plasmids harboring an *YFP* gene fusion with *BnCOL2* driven by the 35S promoter were transiently expressed in the leaf cells of tobacco. (a), A control figure in which no fluorescence can be found because of the absence of laser signal; (b), the fluorescence of chloroplast; (c) significant YFP-fluorescence signal observed from the nucleus in the transformed cells; (d), a figure merging the fluorescence of chloroplast and YFP-fluorescence.

domain was present in only four of the BnCOL proteins. Our results are in accordance with those of Griffiths et al. (2003), who reported the presence of three and four COL proteins with a single B-box domain in rice and *Arabidopsis*, respectively. Therefore, the B-box II domain is not well conserved in the COL family. Previous studies have demonstrated that a functional B-box II domain is required for flower induction (Griffiths et al., 2003; Martin et al., 2004; Campoli et al., 2012). Interestingly, the overexpression of *COL8*, which is a COL TF with a single B-box domain, resulted in a phenotype with delayed flowering under LD conditions in *Arabidopsis* (Takase et al., 2011). Therefore, it seems that the absence of the B-box II domain in some COL TFs does not necessarily affect the regulation of flowering.

In rice, at least four genes involved in flowering regulation have been shown to have a pleiotropic role in regulating plant height and grain yield (Xue et al., 2008; Yan et al., 2011, 2013; Gao et al., 2013; Liu et al., 2013b). Interestingly, all of these genes were commonly involved in flowering-regulated pathway of *Ehd1–Hd3a*. One of these genes, namely, *Ghd7*, was characterized as a *CONSTANS-like* gene that regulated the expression of *Ehd1* (Xue et al., 2008). Among the six BnCOL proteins identified in the present study, the *BnCOL3*- and *BnCOL4*-encoding proteins showed higher levels of homology compared to the *Ghd7*-encoding protein and were classified into group III based on phylogenetic analysis. However, it seems that the expression patterns of *BnCOL3* and *BnCOL4* differ from that of *Ghd7*. The *Ghd7* gene is expressed in some young tissues, including the apical meristem, the root meristem, the epidermal layer of developing stems, and the branch-primordia of developing panicles (Xue et al., 2008); whereas the *BnCOL3* and *BnCOL4* showed less transcript-abundant in the shoot, which mainly consists of young tissues. In addition, the proteins encoded by *BnCOL1*, *BnCOL5*, and *BnCOL6* exhibited relatively high homology with the *DTH2*- and *COL9*-encoding proteins. Previous studies have shown that *COL9* and *DTH2* were constitutively expressed in various organs (Cheng and Wang, 2005; Wu et al., 2013). In the present study, we found that *BnCOL1*, *BnCOL5*, and *BnCOL6* were expressed in all of the investigated tissues, especially in the stem xylem.

4.2. Diurnal expression patterns of BnCOL2 under LD and SD conditions

Hd1, *HvCO1*, and *LpCO* were previously shown to be the closest orthologous genes of *CO* in rice, barley, and ryegrass, respectively (Yano et al., 2000; Martin et al., 2004; Campoli et al., 2012). In addition, *CO* and its three orthologous genes commonly exhibited an oscillating expression pattern in response to photoperiod. Moreover, all three orthologous genes were confirmed to have the same function as *CO* and commonly played key roles in regulating flowering in their respective species (Yano et al., 2000; Suarez-Lopez et al., 2001; Martin et al., 2004; Campoli et al., 2012). In *Arabidopsis*, under LD conditions, the CO protein is rapidly degraded in the dark (Valverde et al., 2004). Thus, in order to make the CO protein stable, mRNA expression and translation of the *CO* gene must be coincident with light. The CO protein is then able to induce expression of the FLOWERING LOCUS T (FT) protein, which further induces the expression of floral meristem identity genes (Abe et al., 2005). However, under SD conditions, *CO* transcription peaks during the dark period and flowering do not occur (Suarez-Lopez et al., 2001). In rice, there is a different model. *Hd1* expression peaks during the dark period and the Hd1 protein appear to behave as a floral activator under inductive SD conditions, thereby positively regulating

Please cite this article as: Liu, T., et al., Identification of a *CONSTANS* homologous gene with distinct diurnal expression patterns in varied photoperiods in ramie (*Boehmeri...*, Gene (2015), http://dx.doi.org/10.1016/j.gene.2015.01.045

the expression of *Heading Date3a* (*Hd3a*), which is the *FT* homolog (Hayama et al., 2003).

Among the six BnCOL TFs identified in the present study, the *BnCOL2*-encoded protein showed the highest level of homology with *CO*/*Hd1*-encoded proteins. Subcellular localization of the BnCOL2 protein showed that significant fluorescence signal caused by the BnCOL2 protein fused with YFP was observed from the nucleus. However, it seemed that there was little fluorescence found on the cell membrane, which probably was caused by a high background luminance of the figure. In this study, the BnCOL2 protein exhibited a low level in the transformed tobacco cells. In order to clearly observe the fluorescence of BnCOL2 protein fused YFP, a high background luminance of the photo was adopted, finally resulting in little fluorescence found on the cell membrane. However, more fluorescence observed from nucleus than cell membrane suggested that BnCOL2 protein was localized in the nucleus, implying its function as a transcription regulator. Analysis of the expression response to photoperiod revealed that, similar to *CO*/*Hd1*, *BnCOL2* showed a distinct diurnal expression pattern. Our results are in accordance with those of previous studies, which reported the expression of *CO*/*Hd1* under LD and SD conditions. Moreover, in the SD plant rice, *Hd1* shows an expression peak during the dark under SD conditions, and the Hd1 protein is a key positive regulator for flowering. Similarly, in the present study, *BnCOL2* showed an expression peak during the dark under SD conditions.

The *CO* gene has been shown to exhibit a wide spatial expression pattern, which includes the young leaves, phloem and protoxylem (An et al., 2004). According to the RiceXPro database, *Hd1* also shows a wide spatial expression pattern, especially in the leaf, stem, embryo, and root (Sato et al., 2010). In the present study, we observed the expression of *BnCOL2* in several of the investigated tissues, especially in the stem and leaf. The leaf is a key location for the functioning of the CO/Hd1 protein as a flowering regulator to modulate the transcription of *FT*/*Hd3a* (Turck et al., 2008); hence, it is not surprising that *CO*/*Hd1* shows a high level of expression in the leaf. Being similar to *CO*/*Hd1*, the *BnCOL2* had a strong expression in leaf. Interestingly, in the present study, *BnCOL2* exhibited a high level of expression not only in the leaf, but also in the stem. Actually, the *CO*/*Hd1* was also observed to exhibit a high transcript accumulation in the stem. The functional role of *CO*/*Hd1*/*BnCOL2* in the stem is still unknown. The homologous sequence and similar expression pattern of *BnCOL2* and *CO*/*Hd1* suggest that *BnCOL2* possibly has a role in flowering modulation, and can be used as a candidate gene for research in the flowering regulation of ramie.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.01.045.

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