Characterization of Mungbean *CONSTANS-LIKE* Genes and Functional Analysis of *CONSTANS-LIKE 2* in the Regulation of Flowering Time in *Arabidopsis*

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CONSTANS-LIKE (*COL*) genes play important roles in the regulation of plant growth and development, and they have been analyzed in many plant species. However, few studies have examined *COL* genes in mungbean (*Vigna radiata*). In this study, we identified and characterized 31 mungbean genes whose proteins contained B-Box domains. Fourteen were designated as *VrCOL* genes and were distributed on 7 of the 11 mungbean chromosomes. Based on their phylogenetic relationships, *VrCOLs* were clustered into three groups (I, II, and III), which contained 4, 6, and 4 members, respectively. The gene structures and conserved motifs of the *VrCOL* genes were analyzed, and two duplicated gene pairs, *VrCOL1/VrCOL2* and *VrCOL8/VrCOL9*, were identified. A total of 82 *cis*-acting elements were found in the *VrCOL* promoter regions, and the numbers and types of *cis*-acting elements in each *VrCOL* promoter region differed. As a result, the expression patterns of *VrCOLs* varied in different tissues and throughout the day under long-day and short-day conditions. Among these *VrCOL* genes, *VrCOL2* showed a close phylogenetic relationship with *Arabidopsis thaliana CO* and displayed daily oscillations in expression under short-day conditions but not long-day conditions. In addition, overexpression of *VrCOL2* accelerated flowering in *Arabidopsis* under short-day conditions by affecting the expression of the flowering time genes *AtFT* and *AtTSF*. Our study lays the foundation for further investigation of *VrCOL* gene functions.

Keywords: mungbean, flowering time, CONSTANS, *VrCOL2*, genome wide

INTRODUCTION

Flowering time is a key factor that influences crop growth and development, and crops achieve higher yields when they flower at the correct time. To regulate flowering time, crops sense the interactions between endogenous and environmental factors to determine the transition from vegetative to reproductive growth (Wickland and Hanzawa, 2015; Beinecke et al., 2018; Eom et al., 2018; Xu and Chong, 2018). Several functional pathways have been identified that regulate the switch from vegetative to reproductive development. These include the photoperiodic, vernalization, ambient temperature, plant hormone, and autonomous flowering pathways

(Boss et al., 2004; Jack, 2004; Baurle and Dean, 2006; Wickland and Hanzawa, 2015; Xu and Chong, 2018; Ronald and Davis, 2019; Taylor et al., 2019; Zhang et al., 2019). A number of genes in these pathways are known to be involved in flowering time regulation, including *CONSTANS-LIKE* (*COL*) genes, phosphatidyl ethanolamine-binding protein (PEBP) genes, and several members of the MADS-box gene family (Gangappa and Botto, 2014; Wickland and Hanzawa, 2015; Beinecke et al., 2018; del-Olmo et al., 2019; Jin et al., 2019, 2020; Jing et al., 2019; Lee et al., 2019; Nam et al., 2019; Ning et al., 2019; Parenicova et al., 2019).

COL genes belong to the zinc-finger transcription factor family and play central roles in plant growth and development (Khanna et al., 2009; Gangappa and Botto, 2014). COL proteins are identified based on their conserved structure, which includes one or two BBX (B-Box) domains and one CCT (CONSTANS, CO-like, and TIMING of CAB1) domain (Khanna et al., 2009; Gangappa and Botto, 2014). The BBX domain can be further divided into two types, B-Box1 and B-Box2, which are recognized by their consensus sequences and the distances between their zinc-binding residues, which are considered to be involved in protein-protein interactions (Khanna et al., 2009). The CCT domain has important functions in transcriptional regulation and nuclear protein transport (Robson et al., 2001; Khanna et al., 2009; Yan et al., 2011; Gangappa and Botto, 2014). The COL proteins are grouped into three classes based on the number and type of their conserved domains. Classes I and II have two distinct BBX domains and one CCT domain, whereas class III has only one BBX and one CCT domain. Classes I, II, and III contain 6, 7, and 4 members in *Arabidopsis*, respectively. In addition, several COL proteins contain valine-proline (VP) motifs in their C termini (Khanna et al., 2009; Gangappa and Botto, 2014).

Among these *COL* members, *AtCO* (*AtBBX1*) and its homologs are well studied in many plant species (Khanna et al., 2009; Gangappa and Botto, 2014; Luo et al., 2018; Luccioni et al., 2019; Serrano-Bueno et al., 2020). *AtCO* is expressed in a rhythmic manner and coordinates light pathway and circadian clock signal inputs in *Arabidopsis* (Putterill et al., 1993, 1995; Andres and Coupland, 2012; Song et al., 2013). Thus, *AtCO* plays an important role in the regulation of flowering time by the photoperiod-dependent pathway. *Atco* mutants exhibit delayed flowering time under long-day conditions (LD), but under short-day conditions (SD), their flowering times are similar to those of wild-type plants. By contrast, *AtCO* overexpression plants show early flowering time under both LD and SD conditions (Khanna et al., 2009; Gangappa and Botto, 2014). The AtCO protein binds to *cis*-acting elements in the promoter region of the flowering activator *FLOWERING LOCUS T* (*AtFT*) to active *AtFT* expression. Moreover, AtCO is regulated by many flowering factors, such as AtGI (GIGANTEA), AtCDF1 (CYCLING DOF FACTOR 1) and AtFKF1 (FLAVIN BINDING, KELCH REPEAT, F-BOX1) (Imaizumi et al., 2005; Sawa et al., 2007). *OsHd1* (*Heading date 1*), the *AtCO* ortholog in rice, accelerates flowering under SD conditions but delays flowering under LD conditions through the regulation of the *AtFT* orthologs *OsHd3a* (*Heading date 3a*) and *OsRFT1* (*RICE FLOWERING LOCUS T1*) (Yano et al., 2000; Komiya et al., 2008, 2009). The

soybean *AtCO* orthologs *GmCOL1*, *GmCOL2*, *GmCOL3*, and *GmCOL4* can complement the late flowering phenotype of *Atco* mutants (Wu et al., 2014). In addition to their functions in flowering time and circadian clock regulation, some COL proteins are also involved in abiotic or biotic stress responses, root development and stomatal opening (Khanna et al., 2009; Gangappa and Botto, 2014).

Mungbean is a diploid legume crop, and its seeds contain proteins and nutrients that are essential for human nutrition (Keatinge et al., 2011). The cultivated mungbean is thought to have been domesticated in India, from which it then spread to other areas (Fuller, 2007). Mungbean is considered to be an SD crop, and flowering time is a critical factor influencing its production (Vas Aggarwal and Poehlman, 1977; Imrie, 1996; Kim et al., 2015). Mungbean plants produce a large number of flowers, but only a few set pods. Approximately 70–90% of the flowers are shed, mainly the later-formed flowers of the racemes (Kumari and Verma, 1983; Mondal et al., 2011). Thus, it has been suggested that the prevention of late flowering is an important way to increase mungbean yield (Isobe et al., 1995; Kuroda et al., 1998; Mondal et al., 2011). The sequencing of the mungbean genome provides genetic resources for the investigation of gene functions (Kang et al., 2014), and the study of mungbean flowering time genes can therefore provide essential information for further modification of mungbean cultivars to increase yield. Until now, there has been limited information on the functions of genes involved in mungbean flowering time regulation. In this study, we identified mungbean *COL* genes and investigated their characteristics, including chromosomal distributions, gene structures, *cis*-acting elements and gene expression patterns. We also analyzed the functions of *VrCOL2* in the regulation of flowering time. Our findings will provide useful information for further characterization of mungbean *COL* gene functions.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The mungbean reference genome variety VC1973A was provided by Suk-Ha Lee at Seoul National University, Seoul, South Korea (Kang et al., 2014) and used for all experiments in this study. Mungbean seeds were geminated in tap water for 1 day and then planted in soil-filled pots. Seedlings were grown in growth chambers with 16 h 25°C light/8 h 25°C dark cycles for LD conditions and 10 h 25°C light/14 h 25°C dark cycles for SD conditions. Leaves of 5-week-old mungbean plants were sampled every 4 h after lights-on and used to analyze the diurnal rhythm of gene expression. Multiple tissues were collected from field-grown mungbean plants sown at the end of May in Qingdao, China, including roots, nodule roots, shoot apices, stems, leaves, flowers, pods and seeds (Shi et al., 2021). Tissues were collected in the afternoon (ZT 10–12) in early July for gene expression analysis, and all samples were stored at −80°C before RNA extraction. *Arabidopsis* plants were grown in growth chambers with 16 h 23°C light/8 h 21°C dark cycles for LD conditions and 10 h 23°C light/14 h
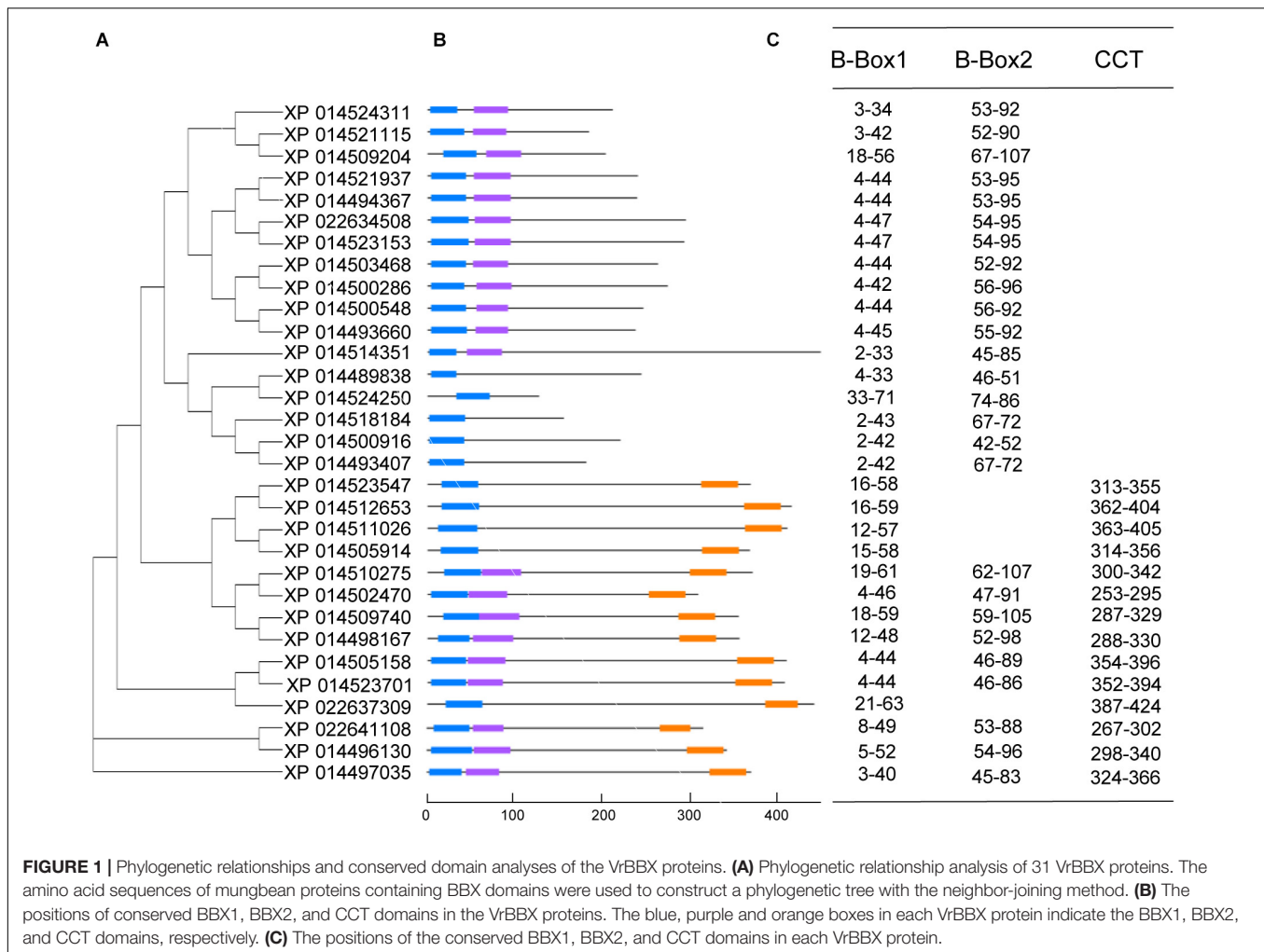
FIGURE 1 | Phylogenetic relationships and conserved domain analyses of the VrBBX proteins. **(A)** Phylogenetic relationship analysis of 31 VrBBX proteins. The amino acid sequences of mungbean proteins containing BBX domains were used to construct a phylogenetic tree with the neighbor-joining method. **(B)** The positions of conserved BBX1, BBX2, and CCT domains in the VrBBX proteins. The blue, purple and orange boxes in each VrBBX protein indicate the BBX1, BBX2, and CCT domains, respectively. **(C)** The positions of the conserved BBX1, BBX2, and CCT domains in each VrBBX protein.

21°C dark cycles for SD conditions. Leaves of 2-week-old *Arabidopsis* were collected every 4 h after lights-on for gene expression analysis.

Identification of Mungbean *VrCOL* Members

The amino acid sequences of *Arabidopsis* BBXs were used as blast queries against the National Center for Biotechnology Information (NCBI) and mungbean genome databases[1] to search for mungbean VrBBX proteins (Kang et al., 2014). The presence of conserved BBX and CCT domains in candidate genes was confirmed using the Pfam database and InterPro program with default parameters (Finn et al., 2017; El-Gebali et al., 2019).

Phylogenetic Analysis

The amino acid sequences of CO and COL proteins from *Arabidopsis*, soybean, *Medicago*, mungbean, rice, and maize were aligned using ClustalW2 (Oliver et al., 2005), and the resulting alignment was used to construct a phylogenetic tree in MEGA

7.0 using the neighbor-joining method with default parameters (Kumar et al., 2016). In addition, VrBBX proteins were aligned separately in ClustalW2 and used to construct a phylogenetic tree in MEGA 7.0 with the neighbor-joining method.

Chromosomal Distribution and Duplication Analyses

The physical positions of *VrCOL* genes were obtained from NCBI, and a chromosomal location map was constructed using MapInspect software (Mike Lischke, Berlin, Germany). Duplicated gene pairs were identified using OrthoMCL software as described by Fischer et al. (2011) and Jin et al. (2020). The duplicated gene pairs were defined as having greater than 60% amino acid sequence similarity and were visualized using Circos software (Krzywinski et al., 2009).

Analyses of Exon-Intron Organization, Conserved Domains, Sequence Logos, Protein Motifs, and *Cis*-Acting Elements
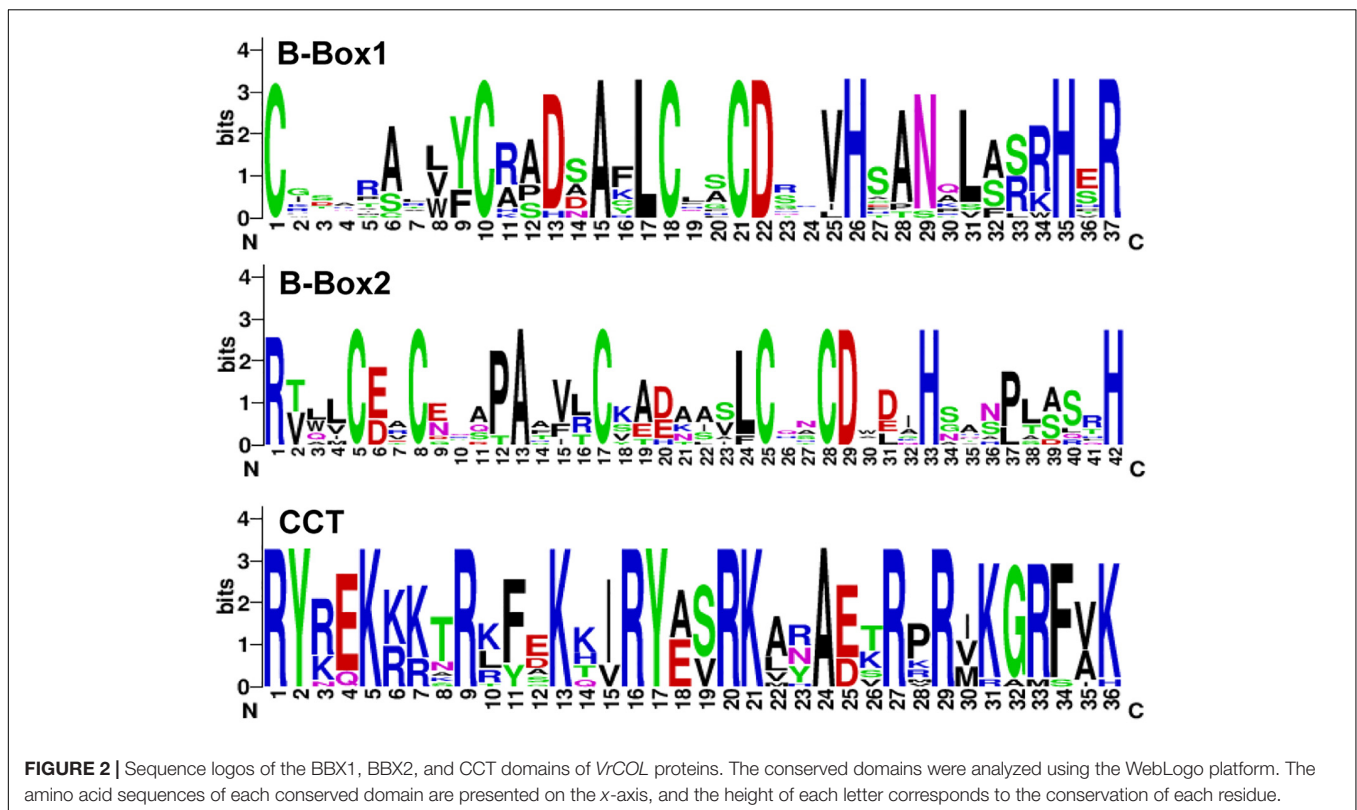
The genomic and CDS sequences of mungbean *VrCOL* genes were obtained from NCBI and used as inputs to the Gene

[1]http://plantgenomics.snu.ac.kr/mediawiki-1.21.3/index.php/Main_Page

TABLE 1 | *VrCOL* genes identified in mungbean genome.

Gene ID	Genomic length/bp	CDS/bp	No. of AA	pI	Mol.Wt/Da	GC%	Chr	Strand	Gene names
XP_014498167	2,481	1,074	357	5.82	39,253.5	42.93	4	−	*VrCOL1*
XP_014509740	2,195	1,071	356	5.27	39,685.26	40.68	1	−	*VrCOL2*
XP_014502470	1,506	933	310	7.01	33,756.82	50.13	5	−	*VrCOL3*
XP_014510275	1,778	1,119	372	6.11	40,396.31	50.39	7	−	*VrCOL4*
XP_014511026	3,495	1,239	412	4.95	46,524.78	36.89	1	+	*VrCOL5*
XP_014505914	2,175	1,110	369	5.64	41,395.12	40.37	7	+	*VrCOL6*
XP_014512653	2,060	1,254	417	5.28	46,964.34	43.83	8	−	*VrCOL7a*
XP_014523547	1,960	1,113	370	9.22	42,031.83	40.10	N/A	+	*VrCOL7b*
XP_014505158	8,864	1,236	411	5.21	45,045.34	34.64	6	+	*VrCOL8*
XP_014523701	14,007	1,230	409	4.86	44,487.59	40.33	5	+	*VrCOL9*
XP_022637309	4,494	1,329	442	6.47	48,806.9	42.28	5	+	*VrCOL10*
XP_014496130	4,536	1,035	344	6.47	38,345.14	45.16	3	−	*VrCOL11*
XP_022641108	3,819	954	317	6.82	35,966.39	40.02	8	−	*VrCOL12*
XP_014497035	3,750	1,119	372	7.00	41,848.9	39.46	4	+	*VrCOL13*

Chr, chromosome number; AA, amino acid; Mol.Wt, molecular weight; pI, isoelectric point; N/A, not applicable.

FIGURE 2 | Sequence logos of the BBX1, BBX2, and CCT domains of *VrCOL* proteins. The conserved domains were analyzed using the WebLogo platform. The amino acid sequences of each conserved domain are presented on the *x*-axis, and the height of each letter corresponds to the conservation of each residue.
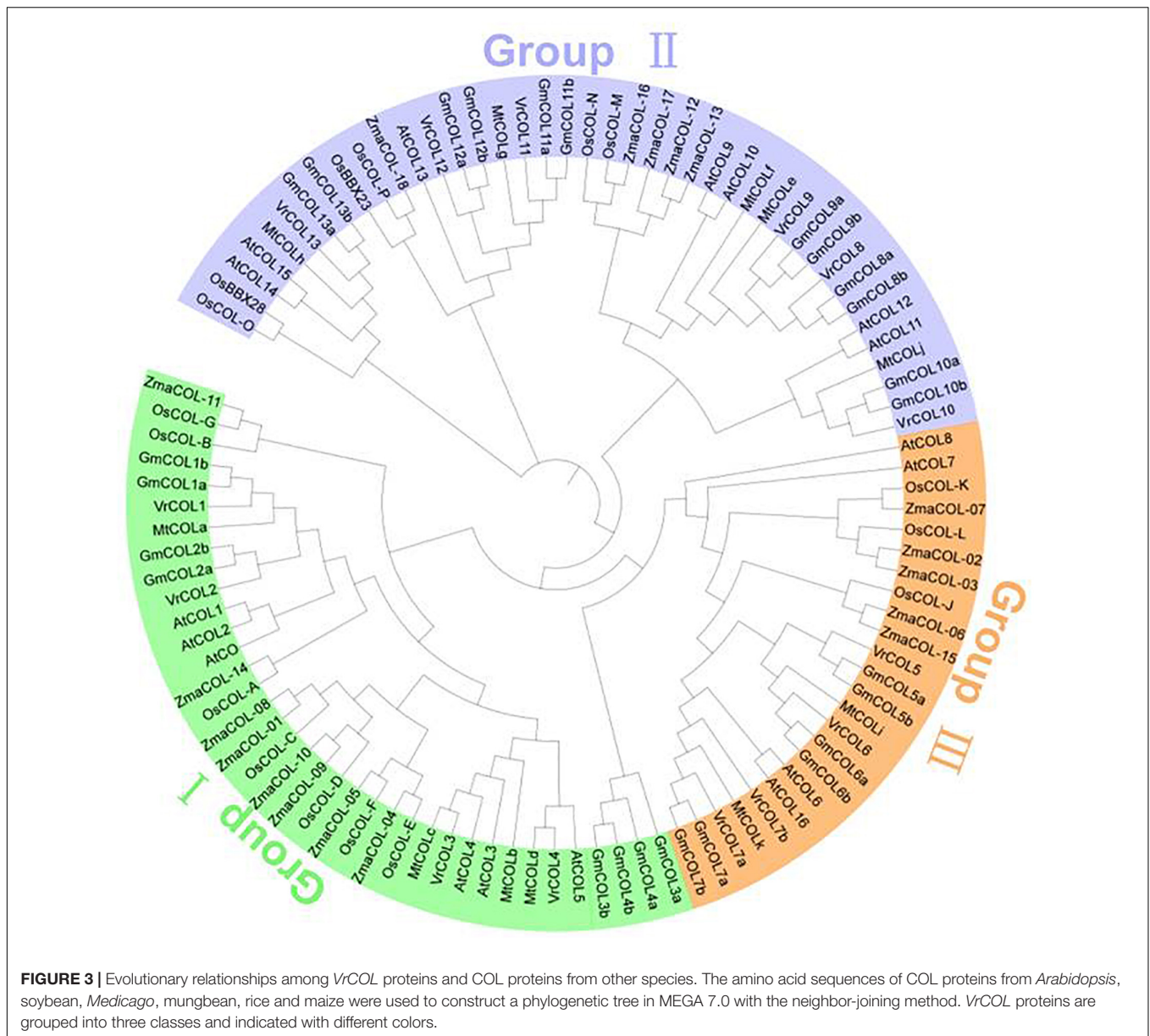
Structure Display Server (GSDS) to analyze their gene structures (Hu et al., 2015). The full-length amino acid sequences of *VrCOL* proteins were used to analyze the positions of the conserved BBX and CCT domains using the InterPro program (Finn et al., 2017). The sequence logos of the conserved BBX1, BBX2, and CCT domains were analyzed using the WebLogo platform (Crooks et al., 2004). The conserved motifs present in the *VrCOL* proteins were identified using MEME tools, with an optimum motif width of 11–50 amino acid residues (Bailey et al., 2009). The *cis*-acting elements in each *VrCOL*

promoter, 2 kb upstream of the initiation codon, were predicted by PlantCARE (Lescot et al., 2002).

Plasmid Construction and Plant Transformation

To investigate the functions of *VrCOL2*, a 35S: CDS-*VrCOL2* plasmid was constructed. The *VrCOL2* CDS was amplified from the cDNA of the sequenced mungbean variety VC1973A using primers with *Xho*I and *Xba*I digestion site sequences.

FIGURE 3 | Evolutionary relationships among *VrCOL* proteins and COL proteins from other species. The amino acid sequences of COL proteins from *Arabidopsis*, soybean, *Medicago*, mungbean, rice and maize were used to construct a phylogenetic tree in MEGA 7.0 with the neighbor-joining method. *VrCOL* proteins are grouped into three classes and indicated with different colors.

The resulting PCR fragment was digested by the restriction endonucleases *Xho*I and *Xba*I to generate sticky ends. The pPTN1171 vector was digested with *Xho*I and *Xba*I to generate a linearized plasmid (Ping et al., 2014). Then the *VrCOL2* and pPTN1171 fragments were ligated using T4 DNA ligase (Promega). The constructed plasmid was verified by sequencing. It was then introduced into *Arabidopsis* using the floral dip method (Bent, 2006), and successful transformation was confirmed by PCR. All primers are listed in **Supplementary Table 1**.

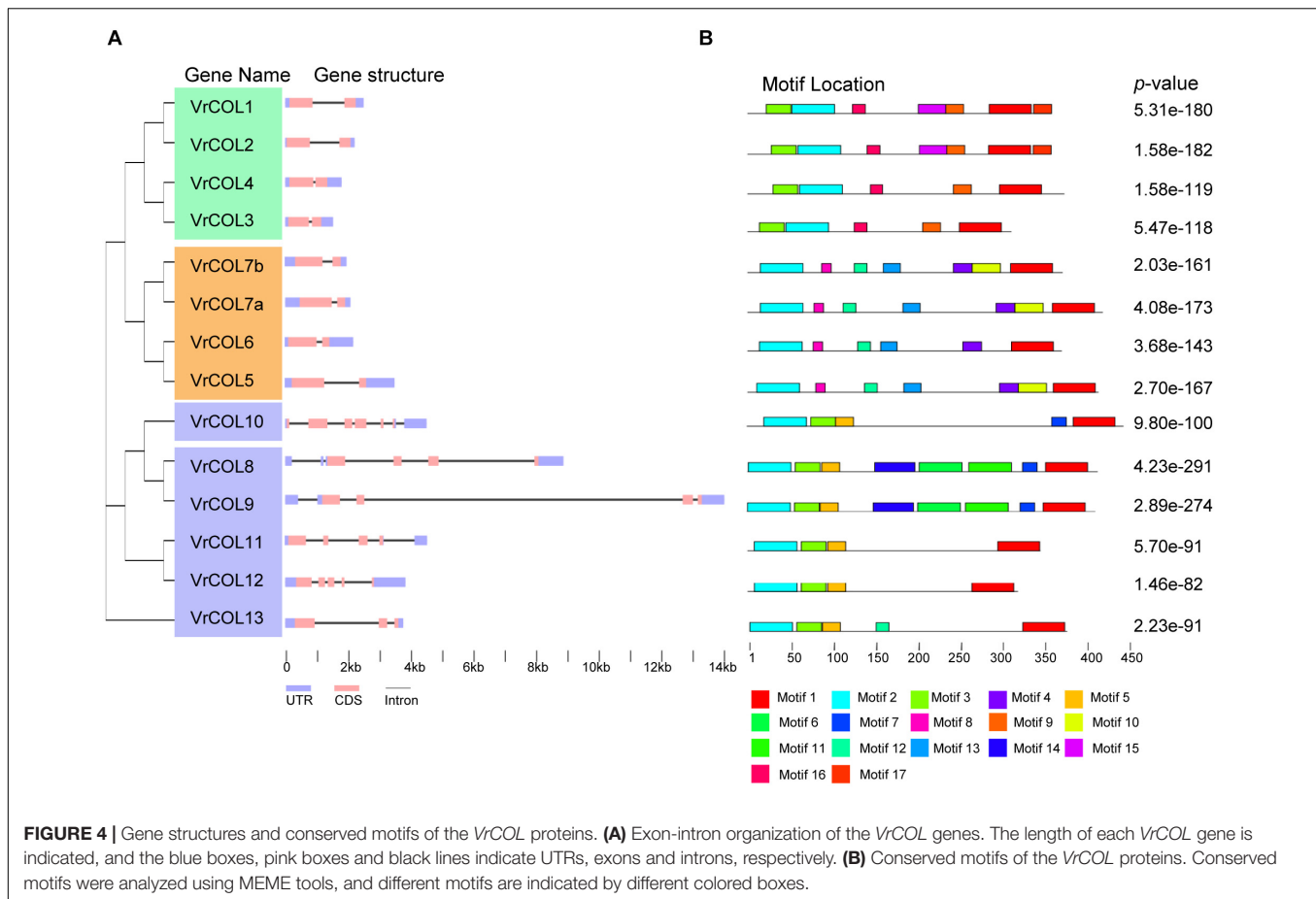## RNA Extraction and Transcription Analysis

RNA isolation and quantitative real-time PCR (qRT–PCR) analysis were carried out as described in Li et al. (2019).

Gene expression levels were normalized to an *Actin* gene from mungbean (*Vradi03g00210*) (Li et al., 2019). Each sample was analyzed using three biological replicates. All primers are listed in **Supplementary Table 1**.

RESULTS

Identification of *VrCOL* Genes in Mungbean

To search for mungbean *VrCOL* genes, we first identified mungbean proteins that contained BBX domains. The amino acid sequences of the conserved BBX domain (PF00643) and of *Arabidopsis* BBX proteins were used as blast queries against the mungbean genome database at NCBI. The presence of

FIGURE 4 | Gene structures and conserved motifs of the *VrCOL* proteins. **(A)** Exon-intron organization of the *VrCOL* genes. The length of each *VrCOL* gene is indicated, and the blue boxes, pink boxes and black lines indicate UTRs, exons and introns, respectively. **(B)** Conserved motifs of the *VrCOL* proteins. Conserved motifs were analyzed using MEME tools, and different motifs are indicated by different colored boxes.
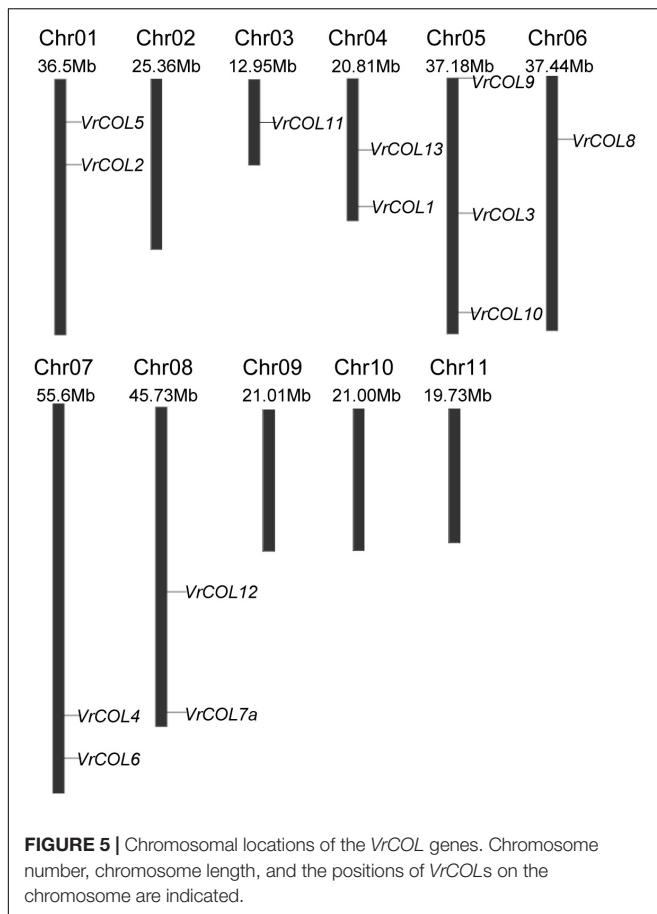
conserved BBX domains in each candidate mungbean gene was confirmed using Pfam and InterPro software, and a total of 31 *VrBBX* genes were identified in the mungbean genome (**Figure 1**). Among the VrBBX proteins, 17 contained only BBX domains, and 14 contained both BBX and CCT domains. The latter were designated *VrCOL* proteins (**Figure 1** and **Table 1**). We then analyzed the numbers and types of BBX and CCT domains in the *VrCOL* proteins, and found two distinct BBX domains (BBX1 and BBX2) and one CCT domain (**Supplementary Figure 1**). Sequence logos of the BBX1 ($CX_2CX_8CX_4AXLCX_2CDX_3HX_8HXR$), BBX2 ($CX_2CX_4AX_3CX_7CX_2CDX_3HX_8H$), and CCT ($RYX_2KX_3RX_3KX_2RYX_2RKX_2AX_2RXR$) domains were produced using WebLogo (**Figure 2** and **Supplementary Figure 1**). Nine *VrCOL* proteins contained one BBX1, one BBX2, and one CCT domain, and five *VrCOL* proteins contained one BBX1 and one CCT domain (**Figure 1** and **Table 1**).

Multiple characteristics of the *VrCOL* members were analyzed based on their genomic and protein sequences (**Table 1**). The genomic lengths of *VrCOL* genes ranged from 1,506 (*XP_014502470*) to 14,007 bp (*XP_014523701*), the CDS lengths ranged from 933 (*XP_014502470*) to 1,329 bp (*XP_022637309*), and the amino acid numbers ranged from 310 to 442. The isoelectric points of *VrCOL* proteins varied from 4.86 (*XP_014523701*) to 9.22 (*XP_014523547*), and their

molecular weights ranged from 33,756.82 Da (XP_014502470) to 48,806.9 Da (XP_022637309). The GC content, which influences gene stability to some degree, ranged from 34.64 to 50.39%, and 12 of the 14 *VrCOL* genes had lower than 50% GC content (**Table 1**).

Phylogenetic Analysis of the *VrCOL* Proteins

To analyze the evolutionary relationships among the *VrCOL* genes and obtain information from well-studied *CO* homologs in other species, a phylogenetic tree was constructed using 17 *Arabidopsis*, 26 soybean, 11 *Medicago*, 16 rice, 18 maize, and 14 mungbean CO and COL proteins (Gangappa and Botto, 2014; Wu et al., 2014, 2017; Hu et al., 2018). The *VrCOL* genes were named *VrCOL1* to *VrCOL13* based on their phylogenetic relationships with their soybean orthologs (**Figure 3** and **Table 1**). The COL proteins were grouped into three classes based on their phylogenetic relationships (Khanna et al., 2009; Gangappa and Botto, 2014; **Figure 3**). Classes I, II, and III contained 4, 6, and 4 *VrCOL* members, respectively (**Figure 3**). The BBX1 and BBX2 domains were located close to one another in the class I and II proteins, with the exception of *VrCOL10* (**Figure 1**), whereas class III proteins contained only one BBX domain (**Figures 1**, **3**).

FIGURE 5 | Chromosomal locations of the *VrCOL* genes. Chromosome number, chromosome length, and the positions of *VrCOL*s on the chromosome are indicated.

Among these *VrCOL* members, *VrCOL1* and *VrCOL2* showed close relationships to *Arabidopsis* AtCO, soybean GmCOL1a, GmCOL1b, GmCOL2a, and GmCOL2b and rice OsHd1 (OsCOL-A), all of which have documented roles in the regulation of flowering time (Khanna et al., 2009; Gangappa and Botto, 2014; Wu et al., 2014). This result suggests that *VrCOL1* and *VrCOL2* may play critical roles in the flowering time regulation of mungbean.

Gene Structures and Conserved Motifs of the *VrCOL* Genes

To investigate the gene structures of the *VrCOL* genes, we downloaded their genomic and CDS sequences from NCBI and analyzed them using the GSDS program (Hu et al., 2015). All the *VrCOL* members contained 5′ UTR and 3′ UTR regions. Their exon numbers ranged from two to six, and their intron numbers ranged from one to six. All the group I and III *VrCOL* members contained two exons and one intron (**Figure 4**). By contrast, group II members contained various numbers of exons (3–6) and introns (2–6), suggesting potential functional diversity among these genes (**Figure 4**). To further investigate the conservation and diversity of *VrCOL* protein structures, we analyzed putative protein motifs in the *VrCOLs*. A total of 17 distinct motifs were identified, and all *VrCOL* proteins contained motifs 1 and 2, which appeared to represent the

conserved BBX1 and CCT domains, respectively (**Figure 4** and **Supplementary Figure 2**). Most members of the same class shared some conserved motifs. For example, class I proteins shared motifs 1, 2, 3, 9, and 16, class II members shared motifs 1, 2, 3, and 5, and class III members shared motifs 1, 2, 4, 8, 12, and 13 (**Figure 4**).
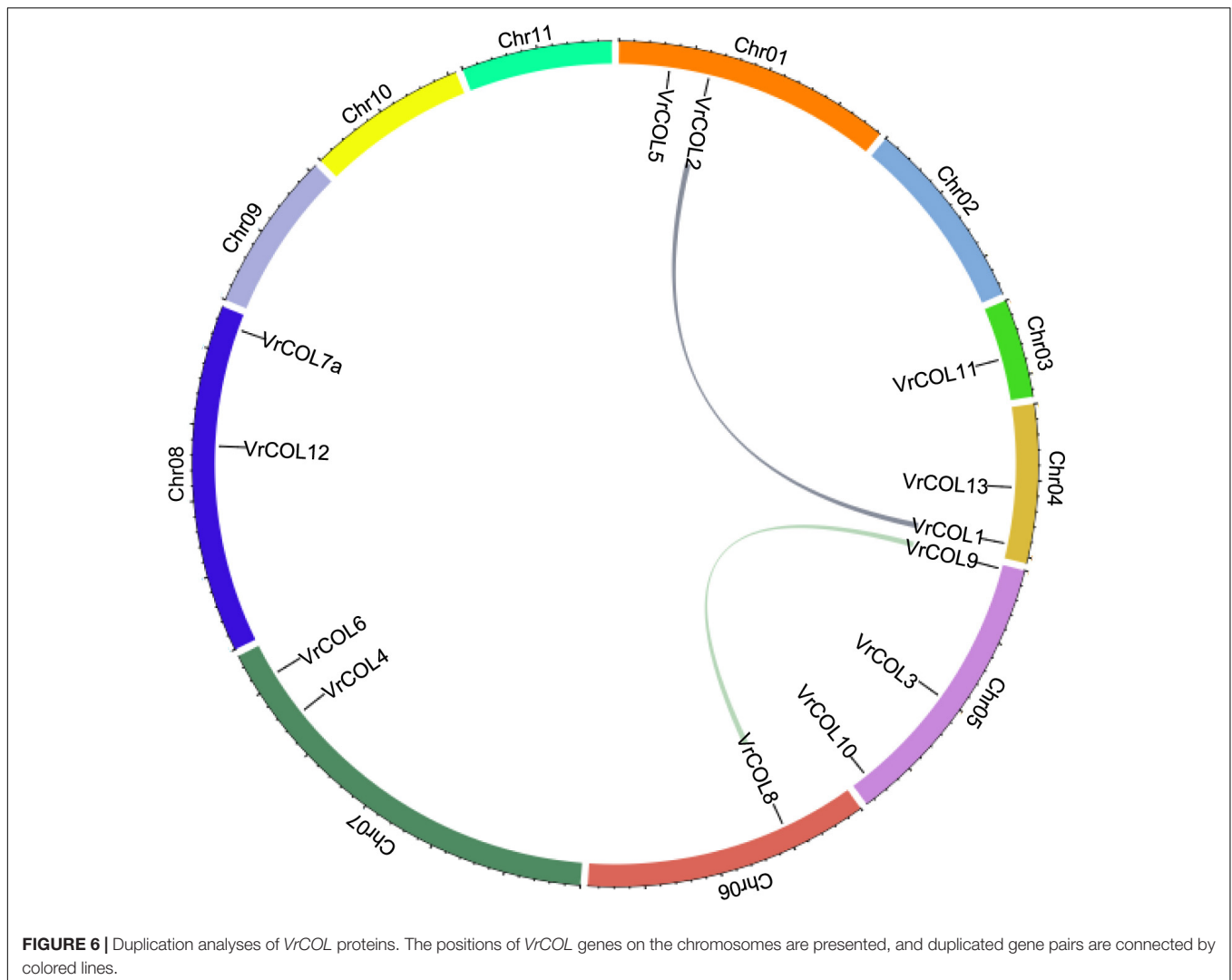
Chromosomal Distribution and Duplication Analysis of the *VrCOL* Genes

Some genes have evolved from common ancestors, and the chromosomal locations of *COL* genes may provide insight into changes in gene distribution during evolution. To visualize the chromosomal locations of the *VrCOL* genes, we mapped them to their physical positions in the mungbean genome. *VrCOL7b* was discarded due to a lack of related positional information. Seven of the 14 *VrCOL* genes were located on the positive strand. Seven of the 11 mungbean chromosomes contained *VrCOL* genes, with the exception of chromosomes 2, 9, 10, and 11 (**Figure 5** and **Table 1**). Chromosome 5 contained the greatest number of *VrCOL* genes (three), followed by chromosomes 1, 4, 7, and 8, with two genes on each. In addition, most of the *VrCOL* genes were located on the relatively long chromosomes (1, 5, 6, 7, and 8). Only three members (*VrCOL1*, *VrCOL11*, and *VrCOL13*) were located on the relatively short chromosomes 3 and 4 (**Figure 5**).

Mungbean has experienced one round of whole-genome duplication that produced many duplicated gene pairs (Kang et al., 2014; Li et al., 2019). To investigate the evolutionary relationships among the *VrCOLs*, we searched for duplicated gene pairs among them. Two interchromosomal duplication events were identified in chromosomes 1, 4, 5, and 6, producing the duplicated gene pairs *VrCOL1/VrCOL2* and *VrCOL8/VrCOL9* (**Figure 6**). The duplicated genes were clustered together in the phylogenetic tree (**Figure 1**). All the duplicated genes contained one BBX1, one BBX2 and one CCT domain and belonged to groups I and II; no duplicated gene pairs were found in group III. The duplicated genes *VrCOL1* and *VrCOL2* showed similar exon-intron organization and similar motifs, as did *VrCOL8* and *VrCOL9* (**Figure 4**), indicating that the duplicates may share similar functions.

Cis-Acting Element Analysis of the *VrCOL* Promoter Regions

To predict the potential expression responses of *VrCOL* genes, we investigated the *cis*-acting elements in their promoters using PlantCARE (Lescot et al., 2002). A total of 82 *cis*-acting elements were found across the 14 *VrCOL* promoter regions (2 kb upstream of the initiation codon) (**Supplementary Table 2**). Forty-five of them had predicted functions, including six development-related elements, four environmental-stress-related elements, three site-binding-related elements, nine hormone-responsive elements, three promoter-related elements and twenty light-responsive elements (**Table 2** and **Supplementary Table 2**). The various *VrCOL* promoter regions had different numbers and types of *cis*-acting elements, highlighting the functional diversity of these genes. All *VrCOL* promoters contained hormone-responsive elements,

FIGURE 6 | Duplication analyses of *VrCOL* proteins. The positions of *VrCOL* genes on the chromosomes are presented, and duplicated gene pairs are connected by colored lines.

light-responsive elements and promoter-related elements. Light-responsive elements were the most abundant element in each *VrCOL* promoter, with the exception of *VrCOL8* (**Table 2**), indicating that *VrCOL* genes may play critical roles in light-dependent signaling pathways. Environmental-stress-related elements were the most abundant element in the *VrCOL8* promoter (nine elements), indicating that *VrCOL8* may function in stress response (**Table 2**). All the *VrCOL* genes contained the promoter-related elements CAAT-Box and TATA-Box, which are basic promoter components. Thirteen of the 14 *VrCOLs* contained the hormone-responsive elements CGTCA-motif and TGACG-motif and the light-responsive element Box 4 (**Supplementary Table 2**), suggesting potential functions of these genes in related signaling pathways.

Transcription Patterns of *VrCOL* Genes in Different Tissues
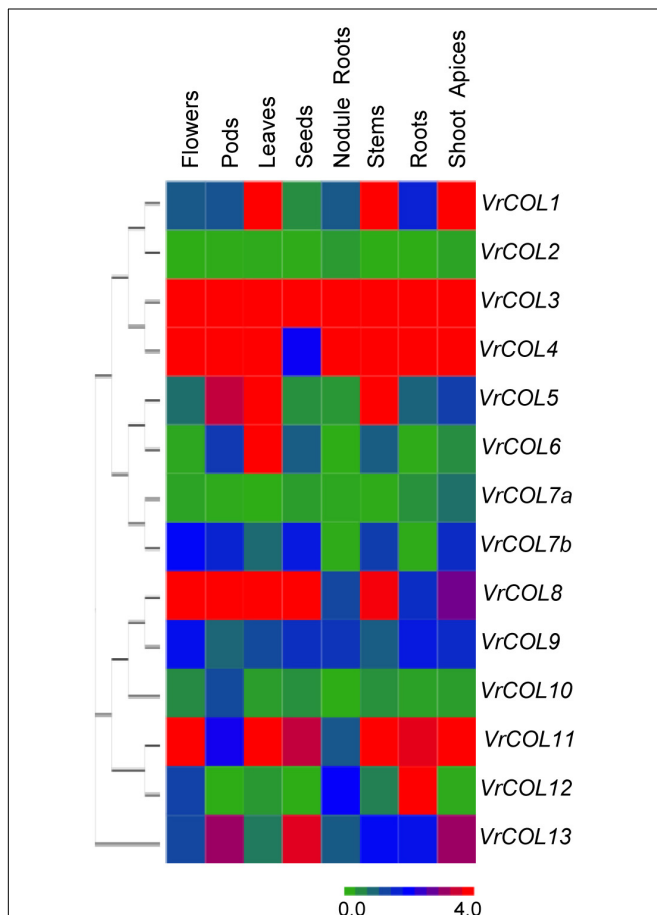
To shed light on the potential functions of *VrCOL* genes during plant development, we analyzed the expression of *VrCOL* genes in different tissues, including roots, nodule roots, shoot apices, stems, leaves, flowers, pods and seeds. *VrCOL* genes showed distinct expression patterns in different tissues (**Figure 7**). For example, *VrCOL3* was highly expressed in all tissues examined, whereas *VrCOL2* and *VrCOL7a* showed low expression in most tissues. Some genes were expressed at high levels in specific tissues, suggesting that they may have critical functions in those tissues. For example, *VrCOL6* showed high expression in leaves but low expression in nodule roots and flowers.

Duplicated genes may retain some common functions and evolve some new functions (Kondrashov et al., 2002; Wang et al., 2015). To investigate the conservation and diversity of duplicated genes, we also analyzed their tissue-specific expression patterns. *VrCOL1* and *VrCOL2* differed in their expression levels across all tissues examined, indicating that they may have different responses to the environment in these tissues. *VrCOL8* and *VrCOL9* showed similar expression levels in roots and nodule roots but different expression levels in other tissues (**Figure 7** and **Supplementary Figure 3**).

TABLE 2 | Numbers and types of *cis*-acting elements in each *VrCOL* promoter region.

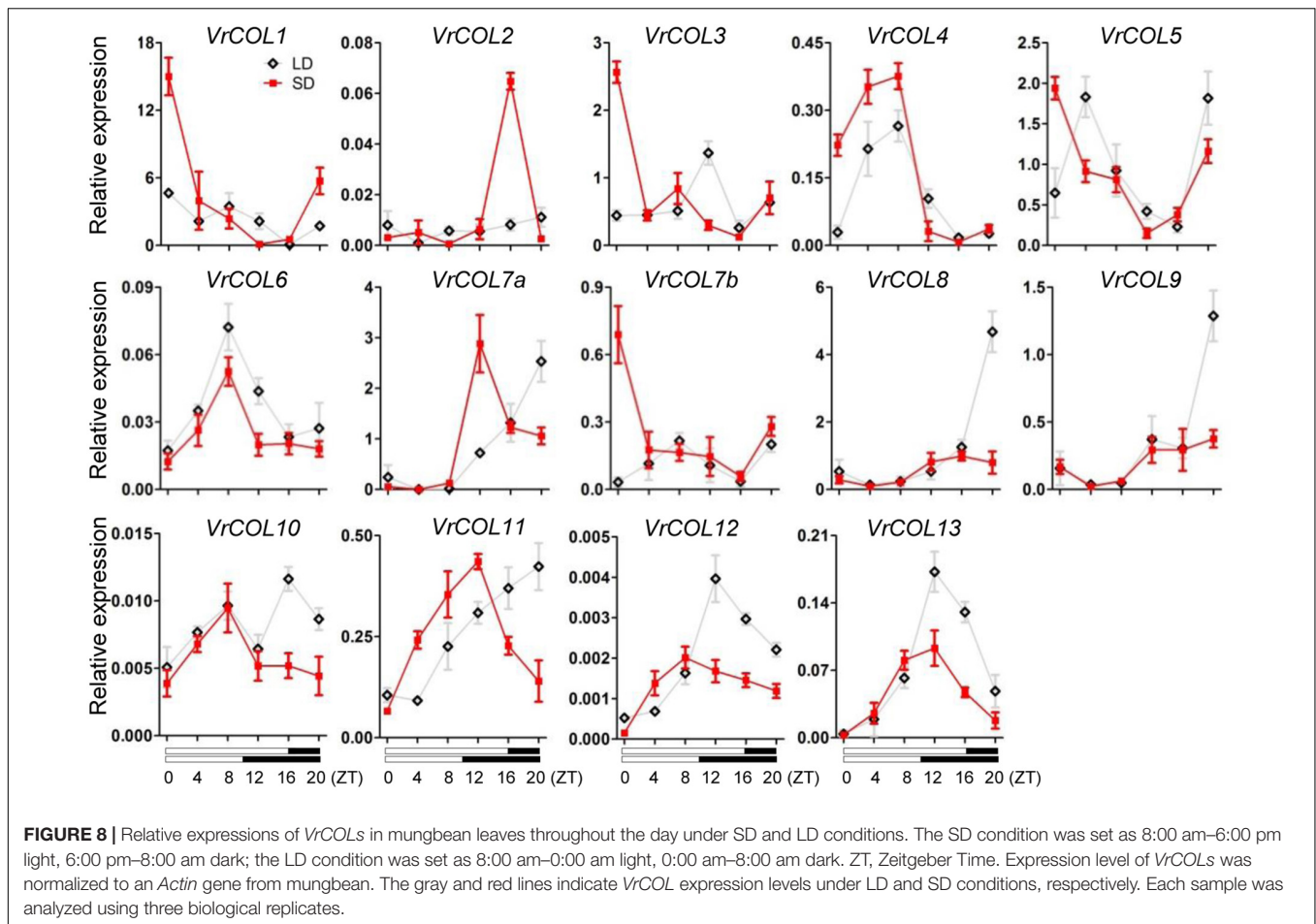| Gene name	Development related elements	Environmental stress related elements	Hormone-responsive elements	Light-responsive elements	Promoter related elements	Site-binding related elements	Others
VrCOL1	0	3	4	11	2	0	18
VrCOL2	1	3	4	6	2	1	18
VrCOL3	2	1	4	8	2	0	19
VrCOL4	1	0	4	11	2	2	17
VrCOL5	1	3	3	6	2	0	17
VrCOL6	1	0	4	11	2	2	17
VrCOL7a	1	1	5	8	2	2	17
VrCOL7b	0	0	4	7	2	0	14
VrCOL8	1	9	4	8	2	0	13
VrCOL9	0	1	5	7	2	0	14
VrCOL10	1	2	4	6	2	1	14
VrCOL11	0	2	5	6	3	0	20
VrCOL12	0	3	4	7	2	1	16
VrCOL13	4	1	4	6	2	2	15

FIGURE 7 | Relative expression levels of *VrCOL* genes in different tissues. The expression levels of *VrCOL* genes were analyzed by qRT–PCR. The expression level of *VrCOL1* in flowers was set to 1, and other values were adjusted accordingly. The gene expression results were visualized using a heatmap generated with Multiple Experiment Viewer 4.9.0 (Saeed et al., 2003). Different colors in the heatmap indicate different expression levels.

Diurnal Rhythm of *VrCOL* Gene Expression

In *Arabidopsis*, the expression levels of *CO*, *COL1*, and *COL2* are regulated by the circadian clock and show diurnal oscillations (Suárez-López et al., 2001; Gangappa and Botto, 2014). We therefore investigated whether *VrCOL* genes exhibited diurnal expression rhythms in mungbean leaves under LD and SD conditions. Gene expression analysis revealed that *VrCOL4*, *VrCOL6*, *VrCOL12*, and *VrCOL13* showed daily oscillations under both LD and SD conditions, whereas *VrCOL1*, *VrCOL2*, *VrCOL5*, *VrCOL7a*, *VrCOL7b*, *VrCOL10*, and *VrCOL11* showed daily oscillations only under SD conditions (**Figure 8**). The duplicated genes *VrCOL8* and *VrCOL9* exhibited similar expression patterns under both LD and SD conditions, whereas *VrCOL1* and *VrCOL2* showed distinct expression patterns under both LD and SD throughout the day (**Figure 8**).

Overexpression of *VrCOL2* Accelerates Flowering in Arabidopsis Under SD Conditions

VrCOL1 and *VrCOL2* displayed close phylogenetic relationships with *AtCO* (**Figure 1**), and the amino acid sequences of *VrCOL*1 and *VrCOL*2 showed 49.35 and 50.93% similarities with AtCO, respectively. We speculated that *VrCOL1* and *VrCOL2* might influence flowering time in mungbean, and we therefore first analyzed the function of *VrCOL2* in the regulation of flowering time in *Arabidopsis* in this study. To investigate the potential functions of *VrCOL2* in flowering time regulation, *VrCOL2* was transformed into *Arabidopsis* under the control of the 35S promoter. The empty vector was also transformed into *Arabidopsis*, and the transgenic plants showed no differences from wild type under both LD and SD conditions (**Supplementary Figure 4**). The *VrCOL2* transgenic *Arabidopsis* lines showed high

FIGURE 8 | Relative expressions of *VrCOLs* in mungbean leaves throughout the day under SD and LD conditions. The SD condition was set as 8:00 am–6:00 pm light, 6:00 pm–8:00 am dark; the LD condition was set as 8:00 am–0:00 am light, 0:00 am–8:00 am dark. ZT, Zeitgeber Time. Expression level of *VrCOLs* was normalized to an *Actin* gene from mungbean. The gray and red lines indicate *VrCOL* expression levels under LD and SD conditions, respectively. Each sample was analyzed using three biological replicates.

levels of *VrCOL2* expression (**Supplementary Figure 5**). The wild-type *Arabidopsis* plants and three *VrCOL2* overexpression lines exhibited approximately 14 rosette leaves after bolting under LD conditions, indicating that they had similar flowering times. By contrast, the wild-type *Arabidopsis* plants and three *VrCOL2* overexpression lines showed approximately 47, 34, 34, and 31 rosette leaves after bolting under SD conditions, suggesting that *VrCOL2* transgenic plants had earlier flowering times than wild-type plants (**Figure 9**). These results indicated that *VrCOL2* regulated flowering time through a photoperiod-dependent pathway.

AtFT and *AtTSF* accelerate flowering and are regulated by *AtCO* in *Arabidopsis* (Khanna et al., 2009; Gangappa and Botto, 2014), and we therefore investigated the expression of *AtFT* and *AtTSF* in wild-type and *VrCOL2* transgenic plants under LD and SD conditions throughout the day. *AtFT* and *AtTSF* showed similar expression levels in *VrCOL2* transgenic and wild-type plants in both light and dark conditions under LD treatment. By contrast, *AtFT* and *AtTSF* showed higher expression levels in *VrCOL2* transgenic plants at several time points than in wild-type plants under SD conditions (**Figure 9**). These results further support the conclusion that *VrCOL2* is involved in flowering time regulation under SD conditions.

DISCUSSION

In recent decades, the investigation of *CO* and *COL* genes in many plant species has greatly increased our knowledge about the molecular mechanisms of flowering time regulation, stress response and root development (Khanna et al., 2009; Gangappa and Botto, 2014). Mungbean is a globally important legume crop, and the mechanisms of its flowering time regulation are still largely unknown. In this study, we identified and characterized 14 *VrCOL* genes from the mungbean genome and investigated the function of *VrCOL2* in flowering time regulation.

The *Arabidopsis*, soybean, *Medicago*, and mungbean genomes contain 17, 26, 11, and 14 *CO* and *COL* members (**Figure 1**; Wong et al., 2014; Wu et al., 2014), and their genome sizes are 125 Mb (Initiative, 2000), 1100 Mb (Schmutz et al., 2010), 500 Mb (Young et al., 2011), and 579 Mb (Kang et al., 2014), respectively. Thus, genome size has no direct relationship with the number of *COL* genes in plants. Soybean has undergone two rounds of whole-genome duplication, whereas mungbean has experienced only one such duplication (Schmutz et al., 2010; Kang et al., 2014). As a result, the *COL* gene number in mungbean is approximately half that of soybean. Seven of the 11 (63.6%) mungbean chromosomes (**Figure 5**), seven of the eight (87.5%) *Medicago* chromosomes
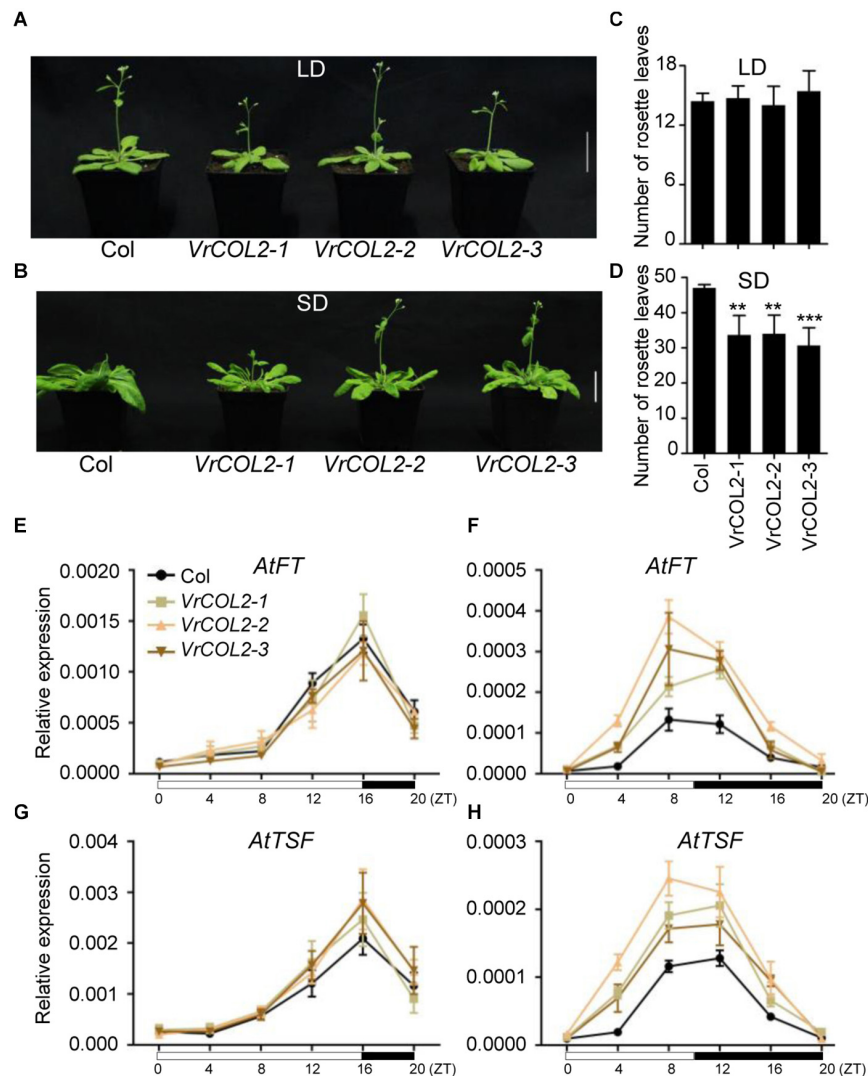
FIGURE 9 | Overexpression of *VrCOL2* accelerates flowering under SD conditions in *Arabidopsis*. Flowering time phenotypes of three *VrCOL2* overexpression transgenic lines and wild-type *Arabidopsis* (Col) plants grown under LD **(A)** and SD conditions **(B)**. Bar = 4 cm. The rosette leaf numbers of *VrCOL2* overexpression transgenic lines and wild-type plants grown under LD **(C)** and SD conditions **(D)** as shown in panels **(A,B)**. The expression levels of *AtFT* and *AtTSF* throughout the day in *VrCOL2* transgenic lines and wild-type plants under LD **(E,G)** and SD **(F,H)** conditions. Leaves of 2-week-old *VrCOL2* overexpression transgenic lines and wild-type plants were sampled every 4 h after lights-on. Expression levels of *AtFT* and *AtTSF* were normalized to an *Actin* gene from *Arabidopsis*. The SD condition was set as 8:00 am–6:00 pm light, 6:00 pm–8:00 am dark; the LD condition was set as 8:00 am–0:00 am light, 0:00 am–8:00 am dark.

and 16 of the 20 (80.0%) soybean chromosomes contained *COL* genes (Wong et al., 2014; Wu et al., 2014), indicating that the distribution of *COL* genes has changed during evolution in legumes.

Plant genome evolution produces many duplicated gene pairs and provides resources for new gene functions (Kondrashov et al., 2002). Two duplicated gene pairs, *VrCOL1/VrCOL2* and *VrCOL8/VrCOL9* (**Figure 6**), were found among the mungbean *VrCOLs*. The duplicated genes showed close relationships in the phylogenetic tree and contained similar motifs (**Figures 1**, **4**), indicating that they evolved from the same origin and may share similar functions. However, the duplicated gene pairs contained different numbers and types of *cis*-acting elements in their promoter regions and exhibited different expression levels in some tissues (**Figure 7** and **Table 2**), suggesting that they might have evolved novel functions compared with their original gene. For example, *VrCOL8* and *VrCOL9* shared similar numbers of several *cis*-acting elements in their promoter regions, including promoter-related elements and site-binding related elements, but differed in the numbers of development-related elements, environmental-stress-related elements, hormone-responsive elements and light-responsive elements (**Table 2** and **Supplementary Table 2**). *VrCOL8* and *VrCOL9* showed similar expression levels in roots and nodule roots, but their expression differed in flowers, pods, leaves, seeds, stems, and

shoot apices (**Figure 7** and **Supplementary Figure 3**). This result suggests that they may have retained some common functions from the original gene in roots and nodule roots but evolved novel functions in other tissues.

The expression of *VrCOL* genes in different tissues provides clues to their potential functions, and many *VrCOL* genes (such as *VrCOL6* and *VrCOL12*) showed tissue-specific expression patterns (**Figure 7**). However, several *VrCOL* genes (including *VrCOL2*, *VrCOL7a*, and *VrCOL10*) showed low expression levels in all tissues tested, despite the fact that their promoter regions contained many *cis*-acting elements (**Figure 7**, **Table 2**, and **Supplementary Table 2**). Gene expression is influenced by many factors. For example, many circadian clock and flowering time regulation genes are controlled by photoperiod. Their expression changes under different photoperiods and during the day and night (Suárez-López et al., 2001; Jack, 2004; Wickland and Hanzawa, 2015; Xu and Chong, 2018). For example, *VrCOL2* appeared to be a daily oscillation gene whose expression changed during the day under SD conditions but was low throughout the day under LD conditions (**Figure 8**). The different field-grown mungbean tissues were collected in the afternoon under relatively LD conditions in July, and that may explain why *VrCOL2* showed low expression levels in the tissue expression analysis (**Figure 7**).

CO and *CO*-homologous genes, such as *OsHd1*, play critical roles in flowering time regulation (Khanna et al., 2009; Gangappa and Botto, 2014). *VrCOL2* showed close relationships with *Arabidopsis CO*, soybean *GmCOL1a*, *GmCOL1b*, *GmCOL2a*, and *GmCOL2b* and rice *OsHd1* (*OsCOL-A*), and accelerated flowering under SD but not LD conditions in transgenic *Arabidopsis* lines (**Figure 9**). AtCO regulates *AtFT* and *AtTSF* to accelerate flowering (Putterill et al., 1993, 1995; Andres and Coupland, 2012; Song et al., 2013), and the expression of *AtFT* and *AtTSF* increased in *VrCOL2* transgenic *Arabidopsis* lines at several time points under SD but not LD conditions (**Figure 9**). Moreover, *VrCOL2* showed daily oscillations only under SD conditions, but not LD conditions (**Figure 8**), indicating that *VrCOL2* might only have functions under SD conditions. *VrCOL2* therefore affects the expression of downstream *AtFT* and *AtTSF* genes *via* photoperiod-dependent pathways. Moreover, AtCO protein accumulation is also regulated by the circadian clock. AtCO mRNA is highly abundant from late afternoon to dawn, but AtCO protein accumulates only in the late afternoon under LD conditions (Putterill et al., 1995; Shim and Imaizumi, 2015; Song et al., 2015; Shim et al., 2017). Although *VrCOL2* was controlled by the 35S promoter and expressed under both LD and SD conditions, the accumulation of *VrCOL2* proteins was unknown. Whether the accumulation of *VrCOL2* protein depends on day length, in turn affecting flowering time by influencing *AtFT* and *AtTSF* expression requires further investigation. In addition, AtCO promotes flowering under LD conditions and suppresses flowering time under SD conditions (Luccioni et al., 2019), but rice *OsHd1* accelerates flowering under SD conditions and delays flowering under LD conditions (Yano et al., 2000; Komiya et al., 2008, 2009). Mungbean (Imrie, 1996; Kim et al., 2015) and rice

are SD plants, and *Arabidopsis* is an LD plant, and this may explain why CO homologs have different functions in different plant species. These results suggest that *CO* and its homologs are involved in flowering time regulation under photoperiod-dependent pathways and have distinct roles in different plant species. Thus, in summer LD conditions, the expression of *VrCOL2* may be low and have little effect on the acceleration of flowering. In the autumn, as days become shorter, the expression of *VrCOL2* may increase and accelerate mungbean flowering. In addition, *VrCOL1* and *VrCOL2* form a duplicated gene pair and show a close relationship with one another (**Figures 1**, **6**), and *VrCOL1* showed high expression levels in many tissues, indicating that *VrCOL1* may share similar functions to *VrCOL2* in flowering time regulation, a possibility that requires further investigation. Much more work is needed to fully elucidate the mechanisms by which *VrCOL2* affects flowering time and circadian clock regulation in mungbean.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SL conceived and designed the research. CL, QZ, HZ, and CC conducted the experiments and analyzed the data. SL and HZ wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.608603/full#supplementary-material

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