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# Research Article Characterization and spatial expression pattern of a novel CONSTANS gene (GbCOL-6) from Ginkgo biloba

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# Abstract

Background and Objective: Ginkgo biloba is an important medicinal plant but the transitional period from the sowing to the flowering of G. biloba is too long, which severely limits its reproduction and application. The CONSTANS (CO), is one of the important transcription factors in the transition from vegetative growth to reproductive growth. The CO plays a central role in photoperiodic flowering control. The aim of this study was to clone and characterize a CONSTANS-like 6 (GbCOL-6) gene from G. biloba. Materials and Methods: A pair of specific primers were designed based on the data of the G. biloba transcriptome. The full-length cDNA of the CO gene was amplified from the total RNA of G. biloba leaves by RT-PCR, named GbCOL-6 (GenBank Accession No. MG251395). The expression of GbCOL-6 gene in different tissues was studied by guantitative RT-PCR. **Results:** Sequence analysis results showed that the cDNA of GbCOL-6 contained a 1479 bp open reading frame (ORF), which encodes a 492 amino-acid protein. The theoretical molecular weight and pl of the GbCOL-6 are 54.65 kDa and 5.08, respectively. Similar to other CO proteins, GbCOL-6 contains two conserved domains (B-box and CCT domain) and the amino acid sequence showed high similarity to other plant CO proteins. Phylogenetic tree analysis revealed that GbCOL-6 belonged to the second group members of the CONSTANS gene family, which has aB-box domain and a CCT conserved domain. These results indicated that GbCOL-6 is a member of the CONSTANS family and belongs to CONSTANS-LIKE 6. Tissue expression profile analysis showed that GbCOL-6 was expressed in all tissues, the highest expression level was detected in the female strobili and the lowest in the roots. Conclusion: This study found that GbCOL-6 isolated from G. biloba was specifically expressed in the female strobili of G. biloba. However, GbCOL-6 rarely was expressed in roots of G. biloba. This laid the foundation for further understanding of the molecular regulation mechanism of the flowering of G. biloba and shortening the juvenile phase of woody plant through the transgenic technology and providing genetic resources for promoting early flowering.

Key words: Ginkgo biloba, CONSTANS, flowering time, gene cloning, expression analysis

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Data Availability: All relevant data are within the paper and its supporting information files.

# **INTRODUCTION**

*Ginkgo biloba* also known as 'gongsun tree'. *Ginkgo biloba* is a relict plant from the Cenozoic Quaternary glacial period, which has a long juvenile phase. *Ginkgo biloba* is a unique peculiar species in China, the general seedling planting 15-20 years to blossom results<sup>1</sup>. *Ginkgo biloba* is a subtropical, temperate deciduous tree species and one of the major economic tree species in China<sup>2</sup>. Its fruits are rich in flavonoids and terpene lactones, which improve cognitive expression, the brain function, antagonize platelet activation factor, scavenging free radicals, inhibition of lipid peroxidation and other effects<sup>3</sup>.

As the juvenile phase of *G. biloba* is particularly long, the selection of fine varieties of *G. biloba* has brought serious obstacles, making the *G. biloba* many economic and social values are subject to a large extent. Although traditional breeding methods such as seedling propagation, grafted seedling propagation, cutting propagation, root tiller and micropropagation<sup>4</sup>, shorten the ginkgo juvenile phase to some extent, it is still relatively longer, therefore, finding new ways to shorten the ginkgo juvenile phase is an urgent problem to be solved.

Flowering is an important signal before plant fruit. The process of plant flowering mainly involves the following regulatory pathways: autonomous pathway, photoperiod pathway, vernalization pathway, gibberellin pathway, carbohydrate induction and flowering inhibition pathway<sup>5</sup>. These regulatory pathways are independent of each other and interact, together constitute plant cell within the precise regulatory network<sup>6</sup>. The CONSTANS (CO) gene is one of important regulatory gene in the photoperiod pathway and its expression is affected by the photoperiod pathway Circadian Clock-associated 1 (CCA1), Late Elongated Hypocotyl (LHY), Zeitlure (ZTL), Timing of Cab Expression 1 (TOC1), Gigantea (GI) and F Lavin Binding Kelch-repeat f-box 1 (FKF1)<sup>7</sup> and other upstream genes. The CO gene control Flower Locus T (FT) gene synthesis, FT as CO target gene, together constitute the CO/FT regulatory elements, regulate the flowering time of plants<sup>8</sup>. The results showed that the up-regulation of CO gene promoted the expression of FT in the long daylight condition and promoted the flowering<sup>9</sup> and the promoting effect was not obvious under the condition of short daylight<sup>10-12</sup>.

At present, the homologous genes of *CO* were obtained in the *Nicotiana tabacum*<sup>13</sup>, *Agapanthus africanus Hoffmgg*<sup>14</sup>, *Fragariaananassa duch*.<sup>15</sup>, *Vitis vinifera*<sup>16</sup>, *Malus pumila* Mil<sup>17</sup>, *Festuca elata*<sup>18</sup>, *Pyruspyrifolia nakai*.<sup>19</sup>, *Solanum lycopersicum*<sup>20</sup> and *G. biloba*<sup>21</sup>, but their functions were different. The control route of the CO homologous gene Vv CO from V. vinifera is similar to that of Arabidopsis thaliana. However, there gulation pathway of MdCOL1 and MdCOL2 in M. pumila Mil is different from that of A. thaliana, which shows organ specificity and plays an important role in growth and development of reproductive organs. Based on the data of G. biloba transcriptome, the GbCOL-6 cDNA of G. biloba was cloned and the bioinformatics analysis was carried out to predict its function. In addition, the expression of GbCOL-6 gene in G. biloba was analyzed.

# **MATERIALS AND METHODS**

**Materials:** The materials were taken from 31 years old trees of the *G. biloba* cultivar 'Jiafoshou', in the Ginkgo Science and Technology Garden, Yangtze University. The tree was in good condition and normal growth. From February, 2017, the materials of *G. biloba* were collected and the recovered material was stored after liquid nitrogen in a -80°C refrigerator.

PrimeScript<sup>™</sup> 1st Strand cDNA Synthesis Kit, Agarose Gel DNA Purification Kit Ver. 4.0, MiniBEST Plant RNA Extraction kit, Taq DNA polymerase RNase, dNTP, pMD18-T vector were purchased from Takara Company (Dalian, China). In this experiment, both the primers synthesis and DNA sequencing were completed by Shanghai Sangon Biotechnology Company, China.

Cloning of GbCOL-6: Total RNA was isolated from frozen leaves of G. biloba using MiniBEST Plant RNA Extraction kit. The extracted RNA was reverse transcribed into cDNA using PrimeScript<sup>™</sup> 1st Strand cDNA Synthesis Kit. The Specific Primers C1 and C2 were designed based on the COL-6 unigene sequence of *G. biloba* transcriptome data (Table 1). The GbCOL-6 gene was amplified by reverse transcription polymerase chain reaction (RT-PCR) using cDNA obtained by RNA reverse transcription. The PCR reaction system was 1 µL upstream of the primer (10 umol  $L^{-1}$ ), Exug polymerase (5 U  $\mu$ L<sup>-1</sup>) 0.5  $\mu$ L, 10×PCR buffer 2.5  $\mu$ L, PCR Enhancer 4  $\mu$ L, dNTP (2.5 mmol L<sup>-1</sup>) 2  $\mu$ L, total cDNA (1  $\mu$ g  $\mu$ L<sup>-1</sup>) 1  $\mu$ L, ddH<sub>2</sub>O make up 25 µL. The reaction procedure is: Pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 64.5°C for 30 sec, extension at 72°C for 90 sec, a total of 35 cycles, finally at 72°C for 10 min. The amplified products were detected by 1% gel electrophoresis and the PCR products were recovered by Agarose Gel DNA Purification Kit Ver.4.0. The purified product was cloned into the pMD18-T vector, then transformed into *Escherichia coli* DH5a strain. Positive clones were selected and sent to Shanghai Biotechnology Biology Company for sequencing.

Table 1: Primer sequences used in this study

-	•
Primers	Sequence (5'-3')
GbCOL-6-up C1	TGAAGAAGATGGAGGGTAGTGAA
GbCOL-6-down C2	TGCGTGAGTTCATTCAAGAGAC
qRT-PCR upstream primer P1	ATGAAGATGTTGTAATGGCCTGGTC
qRT-PCR downstream primer P2	ACTCTTGAAAACCTTCTGTTCCATC
18S upstream primer H1	TTGGTCTCCCGTGCTAATGG
18S downstream primer H2	CGAAGCGTCATCCTAAGACAACA

**Bioinformatics analysis:** Using the BLAST tool on the NCBI website to find the sequence with the highest similarity to the nucleotide and deduced amino acid sequences obtained in this experiment. Sequence splicing, protein translation and amino acid sequence multiple comparisons were performed using Vector NTI 11.5 and DNAMAN. The deduced amino acid sequence was analyzed by CLUSTAL X 2 and MEGA6 software. The Neighbor-Joining (NJ) method was used to construct phylogenetic tree and the ExPASy online server ProtParampredicts the molecular weight and theoretical isoelectric point of the protein.

Quantitative real-time PCR analysis: The RNA was extracted from samples including root, stem, leaf, female strobili, male strobili and fruit and cDNA was reverse transcribed. According to the known sequence, real-time quantitative primers P1 and P2 (Table 1) were designed to amplify the GbCOL-6 gene fragment. Reverse transcribed cDNA was used as a template and 18S was selected as an internal reference gene for gRT-PCR amplification<sup>22</sup>. The upstream and downstream primers of 18S were H1 and H2, respectively (Table 1). Reaction system 25 µL, the reaction program with reference TaKaRa real-time quantitative instructions (PrimeScriptTM RT reagent Kit Perfect Real Time) was carried out. Each sample was set up three times to repeat the ultrapure water as the negative control, 18S to do the internal reference gene. Data processing using the relative guantitative method, concerning  $2^{-\Delta\Delta Ct}$  method for the results of analysis<sup>23</sup>.

#### RESULTS

**Analysis of the** *GbCOL-6* **gene sequence:** Ginkgo cDNA was used as a template for PCR amplification of the *GbCOL-6* gene. The specific primers C1 and C2 (Table 1) were designed and the PCR product was subjected to electrophoresis in 1% agarose gel to obtain a DNA fragment of about 1500 bp (Fig. 1). The product was purified and sent to Shanghai Biotechnology Company for sequencing. The sequencing results showed that the cDNA of GbCOL-6 was 1594 bp in



# Fig. 1: Electrophoresis of RT-PCR of ORF in *GbCOL-6* gene: M, DL2000 marker

Table 2: Similarity comparison of GbCOL-6-coding protein and other known CO proteins

Species	Accession number	Homology (%)	E value
Populuseuphratica	XP_011016373.1	48	3e-24
Vitis vinifera	CBI27020.3	34	3e-46
Nicotiana tabacum	XP_016463604.1	33	1e-41
Herraniaumbratica	XP_021298869.1	33	1e-46
Ricinus communis	EEF30756.1	33	4e-36
Sesamumindicum	XP_020547025.1	33	3e-41
Pyrus x bretschneideri	XP_009356747.1	32	6e-40
Dorcocerashygrometricum	KZV40687.1	32	4e-40

length. The results of Vector NTI 11.5 showed that GbCOL-6 contained a 1476 bp ORF encoding 492 amino acids (Fig. 2, GenBank accession number MG251395). The on-line analysis of ExPASy-ProtParam showed that the theoretical molecular weight and Pi of the GbCOL-6 protein were 54.65 kDa and 5.08, respectively.

**Analysis of GbCOL-6 protein:** The amino acid sequence of GbCOL-6 was compared on NCBI website to obtain protein sequences with high similarity. The homologous alignment results of the deduced amino acid sequence of GbCOL-6 with BLAST-protein on NCBI site showed that the GbCOL-6 protein sequence had high homology with the COL protein sequences of other plants and their similarities ranged from 32-48%. The sequence similarities of GbCOL6 protein and other COL proteins were as follows: *Populus euphratica, V. vinifera, N. tabacum, Herrania umbratica, Ricinus communis, Sesamum indicum, Pyrus x bretschneideri* and *Dorcoceras hygrometricum* were 48, 34, 33, 33, 33, 33, 32 and 32%, respectively (Table 2). In addition, the GbCOL-6 protein has a conserved B-box structure and a CCT domain of the CO protein family Fig. 3.

_	TGA	AGA	AGA	TGG.	AGO	GTA	GTG.	<u>AA</u> G	ATG	CAG	ATG	GGT	CGA	IGO	GGA	AAA(	CAGO	CTA	AAA	ATA	GTA	ATA	AAA'	rga(	.AA
1				M I	E (	G :	S 🔅	E	D.	A I	D (	G	s :	a I	RI	3	ΓÆ	A (	К	N I	5	N I	K 3	ε 1	r
76	TGG	CCA	TAG	GGO	GAA	GAA	GAG	CTC	GTC	CAT	GTG	ATG	ICT	GCG	GAG	AAC.	AGCI	FCG	CTC	GAT	GGT.	ACT	GTG	FTGC	LAG
25	М	A	Ι	G	G	R	R	А	R	Ρ	С	D	v	С	G	Е	Q	L	A	R	П	Y	С	G	А
151	ACC	AAG	CAA	ACC	TAT	GCG.	ACT	CCT	GC G.	ACG	GAT	αG	TTC.	ACA	CTG	CCA	ATTO	CAG	TCG	CCI	GCA	GAC	ACG	AAA(	JAG
<b>5</b> 0	D	ଘ	A	N	L	С	D	s	С	D	G	S	v	H	Т	A	N	S	τ	А	С	R	H	Е	R
226	TCA	GAC	TGA	CGO	CGA	CGC.	AGG	TTC	GAT	CCG	CAG	GAG	CAC	CAT	TAA	CAA	ATC/	٩TT	СТТ	CCG	ccc	TTC	TAA	CAGO	CTA
7ā	7	R	Ľ.	Т	Ρ	Т	Q	7	R	s	А	G	А	P	L	Т	N	H	s	s	A	L	L	Т	A
301	CTC	GAA	GCT	CCA	FAT	GGO	GCC.	ACG	CTT	CTC	GAA.	AAC	GAA	STO	GGCI	CTT	CCAC	GTO	GAC	CGC	ATC	CGG	ACT/	ICC!	AGA
100	Т	R	S	S	Ι	W	R	Н	А	S	R	К	R	s	R	₽	s	S	R	Р	H	Ρ	D	Y	Q
376	GAC	AGA	CAG	ACG	AAA'	TTA	TAA	CGA	AGC	TCG	GTC	GAC	TCA.	4AC	CCI	CCA:	ICA	AAG	тст	TCG	ACT	TCA	TGT	CGAO	CTA
125	R	ଘ	Т	D	Е	Ι	I	Т	К	L.	G	R	Ľ.	К	Ρ	s	Ι	K	Т	F	D	F	М	S	Т
451	CTG	CAG	AAA	GTA	ATG	GCG.	ACG.	ACG	ACT.	ATC	CGC	TGC.	ACC.	AAG	TGC	CAA'	ITT	FGG	CCG	ACG	AAT	TCG	ACT	[TA]	ΓGA
150	Т	A	Е	s	N	G	D	D	D	Y	Ρ	L.	H	Q	Т	Ρ	Ι	L	A	D	Е	F	D	F	М
526	ATC	GGC	CGA	CTO	CCL	ACT	CTA	CGC	TCC	CAT	CGT	CGA	CCG	сто	CTA	A TG	AGA/	ATT	CCA	GTG	CCG	GTO	CTA	rgg(	GOG
175	N	R	Ρ	Т	A	Y	s	Т	L	Р	S	s	Т	А	Ρ	X	Е	X	S	S	А	G	Р	М	G
601	GCA	CGG	GTA	GAG	CCT	CAT	GCG.	AAG	AGC	TGC	CAG	TGG.	AGT	TTC	TAG	AGT	CGAŁ	AGG	CTG	TCG	ATC	TGT	GTG	\TTI	TG
200	G	Т	G	R	A	s	С	Е	Е	L.	Ρ	v	Е	F	L	Е	s	Κ	А	7	D	L	С	D	F
676	AAG	TGG	AGA	TGA	GTT	TGG	GGT	GTA	GCT	TCA	TTG	GOC	CGG.	ATG	CCC	A TG	IGAC	CCG	TCG	ACG	ACT	TGG	GAG	50C]	l'AG
225	Е	r	Ε	М	s	L	G	С	S	F	Ι	G	Ρ	D	А	H	Т	Т	τ	D	D	L	G	G	L
731	GCT	TGT	ATA	GTT	ICA	GTA	AAC	CCT.	ACG	CCG	ACC	TAG.	ATG	CTC	ATG	ATG	GCGI	rcg	GGT	TCT	TTG.	ATD	CTC	AGA(	FAC
250	G	L	Ÿ	s	F	S	К	Р	Y	А	D	Ľ.	D	А	H	D	G	Т	G	F	F	D	S	ର	R
826	ACA	ACT	GCG	GTT	GTT	CTG.	AGA	CGG.	ATC	ATT(	GCG.	AGG	GCT	CGA	GTC	CCA:	IGG/	١TG	AAA	AGA	ATG	GGG	AAA'	TTCC	LAC
275	Н	Χ	С	G	С	S	Е	Т	D	Η	С	Е	G	s	S	Ρ	М	D	Е	Κ	Х	G	Е	Ι	Ρ
901	TTA	GCT	GGC	TTT	GCA	ATO	GGG	TTA	AAG	TCG	AGG.	AAA	GCG.	AGG	ACG	I CG.	ACC	١TG	TGT	TCG	GTA	GCA	GTG	ACA]	[CA
300	L	s	$\underline{R}$	L	С	N	R	Ľ	К	v	Е	E	s	Ε	D	τ	D	H	τ	F	G	s	S	D	Ι
976	AAG	TCG	AGG	AGC	AAG	AGG.	ATA	TAG	ACC	TGT	GTG.	ACA	TAA	CTG	TGA	ACG	₹₩Ġ	<b>\</b> TG	TGG	AGG	TGA	AGA	TTA	AGC 1	ſGG
325	Κ	٣	Ε	Ε	Q	Е	D	I	D	Ľ.	Ç	D	I	Т	τ	N	Ε	D	τ	Е	۲.	Κ	I	Κ	L.
1051	ATT	TTG	ATG	ATT	ATT	TCC.	AAG.	AGG.	AAG.	AGA	AGA.	AAA	TAT	GTC	TGA	GAT:	IGG	\TT	ATG	AAG	ATG	TGT	TAA	r tga	CT
350	D	F	D	D	Y	F	Q	Е	Е	Е	K	К	Ι	С	L	R	L	D	¥	Е	D	T	L	Ι	А
1126	GGT	CTG	ATO	GA0	GTT	CIC	TTT	GGG	CGG.	ATA	ATA.	AGA	CTA	L'AC	AAG	CCT	ITG	FGG	ATG	ACA	CCA	GCT	CCG	ATG(	JAA
375	11.	S	D	R	G	S	L	П	А	D	N	К	Т	I	Q	A	F	٣	D	D	Т	S	S	D	G
1201	CAG	AAG	GTT	TTC	AAG	AGT.	ATG	GAA	TTG	TTC	CTG.	ATT	TGA	κт	TAG	GAA'	ITG	CAC	GCG	GGG	CCT	CGG	GAG	۸TC/	AG
400	Т	Ε	G	F	Q	Е	Y	G	Ι	۲.	Ρ	D	L	ŝ	L	G	Ι	A	R	G	A	S	G	D	Q
1276	GGG	AGC	AAG	TGO	CTG	TGA	TGA.	ATG	GCG	GAGI	GAG.	ATC.	ATG.	AAG	GGA	GC	ATG	GAG	AAG	GAA	GAG	AGG	CTC	GCGI	ΓTA
425	G	Е	Q	r	₽	τ	М	N	G	G	G	D	Н	Ε	G	R	H	G	E	G	R	Е	А	R	v
1351	TGA	GGT	ACO	GTG	AGA	AAO	GTC	GAA	GCA	GAC	TTT	ICT	CAA.	AGA	AGA:	I CA(	GAT/	ACG	AAG	TTO	GCA	AAC	TCA/	1.CGG	.AG
4ā0	М	R	Y	R	Е	K	R	R	s	R	L	F	s	K	K	Ι	R	¥	Е	r	R	Κ	L	N	А
1426	AAA	AGC	GAO	CGO	GCA'	TGA.	AGG	GTC	GAT	TTG	TGA.	AAA	GAAJ	CGO	CAG	GTC:	IGTO	CTC	ттd	AAT	GAA	CTC	ACG	CA	
475	Е	Κ	R	Ρ	R	М	К	G	R	F	v	К	R	Т	Ρ	G	L	S	L	Е				_	

Fig. 2: Nucleotide sequence and deduced amino acid sequence of GbCOL-6. The start codon and termination codon are indicated by the box, the primers are indicated by the underline

Phylogenetic analysis of GbCOL-6: In order to understand the molecular evolution of GbCOL-6 and other plant CO proteins, the GbCOL-6 protein sequence was analyzed by CLUSTAL X 2 and MEGA6 software. The GbCOL-6 protein sequence was homologous analysis with the CO protein of other species on GenBank and the phylogenetic tree was constructed by NJ method (The accession numbers are shown in Table 3). The CONSTANS gene is a multi-copy gene family in the plant genome, 17 members in A. thaliana<sup>24</sup> and 16 members in Oryza sativa<sup>25</sup>. As Fig. 4 shown, the phylogenetic tree is divided into three major branches, one for CONSTANS LIKE 6-8 and CONSTANS LIKE16, one for CONSTANS LIKE 1-5 and CONSTANS and one for CONSTANS LIKE 9-15. This is consistent with the results of the CO gene family classification in A. thaliana.

Genetic studies of *A. thaliana* CO show that the CO gene family is divided into three major categories<sup>24</sup>. Class I contains CO and COL1-COL5 (Fig. 4, Group1) and Class I can be further divided into class Ia (CO, COL1 and COL2) and class I<sub>0</sub> (COL3-COL5), Class II contains COL6-COL8 and COL16 (Fig. 4, Group 2), Class III contains COL9-COL15 (Fig. 4, Group 3). Its classification is mainly N-terminal B-box structural features. The N-terminus of Group 1 has two B-boxes, the N-terminus of Group 2 has only one B-box, the N-terminus of Group 3 has a normal B-box and a secondary structure-modified B-box zinc finger structure<sup>24</sup>. The GbCOL-6 has a normal B-box structure and a CCT conserved domain and it shows that GbCOL-6 is a Class II member of the CO family.

**Expression pattern of GbCOL-6 in different tissues:** As shown in Fig. 5, GbCOL-6 was expressed in roots, stems, leaves, male strobilis, female strobilis and fruits, but the expression of GbCOL-6 was various in different tissues. It was the highest expression in female strobilis, followed by stems, leaves, fruits, male strobilis and the lowest level of expression in roots.

Ginkgo biloba	(1) MEGSEDADGSMRKTAKNSN MIMALG RR RPCDVCGEQLAR CCGADQANLCDSCDGSVHTANSVACRH RV LIPTQVRSAGA
Dorcoceras hygrometricum	(1)M N A LSSA A S R CD C K AR C AD A LC CD VH AN A RH RVCL I S IKSLD
Sesamum indicum	(1)MQELKP V DR L VA GGES R CD CV REAR OC AD A LC CD SVH AN LARH RV I GTA ISSFD-D
Nicotiana tabacum	(1)V DRAL SAMOKT R CDAC RKAR CPAD A LCANCD SVH AND A RH RV L SPLKESSPDENFP
Populus euphratica	(1)
Pyrus x bretschneideri	(1) N S V HAVGET R CD C K VAR IC AD A LCEACD DVH AND A RH RVLL VAS SLKPS-NKDDR
Ricinus communis	(1)VI NOV VA A & T R CDSCV WAAR C AD A LCSSCD SVH ANY A RH RVIL IS -LKSLDI
Vitis vinifera	(1)VI ENVIALGET R CD-C PREARF C AD A LC ACDM VH AND A RH RV LL ASLKL
	B-box
Ginkgo biloba	(86) PLTNHSSALLTATRSSIWRH SRKRSRPSSRPHPDYQRQTD IITKLGR KPS KVF FM TTAESNGD-DUYPLHQVP LADEFDF
Dorcoceras hygrometricum	(72)AKLQT VPSWHQGFTRARTPRQK-KQSKFNFSDESTARVOVPH VPS YE N HEARE
Sesamum indicum	(78) GSKAGLQI SV SKHQGFTRAAKTPRQK-KPGLINNSEDFINRISH H VPG C-SE Q HESNEB LIKVP TEDETVAE
Nicotiana tabacum	(77) NLESPVSV SLOSWIR OF RAARDERNGRKVSSTEEEEEEVILKYPIH YPO L-SOLN SLOENE LIKVP (PDP) VAE
Populus euphratica	(72) SLNTDQDY P A HOGE RAARGERINSNSN SLLVQKLLKDDR KVL 9525 VP3 G - E EPNMAADNEDQ LC VPV 505 A K
Pyrus x bretschneideri	(76) TTAVSKTSAA-SWER OF IR PROFRGGSMTHHGKSK GIR LF Q P V GD N YD ENED Q L KVP PD S AT
Ricinus communis	(72) ISKGSSVITV SWOOT ROACT RNG-HGNPLLSLLKIE KSRN I TO P V SD OI HED NBEQ LOVP DEPENDE
Vitis vinifera	(69) PGADSLEN M SELECT REAL FROM HER PHARPAF SD LTRAD FOR POLICATED YDDNO L CVP DDDY AF
Ginkgo biloba	(172) MNRPTAY TLPSS APNENSSAGPM GTGRASCEELPVEFLESKAVDLCDFEVEMSLGCSFIGPDAHUTVDDLGGL VSFSKPYADLDA
Dorcoceras hygrometricum	(148) C CEAMNNSNI AESIKQ FGNDQSK LSNDLSPDMKR V LISEA DARDAADVISLI GAGUST LD
Sesamum indicum	(158) CT AN- P VLT TFQINDE NKRFG-HESK AANG SQEMNH OF LESDHOLABBAAUVESL GAGLEDSCH
Nicotiana tabacum	(158) CT ATRADOIENVQTATETDPEFKLESKEMQKHDICNDDLNRFR MLSEE BABPAALVISL GAGLEPSID
Populus euphratica	(159) M DIVTSEDGNMAVEVYGE
Pyrus x bretschneideri	(157) USNSGN S DAAANYEANNLSTVSDNVGSQSK VSS- PNCNGHDGE LCSDOD AD AAVYD L GRUND
Ricinus communis	(155) CTPTTLIN ADD TTAPFTALGONNDTDOTY GANG JESKASY DELCETOLD AN VOLL GRUTNSTG
Vitis vinifera	(147) LANTA-N AVI VANDIETADVTSETKALV GRGHDVDS UP LISUND AU ANVIS LIG OF DASEG
Ginkgo biloba	(262) HD V FF SQRHNCGCSETDHCEGSSPMD KNGIPLSWLCNRVKVEESEDVDHVFG SDIKV GEDIDLCDITV
Dorcoceras hygrometricum	(219) DEGLET MODDAY RKNSDSFEIERV V E YITLEHNHDQVGV DLGT - DNDOD - IASO EN AAQSGDQ
Sesamum indicum	(233) HERE REPORTED KGKELHDCSNLLPS-GRVKVD DVALMA-MGHENHHLVVF MGVT-REFKIDVG - VALUE DI AQNE-ER
Nicotiana tabacum	(233) degler LGGCNKE NSMECSMVSNEKVLI D VVMDTTTTSHD I I GDT- OFK DADS - INING D VGEVVMKI
Populus euphratica	(218) TKD E LUSKGEDEGQFCFADKV MKD QEM TIIDCHF QDFN ANSLGWN DETLVDGD VE RKVPVPET
Pyrus x bretschneideri	(234) HEG OLROCK - V SKGR L EDDDDDGIDGVDFMGCQLGES DV MK PFVLNFE VVDS AS G ODD MIVGIDS
Ricinus communis	(232) HED VOK K E OFKMQEREFSLASGQVI LEEEKEE-EAVRECHINTS ON AK PPORS DOGAT GOD SVATPORD
Vitis vinifera	(220) HEG. G. LUK K - V SVEYSLHSGRV LEEDIGGVMACQA A DOUTREP- TH NOVG - AT CORE VAVGAMD
Ginkgo biloba	(338) EDVEVKIKLDFDDYFQEEE - CLALDYED LIAWSDR - LWADNKTIQAFVD TSSDGTEGFQEYGIVPDLSL IARGASGDQGEQ
Dorcoceras hygrometricum	(297) LINIVEDSYKAGDDINKVNFL LDYEGLM AWDG K- W T EVELI FG COUDCMGTCGAM AYGEM
Sesamum indicum	(316) IGWNSCFQECKMGDSS TFL LDYEG LSAWDDKK- W TEEN NUSCOW DHCLGTSGAM VYGEM
Nicotiana tabacum	(309) GEDGVVGDGNYNE K LLNLDYES L AW D R- W N EXPEDIS NOW DC
Populus euphratica	(297) EMMTSTEYKEM R VSL LDYES IAW Not -C W TOSKORI ADUS IDS
Pyrus x bretschneideri	(312) CTEDHEHEEADEDAIDNSK R TILLDYEA AWDGS W S D DF-SE L DY
Ricinus communis	(316) IKSNNGEYKDNTRSKNDNKKKR LLSLDYEA (AW TW TW TW W D T RDV P EC QDC
Vitis vinifera	(296) MNNKVDDPKK NK LL-LDYEAL (AW SK - KW NCHRPEL POKK DCL T GIQV H VGE
	CCT domain
Ginkgo biloba	(426) VPVMNGGGD EGRHGE REARVMRY EKRRSRLFSK IRYEVRKLNAEKRPRMKGRFVKRTPGLSLE
Dorcoceras hygrometricum	(367) VVASCHGGANLUCCTREARVERYEKKRERLESKEIRYEVKKLNAEKRPRMKGREVKRESEVTPAPPLIPFAU
Sesamum indicum	(385) GRMS HAA <mark>NAANS REARV RY EKRR RLFSK IRYEVRKLNAEKRPRMKGRFVKREN</mark> APPAA AAAA AAFSFN
Nicotiana tabacum	(375) -IMN TT I R REARV RY EKRR RLFSKTIRYEVRKLNAEKRPRMKGRFVKRSNLVPNYPLLK
Populus euphratica	(362) LIS' TRG <mark>GIX E</mark> REARVM <mark>RYKEKRR RLFSK IRYEVRKLNAEKRPRMKGRFVKRT</mark> LM(-TTD
Pyrus x bretschneideri	(384) LN ICV PANVDCCREARV RY EKRR RLFSK IRYEVRKLNAEKRPRMKGRFVKRSSIACSAUDVLAS
Ricinus communis	(392) TN L/GVGGGG\P\\AG>CREARVSRYEKKRERLFSKSIRYEVRKLNAEKRPRMKGRFVKRET AFGPSAFELHAN
Vitis vinifera	(361) FG MGEQQANAGDGCREARVSRYCEKRRIRLFSKKIRYEVRKLNAEKRPRMKGRFVKRISSGG-PAADDLLN

Fig. 3: Sequence multi-alignment of the deduced GbCOL-6 protein with other plant CO proteins. The completely identical amino acids are indicated with red foreground and yellow background, the conservative amino acids are indicated with yellow foreground and cyan background, the block of similar amino acids are indicated with blue foreground and yellow background, the weakly similar amino acids are indicated with gray foreground and white background, the non-similar amino acids are indicated with black foreground and white background

# DISCUSSION

*Ginkgo biloba* is a perennial woody plant with a long juvenile phase. The transformation from vegetative growth to

reproductive growth is regulated by complex gene network. The CO plays a key role in the plant photoperiod pathway and its expression is precisely regulated by the biological clock. In this study, the full-length cDNA of the *CO* homologous gene



# Fig. 4: Phylogenetic tree of CONSTANS from different plants. The numbers of nodes represent the percentage of boot strap value obtained from 1000 sampling

was cloned from *G. biloba* leaves and named as GbCOL-6. The full length cDNA of GbCOL-6 was 1479 bp and encodes 492 amino acids. Protein analysis showed that GbCOL-6 contained one B-box structure and one CCT motif structure (Fig. 3). Tissue expression profile analysis showed that the *GbCOL-6* gene was expressed in all tissues of *G. biloba*. However, the expression level of GbCOL-6 was different and GbCOL-6 was highest expressed in female strobili.

Phytochrome (PhyA, PhyB, PhyC, PhyD, PhyE) and Cryptochrome (Cryl, Cry2) are the photosynthetic receptor for *CO* gene of plant. This photosynthetic receptor mainly used

to accept the light signal. It affects the stability of protein encoded by CO gene to control the expression of CO gene, so that the abundance of CO protein in the day showed a rhythmic change.

The CO protein regulates the expression of downstream genes *FT* and *SOC1* to control flowering time, which reflects the biological clock rhythm of CO gene expression<sup>26-28</sup>.

The CO homologous genes have been cloned in many species, most of which contain conserved B-box structures and CCT domains<sup>29,30</sup>. B-box was a zinc finger protein that could bind to DNA or protein-protein interaction<sup>31-33</sup>, B-box



Fig. 5: qRT-PCR relative expression of GbCOL-6 gene in different tissues

Table 3: Sequence accession numbers of Gene	Table 3: Sec	quence accession	numbers of	Gene
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Gene	Species	Accession number
PaCOL1	Piceaabies	CAK26109.1
PpCOL1	Pinus pinaster	AFV79556.1
CaCOL2	Capsicum annuum	XP_016561206.1
SpCOL2	Solanum pennellii	XP_015065855.1
TpCOL3	Thespesia populneoides	AJR28758.1
GhCOL3	Gossypium hirsutum	AJR28713.1
PdCOL4	Phoenix dactylifera	XP_008789698.1
AtCOL4	Amborella trichopoda	XP_006845571.1
RsCOL5	Raphanus sativus	XP_018452091.1
BnCOL5	Brassica napus	XP_013683363.1
RcCOL6	Ricinus communis	XP_002533412.1
CsCOL6	Camelina sativa	XP_010470801.1
GbCOL-6	Ginkgo biloba	MG251395
AtCOL7	Arabidopsis thaliana	NP_177528.1
GhCOL7	Erycinapusilla	AGI62031.1
CsCOL8	Camelina sativa	XP_010479338.1
RsCOL8	Raphanus sativus	XP_018490515.1
AtCOL9	Arabidopsis thaliana	NP_001118599.1
CsCOL9	Camelina sativa	XP_010486334.1
CsCOL10	Camelina sativa	XP_010493639.1
BrCOL10	Brassica rapa	XP_009151626.1
GaCOL11	Gossypium arboretum	KHG29511.1
GarCOL11	Gossypium arboretum	KHG24248.1
GaCOL12	Gossypium arboretum	KHG03964.1
GhCOL12	Gossypium hirsutum	XP_016666417.1
GaCOL13	Gossypium arboretum	KHG14594.1
GhCOL13	Gossypium hirsutum	XP_016737926.1
GaCOL14	Gossypium arboretum	KHG22096.1
GhCOL14	Gossypium hirsutum	XP_016727140.1
PeCOL15	Populus euphratica	XP_011020834.1
CmCOL15	Cucumismelo	XP_008454034.1
GrCOL16	Gossypium raimondii	XP_012467882.1
GhCOL16	Gossypium hirsutum	XP_016706545.1
AtCOL	Arabidopsis thaliana	CAA71587.1
StCOL	Solanum tuberosum	NP_001274795.1

domain has two conserved amino acid residues (Cys and His) that bind to the zinc atom to tightly fold the entire zinc finger protein, indicating that GbCOL-6 has similar characteristics as the class II CO gene of the CO family of *A. thaliana* and has a normal B-box structure and a CCT conserved domain, which belongs to the CO homologous gene (Fig. 3). Therefore, it is presumed that the main function of the B-box structure of GbCOL-6 is to maintain the stability of the protein.

The number of members of the CO gene family is also divers in different plants. There are 17 members of *A. thaliana*<sup>24</sup>, 16 members of *O. sativa*<sup>25</sup> and 14 inferred CO members in *V. vinifera*<sup>16,25,34</sup>. According to Yan *et al.*<sup>21</sup>, the CO gene also contains multiple members in *G. biloba*. The CO genes have been cloned from *A. thaliana*<sup>33</sup>, *O. sativa*<sup>34</sup> and *Triticum aestivum*<sup>35</sup> and they have been found to have similar photoperiod regulation and transcription patterns, but the function of plants is different. Therefore, the structure, regulation and function of *CO* genes in each plant need to be further studied.

This study found that GbCOL-6 isolated from *G. biloba* was specifically expressed in the female strobilis of *G. biloba*. However, GbCOL-6 rarely was expressed in roots of *G. biloba*. In *V. vinifera*, the expression of *VvCO* and *VvCOL1* are also the lowest in roots<sup>16</sup>. According to Ye *et al.*<sup>20</sup> in the study of *S. lycopersicum CONSTANS* homologous gene expression in different tissues and organs found that *SLCOL1* in roots, stems and leaves are also expressed, but the highest expression of leaves of *S. lycopersicum*. The reason may be due to the ecological habits and developmental status of different plants.

In this study, the cloning and expression analysis of GbCOL-6 will contribute to the development of flowering gene networks of *G. biloba*. Nevertheless, the flowering regulatory mechanism of GbCOL-6 only stays at the theoretical stage and lacks specific verification. In the following experiment, the specific role of *GbCOL-6* gene can be further verified by transgenic technology.

# CONCLUSION

In conclusion, a CO family gene (*GbCOL-6*) in the photoperiod pathway from *G. biloba* was cloned for the first time. The full length cDNA of GbCOL-6 was 1479 bp and encodes 492 amino acids. The GbCOL-6 protein contains two conserved domains (B-box and CCT domain) and the amino acid sequence showed high similarity to other plant CO proteins. Phylogenetic analysis classified GbCOL-6 as ClassII of the CO gene family. The GbCOL-6 is specifically expressed in female strobilis. The flowering regulatory mechanism of GbCOL-6 was elucidated. This finding can provide genetic resources for shortening the juvenile phase of *G. biloba* and other woody plants and it can also lay a theoretical foundation for further study on the molecular mechanism of the flowering pathway of *G. biloba*.

# SIGNIFICANCE STATEMENT

This study discovers a *CO* family gene (*GbCOL-6*) from *G. biloba* that can be beneficial for shortening the juvenile phase of woody plants. The GbCOL-6 is a key flowering gene in the photoperiod pathway of plants and can be used to regulate the flowering time of *G. biloba* to obtain terpenoids in fruits. This study will help the researcher to uncover the critical areas of key flowering genes in the flowering pathway from *G. biloba* that many researchers were not able to explore. The study may be helpful the extraction of natural terpenoids in *G. biloba*.

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# REFERENCES

1. Chen, L., F. Xu, R. Cai and S.Y. Cheng, 2008. Research advances in regulations of flowering genes and genetic improvement of the juvenile phase of *Ginkgo biloba*. J. Henan Agric. Sci., 37: 13-16.

- 2. Cheng, S.Y., F. Xu and Y. Wang, 2009. Advances in the study of flavonoids in *Ginkgo biloba* leaves. J. Med. Plants Res., 3: 1248-1252.
- 3. Smith, J.V. and Y. Luo, 2004. Studies on molecular mechanisms of *Ginkgo biloba* extract. Applied Microbiol. Biotechnol., 64: 465-472.
- Jia, L., C.D. Li and B.Z. Bai, 2004. Research progress on cultivation and propagation pattern of *Ginkgo biloba*. J. Agric. Technol., 6: 74-76.
- 5. Jack, T., 2004. Molecular and genetic mechanisms of floral control. Plant Cell, 16: S1-S17.
- 6. Jung, C. and A.E. Muller, 2009. Flowering time control and applications in plant breeding. Trends Plant Sci., 14: 563-573.
- 7. Jackson, S.D., 2009. Plant responses to photoperiod. New Phytol., 181: 517-531.
- Chen, F.L., Y.F. Fu and C.T. Lin, 2009. Research progress on CO/FT regulon and its role in adjusting plant flowering. J. Agric. Sci. Technol., 11: 17-22.
- 9. Wu, L.C., L.L. Chang, X. Chen and L. Ku, 2010. Expression and regulation of plant gene photoperiod CO reaction. Chin. Agri. Sci. Bull., 26: 116-121.
- Samach, A., H. Onouchi, S.E. Gold, G.S. Ditta, Z. Schwarz-Sommer, M.F. Yanofsky and G. Coupland, 2000. Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. Science, 288: 1613-1616.
- 11. Suarez-Lopez, P., K. Wheatley, F. Robson, H. Onouchi, F. Valverde and G. Coupland, 2001. CONSTANS mediates between the circadian clock and the control of flowering in *Arabidopsis*. Nature, 410: 1116-1120.
- 12. Yanovsky, M.J. and S.A. Kay, 2002. Molecular basis of seasonal time measurement in *Arabidopsis*. Nature, 419: 308-312.
- Lu, Y., Y. Liu, Y. Sun, J. Mu, M. Ren, X. Zhang and Z. Wang, 2013. Molecular cloning and analysis of a CONSTANS homolog from *Nicotiana tabacum*. J. Anim. Plant Sci., 23: 1360-1365.
- Shi, Y.B., D. Zhang, X.H. Shen, L. Wang and L.H. Zhuo, 2014. Cloning and expression analysis of *CONSTANS* homologous gene in *Agapanthus africanus* Hoffmgg. J. Beijing Forest. Univ., 36: 113-120.
- 15. Zhao, Z.Q., L.J. An, F. Li and T.H. Li, 2010. Cloning and expression analysis of CO gene from *Fragaria ananassa* Duch. J. China Agric. Univ., 15: 19-24.
- Almada, R., N. Cabrera, J.A. Casaretto, S. Ruiz-Lara and E.G. Villanueva, 2009. *VvCO* and *VvCOL1*, two *CONSTANS* homologous genes, are regulated during flower induction and dormancy in grapevine buds. Plant Cell Rep., 28: 1193-1203.
- 17. Jeong, D.H., S.K. Sung and G. An, 1999. Molecular cloning and characterization of constans-like cDNA clones of the fuji apple. J. Plant Biol., 42: 23-31.
- Wang, X.L., X.L. Liu, Y.C. Yang, W. Chen, W.C. Li and J.H. Wu, 2010. Cloning and analysis of transient regulatory gene CONSTANS of *Festuca elata*. Mol. Plant Breed., 8: 45-52.

- Zhang, J.X., D.L. Li, R. Wang, C.L. Liu, Y.B. Yuan and C.H. Ma, 2014. CONSTANS gene cloning from Pyrus pyrifolia Nakai and its prokaryotic expression. J. Plant Genet. Resour., 15: 824-830.
- Ye, X.L., Z.Y. Liu, X.X. Wang, Y.C. Du and Y.M. Guo, 2008. Molecular cloning and expression analysis of a CONSTANS-like gene SLCO1 from *Solanum lycopersicum*. Acta Hortic. Sin., 35: 1607-1612.
- Yan, J., D. Mao, X. Liu, L. Wang and F. Xu *et al.*, 2017. Isolation and functional characterization of a circadian-regulated *CONSTANS* homolog (*GbCO*) from *Ginkgo biloba*. Plant Cell Rep., 36: 1387-1399.
- 22. Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol., 25: 169-193.
- 23. Schmittgen, T.D. and K.J. Livak, 2008. Analyzing real-time PCR data by the comparative  $C_T$  method. Nat. Protoc., 3: 1101-1108.
- Robson, F., M.M.R. Costa, S.R. Hepworth, I. Vizir, P.H. Reeves, J. Putterill and G. Coupland, 2001. Functional importance of conserved domains in the flowering time gene *CONSTANS* demonstrated by analysis of mutant alleles and transgenic plants. Plant J., 28: 619-631.
- 25. Griffiths, S., R.P. Dunford, G. Coupland and D.A. Laurie, 2003. The evolution of *CONSTANS-like* gene families in barley, rice and arabidopsis. Plant Physiol., 131: 1855-1867.
- Mockler, T., H. Yang, X.H. Yu, D. Parikh, Y.C. Cheng, S. Dolan and C. Lin, 2003. Regulation of photoperiodic flowering by *Arabidopsis* photoreceptors. Proc. Natl. Acad. Sci. USA., 100: 2140-2145.

- 27. Valverde, F., A. Mouradov, W. Soppe, D. Ravenscroft, A. Samach and G. Coupland, 2004. Photoreceptor regulation of CONSTANS protein in photoperiodic flowering. Science, 303: 1003-1006.
- 28. Klejnot, J. and C. Lin, 2004. A CONSTANS experience brought to light. Science, 303: 965-966.
- 29. Putterill, J., F. Robson, K. Lee, R. Simon and G. Coupland, 1995. The CONSTANS gene of *Arabidopsis* promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell, 80: 847-857.
- Yano, M., Y. Katayose, M. Ashikari, U. Yamanouchi and L. Monna *et al.*, 2000. *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. Plant Cell, 12: 2473-2483.
- Borden, K.L.B., 1998. RING fingers and B-boxes: Zinc-binding protein-protein interaction domains. Biochem. Cell Biol., 76: 351-358.
- 32. Torok, M. and L.D. Etkin, 2001. Two B or not two B? Overview of the rapidly expanding B-box family of proteins. Differentiation, 67: 63-71.
- Khanna, R., B. Kronmiller, D.R. Maszle, G. Coupland, M. Holm, T. Mizuno and S.H. Wu, 2009. The *Arabidopsis* B-box zinc finger family. Plant Cell, 21: 3416-3420.
- Ben Naim, O., R. Eshed, A. Parnis, P. Teper Bamnolker and A. Shalit *et al.*, 2006. The CCAAT binding factor can mediate interactions between CONSTANS like proteins and DNA. Plant J., 46: 462-476.
- 35. Ran, C., H. Shao, J. Yu, C. Kou, Y. Li, L.Q. Li and X.J. Li, 2014. Cloning and analysis of *CO-like* gene *TaCO9* from wheat (*Triticum aestivum* L.). J. Tritic. Crop, 34: 1319-1326.