Functional characterization of mungbean *CONSTANS-LIKE* genes reveals a key role for *CONSTANS-LIKE 2* in the control of flowering time in *A. thaliana* under short-day conditions

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Abstract

Background: *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 investigations have examined *COL* genes in mungbean (*Vigna radiata*).

Results: In this study, we identified and characterized a total 14 of *VrCOL* genes from mungbean, which distributed on 7 of the 11 mungbean chromosomes. Based on their conserved domains, *VrCOLs* were clustered into three groups (I, II and III), which contained 4, 5 and 5 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 under long day and short day conditions throughout the day. Among these *VrCOL* genes, *VrCOL2* showed a close phylogenetic relationship with *Arabidopsis thaliana* (*A. 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 *A. thaliana* under short day conditions by activating the expression of flowering time gene *AtFT* and *AtTSF*.

Conclusion: Overall, we identified 14 *VrCOL* genes from mungbean using genome-wide identification. Characteristics and transcription pattern analysis of *VrCOL* genes revealed their important roles in plant growth and development, and our results suggested that *VrCOL2* regulate flowering time under short day conditions in *A. thaliana*. Our study lays the foundation for further dissection of *VrCOL* gene functions.

Background

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 determinate the transition from vegetative to reproductive growth [1-4]. 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 [1, 4-10]. Within these pathways, a number of genes have been identified as involved in flowering time regulation, including *CONSTANS-LIKE (COL)* genes, phosphatidyl ethanolamine-binding protein (PEBP) genes and several members of MADS-Box gene family [1-2, 11-19].

COL genes belong to the zinc-finger transcription factor family and play central roles in plant growth and development [11, 20]. *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 [11, 20]. 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 [20]. The CCT domain has important functions in transcriptional regulation and nuclear protein transport [11, 20-22]. The COL proteins are classified into three classes based on the number and type of their conserved domains. Class 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, III contain 6, 7 and 4 members in *Arabidopsis thaliana* (*A. thaliana*), respectively. In addition, several COL proteins contain valine-proline (VP) motifs in their C termini [11, 20].

Among these *COL* members, *AtCO* (*AtBBX1*) and its homologs are well-studied in many plant species [11, 20, 23-25]. *AtCO* is expressed in a rhythmic manner and coordinates light pathway and circadian clock signal inputs in *A. thaliana* [26-29]. Thus, *AtCO* plays an important role in the regulation of flowering time via 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 [11, 20]. 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* (CYCKLING DOF FACTOR 1) and *AtFKF1* (FLAVIN BINDING, KELCH REPEAT, F-BOX1) [30-31]. *OsHd1* (*Heading date 1*), the *AtCO* ortholog in rice, accelerates flowering under SD but delays flowering time under LD conditions through the regulation of the *AtFT* orthologs *OsHd3a* (*Heading date 3a*) and *OsRFT1* (*RICE FLOWERING LOCUS T1*) [32-34]. The soybean *AtCO* orthologs *GmCOL1*, *GmCOL2*, *GmCOL3* and *GmCOL4* can complement the late flowering phenotype of *Atco* mutants [35]. 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 [11, 20].

Mungbean is a diploid legume crop, and its seeds contain proteins and nutrients that are essential for human nutrition [36]. The cultivated mungbean is considered to have been domesticated in India, from which it then spread to other areas [37]. Mungbean is considered to be a SD crop, and the flowering time is a critical factor influencing its production [38-40]. Mungbean plants produce a large number of flowers, but only a few set pods. Approximately 70-90% of the flowers are shed, which mainly occurs in later formed flowers of the racemes [41-42]. Thus, it is suggested that the prevention of late flowering is an important way to increase mungbean yield [42-44]. The sequencing of mungbean genome provides genetic resources for the investigation of gene functions [45], and the investigation of mungbean flowering time genes can therefore provide essential information for further modification of mungbean cultivars to increase yield. Until now, limited information about gene functions has been identified 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 under SD conditions. Our findings will provide useful information for further characterization of mungbean *COL* gene functions.

Results

Identification of *VrCOL* genes in mungbean

To search for mungbean *VrCOL* genes, we used the amino acid sequences of AtCO and AtCOL proteins as blast queries against the mungbean genome database and the NCBI database. The conserved BBX and CCT domains in each candidate gene were confirmed using Pfam and InterPro, and a total of 14 *VrCOL* members were identified in the mungbean genome. 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 (*XP_014502470*) to 48,806.9 (*XP_022637309*) Da. The GC content, which influences gene stability to some degree, ranged from 34.64% to 50.39%, and twelve of the fourteen *VrCOL* genes had lower than 50% GC content (Table 1).

Phylogenetic and classification analysis of *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 *A. thaliana*, 26 soybean, 11 *Medicago*, 16 rice, 18 maize and 14 mungbean CO and COL proteins [11, 35, 46-47]. The *VrCOL* genes were named from *VrCOL 1* to *VrCOL 13* based on their phylogenetic relationships with the soybean orthologous genes (Fig. 1, Table 1). Among these *VrCOL* members, *VrCOL 1* and *VrCOL 2* showed close relationships to *A. thaliana*AtCO, soybean *GmCOL 1a*, *GmCOL 1b*, *GmCOL 2a*, and *GmCOL 2b* and rice *OsHd1* (*OsCOL-A*), all of which have documented roles in the regulation of flowering time [11, 20, 35]. This result suggests that *VrCOL 1* and *VrCOL 2* may play critical roles in the flowering time regulation of mungbean.

The COL proteins were classified into three groups based on differences in the numbers and types of conserved BBX and CCT domains [11, 20]. We analyzed the *VrCOL* BBX and CCT domains and found two distinct BBX domains (BBX1 and BBX2) and one CCT domain (Additional file 1). The sequence logos of the BBX1 (CX₂CX₈CX₄AXLCX₂CDX₃HX₈HXR), BBX2 (CX₂CX₄AX₃CX₇CX₂CDX₃HX₈H) and CCT (RYX₂KX₃RX₃KX₂RYX₂RKX₂AX₂RXR) domains were determined using WebLogo (Fig. 2, Additional file 1). Nine *VrCOL* proteins contained one BBX1, one BBX2 and one CCT domain, and five *VrCOL* proteins contained one BBX1 and one CCT domain (Fig. 3). The *VrCOL* proteins were further classified into three groups based on differences in these conserved domains (Fig. 1 and 3). Classes I, II and III contained 4, 5 and 5 *VrCOL* members, respectively. The BBX1 and BBX2 domains were located close to one another in the class I and II genes (Fig. 3). Most of the *VrCOL* genes from the same class were clustered into the same clade in the phylogenetic tree, with the exception of class III member *VrCOL 10*, which showed a closer relationship with class II members (Fig. 1 and 3).

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 the NCBI and analyzed them using the GSDS program [48]. All the *VrCOL* members contained 5' UTR and 3' UTR regions. Their exon numbers ranged from 2 to 6, and their intron numbers ranged from 1 to 6. Most group I and III *VrCOL* members contained two exons and one intron, suggesting conserved functions of the genes within each group. An exception was class III member *VrCOL 10*, which contained 6 exons and 6 introns and had a close relationship with group II members (Fig. 3-4). By contrast, group II members contained various numbers of exons (3 to 5) and introns (2 to 5), suggesting potential functional diversity among these genes (Fig. 4). To further investigate the conservation and diversity of *VrCOL* protein structures, we analyzed putative protein motifs in the *VrCOL*s. A total of 17 distinct motifs were identified, and all *VrCOL* proteins contained motifs 1 and 2, which appeared to represent the conserved CCT and BBX1 domains, respectively (Fig. 4, Additional file 2). Most members of the same group shared some conserved motifs. For example, group I proteins shared motifs 1, 2, 3, 9 and 16, group II members shared motifs 1, 2, 3, and 5, and most group III members shared motifs 1, 2, 4, 8, 12, and 13 (except for *VrCOL10*) (Fig. 4).

Chromosomal distribution and duplication analysis of *VrCOL* genes

Plant *COL* genes evolved from several common original genes, and the chromosomal locations of *COL* genes can represent the alteration of gene distributions during evolution. To visualize the chromosomal locations of the *VrCOL* genes, we mapped them to their physical positions in the mungbean genome. *VrCOL 7b* was discarded due to a lack of related chromosome information. Seven of the fourteen *VrCOL* genes were located on the positive strand. Seven of the eleven mungbean chromosomes contained *VrCOL* genes, with the exception of chromosomes 2, 9, 10 and 11 (Fig. 5, Table 1). Chromosome 5 contained the most 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 (*VrCOL 1*, *VrCOL 11* and *VrCOL 13*) were located on the relatively short chromosomes 3 and 4 (Fig. 5).

Mungbean has experienced one round of whole-genome duplication that produced many duplicated gene pairs [45, 49]. To investigate the evolutionary relationships among the *VrCOL*s, we searched for duplicated gene pairs among them. Two interchromosomal duplication events were identified in chromosomes 1, 4, 5 and 6, including the duplicated gene pairs *VrCOL 1/VrCOL 2* and *VrCOL 8/VrCOL 9* (Fig. 6). The duplicated genes were clustered together in the phylogenetic tree (Fig. 3). 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 *VrCOL 1* and *VrCOL 2* showed similar exon-intron organization and similar motifs, as did *VrCOL 8* and *VrCOL 9* (Fig. 4), indicating that the duplicates may share similar functions.

Cis-acting element analysis of the *VrCOL* promoter regions

To predict the potential expression response of *VrCOL* genes, we investigated the *cis*-acting elements in their promoters using PantCARE [50]. A total of 82 *cis*-acting elements were found across the 14 *VrCOL* promoter regions (2 kb upstream of the initiation codon) (Additional file 3). 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, Additional file 3). 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, light-responsive elements and promoter related elements, and 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 *VrCOL*s contained the hormone-responsive elements CGTCA-motif and TGACG-motif and the light-responsive element Box 4 (Additional file 3), 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 (Fig. 7). For example, *VrCOL3* was highly expressed in all the tested tissues, 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 these tissues. For example, *VrCOL6* showed high expression in leaves but low expression in nodule roots and roots.

Duplicated genes may retain some common functions and evolve some new functions [51-52]. 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 the tissues we examined, indicating that they had undergone functional divergence. *VrCOL8* and *VrCOL9* showed similar expression levels in roots and nodule roots, but they exhibited different expression levels in other tissues (Fig. 7, Additional file 4).

Diurnal rhythm of *VrCOL* gene expressions

In *A. thaliana*, the expressions of *CO*, *COL1* and *COL2* are regulated by the circadian clock and show diurnal oscillations [11, 53]. 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, while *VrCOL1*, *VrCOL2*, *VrCOL5*, *VrCOL7a*, *VrCOL7b*, *VrCOL10* and *VrCOL11* only showed daily oscillations under SD conditions, but not LD conditions (Fig. 8). The duplicated genes *VrCOL8* and *VrCOL9* exhibited similar expression patterns under both LD and SD conditions, while *VrCOL1* and *VrCOL2* showed distinct expression patterns under both LD and SD throughout the day (Fig. 8).

Overexpression of *VrCOL2* accelerates flowering in *A. thaliana* under SD conditions

VrCOL1 and *VrCOL2* displayed close phylogenetic relationships with *AtCO* (Fig. 1), and the amino acid sequences of *VrCOL1* and *VrCOL2* showed 49.35% and 50.93% similarities with *AtCO*, respectively, thus *VrCOL2* was selected for further analysis. To investigate the potential functions of *VrCOL2* in flowering time regulation, *VrCOL2* was transformed into *A. thaliana* under the control of the 35S promoter. The empty vector was also transformed into *A. thaliana*, and the transgenic plants showed no difference with wild type under both LD and SD conditions (Additional file 5). The *VrCOL2* transgenic *A. thaliana* lines showed high levels of *VrCOL2* expression (Additional file 6). The *VrCOL2* overexpression lines exhibited similar flowering time to wild-type plants under LD conditions but exhibited earlier flowering time than wild-type plants under SD conditions (Fig. 9), indicating that *VrCOL2* regulates flowering time under a photoperiod-dependent pathway.

AtFT and *AtTSF* accelerate flowering and are regulated by *AtCO* in *A. thaliana* [11, 20], and we therefore investigated the expression of *AtFT* and *AtTSF* in wild-type and *VrCOL2* transgenic plants under LD and SD conditions. *AtFT* and *AtTSF* showed similar expression levels in *VrCOL2* transgenic and wild-type plants under LD conditions. By contrast, *AtFT* and *AtTSF* showed higher expression levels in *VrCOL2* transgenic plants than in wild-type plants under SD conditions (Fig. 9). In addition, the expressions of two mungbean *FT* and *TSF* homologous genes (*XP_014496932*, *XP_014497364*), which contained CORE *cis*-acting elements in their promoter regions, increased in *VrCOL2* transgenic hair roots under SD conditions, but not LD condition (Additional file 7). 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 [11, 20]. 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 *A. thaliana*, soybean, *Medicago* and mungbean genomes contained 17, 26, 11 and 14 *CO* and *COL* members, respectively (Fig. 1) [35, 54], and their genome sizes are 125 Mb [55], 1100 Mb [56], 500 Mb [57] and 579 Mb [45], 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 [45, 56]. As a result, the *COL* gene number in mungbean is approximately half that of soybean. Seven of the eleven (63.6%) mungbean chromosomes (Fig. 5), seven of the eight (87.5%) *Medicago* chromosomes and sixteen of the twenty (80.0%) soybean chromosomes contained *COL* genes [35, 54], indicating that the distribution of *COL* genes has changed much during evolution in legumes. The *COL* genes were clustered into three groups based on their conserved domains, and most of *VrCOL* genes in each group were clustered into the same clade in the phylogenetic tree, with the exception of the group III member *VrCOL 10*, which contained one BBX and one CCT domain and showed a close relationship with group II members (Fig. 1 and 3). *VrCOL10* protein lacked motifs 4, 8, 12, and 13, which were found in all other *VrCOL* group III members, but it did contain motifs 3 and 5, which were found in all *VrCOL* group II members (Fig. 4), suggesting that *VrCOL10* may derive from a group II ancestor and that one BBX domain may have been lost during evolution.

Plant genome evolution produces many duplicated gene pairs and provides resources for new gene functions [51]. Two duplicated gene pairs, *VrCOL 1/VrCOL 2* and *VrCOL 8/VrCOL 9* (Fig. 6), were found among the mungbean *VrCOL*s. The duplicated genes showed close relationships in the phylogenetic tree and contained similar motifs (Fig. 3-4), indicating that they evolved from the same origin and likely shared 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 (Fig. 7, Table 2), suggesting that they might have evolved novel functions compared with their original gene. For example, *VrCOL 8* and *VrCOL 9* 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, Additional file 3). *VrCOL 8* and *VrCOL 9* showed similar expression levels in roots and nodule roots, but their expression differed in flowers, pods, leaves, seeds, stems and shoot apices (Fig. 7, Additional file 4). 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 *VrCOL 6* and *VrCOL 12*) showed tissue-specific expression patterns (Fig. 7). However, several *VrCOL* genes showed low expression levels in all tissues tested, despite the fact that their promoter regions contained many *cis*-acting elements, including *VrCOL 2*, *VrCOL 7a* and *VrCOL 10* (Fig. 7, Table 2, Additional file 3). 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 [1, 4, 6, 53]. For example, *VrCOL 2* appeared to be a daily oscillation gene whose expression changed during the day under SD conditions but showed low expression throughout the day under LD conditions (Fig. 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 (Fig. 7).

CO and *CO*-homologous genes, such as *OsHd1*, play critical roles in flowering time regulation [11, 20]. *VrCOL2* showed higher expression levels at the stages of during flowering, and after flowering, than that at the stage of before flowering, indicating that *VrCOL2* played an important role in flowering time regulation (Additional file 8). *VrCOL2* showed close relationships with *A. thaliana CO*, soybean *GmCOL1a*, *GmCOL1b*, *GmCOL2a* and *GmCOL2b* and rice *OsHd1* (*OsCOL-A*), and accelerated flowering under SD but not LD conditions in transgenic *A. thaliana* lines (Fig. 9). *AtCO* regulates *AtFT* and *AtTSF* to accelerate flowering [26-29], and the expression of *AtFT* and *AtTSF* increased in *VrCOL2* transgenic *A. thaliana* lines under SD but not LD conditions (Fig. 9), indicating that *VrCOL2* regulates downstream genes via photoperiod-dependent pathways. In addition, *AtCO* protein accumulation is also regulated by circadian clock. *AtCO* mRNA abundance is highly expressed from late afternoon to the dawn, but *AtCO* protein only accumulates in the late afternoon under long day conditions [27, 58-60]. Although *VrCOL2* is controlled by 35S promoter and can be expressed under both LD and SD conditions, the accumulation of *VrCOL2* proteins might be low under LD condition, which might be the reason why *VrCOL2* had no effect on flowering time under LD conditions. Overexpression of *AtCO* accelerates flowering under both LD and SD conditions [11, 20], while in rice *OsHd1* accelerates flowering under SD but delays flowering time under LD conditions [32-34]. Mungbean [38-39] and rice are short day plants, and *A. thaliana* is a long day plant, which might be the reason why *CO* homologous genes 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 (Fig. 1 and 6), indicating that *VrCOL1* may have similar functions to *VrCOL2* in flowering time regulation. Much more work is needed to fully elucidate the mechanisms by which *VrCOL2* affects flowering time and circadian clock regulation in mungbean.

Conclusion

In this study, we identified and characterized 14 *VrCOL* genes from mungbean genome using genome-wide analysis, and many characteristics of these *VrCOL* genes were investigated, including chromosomal distributions, sequence logos, classifications, phylogenetic relationships, gene structures, conserved motifs, duplicated gene pairs, *cis*-acting elements, and expression profiles. Among these *VrCOL* genes, *VrCOL2* showed a close relationship with *AtCO* and the expression of *VrCOL2* exhibited daily oscillations under SD conditions. Further investigation revealed that *VrCOL2* accelerated flowering under SD conditions by activating the expression of *AtFT* and *AtTSF*, but not LD conditions.

Methods

Plant materials and growth conditions

The reference mungbean genome variety VC1973A, stored at Seoul National University, was identified and supplied by Suk-Ha Lee at Seoul National University, Seoul, Korea [45], and the variety was used for all experiments in this study. Mungbean seeds were germinated 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 and 10 h 25 °C light/14 h 25 °C dark cycles for LD and SD photoperiods, respectively. Leaves of 5-week old mungbean plants were sampled every 4 hours after lights-on and used to analyze the diurnal rhythm of gene expression. Multiple tissues were collected from field-grown mungbean plants sown in Qingdao, China, at the end of May, including roots, nodule roots, shoot apices, stems, leaves, flowers, pods and seeds. Tissues were collected in the afternoon, which might be ZT 10-12, in early July for gene expression analysis, and all samples were stored at -80 °C before RNA extraction. Moreover, mungbean leaves at three different stages, before flowering (S1), during flowering (S2, the first flower occurred), and after flowering (S3, after the first flower occurred), were collected from field-grown plants for gene expression analysis. *A. thaliana* plants were grown in growth chambers with 16 h 23 °C light/8 h 21 °C dark and 10 h 23 °C light/14 h 21 °C dark cycles for LD and SD photoperiod treatments, respectively. Leaves of 3-week-old *A. thaliana* were collected for gene expression analysis.

Identification of mungbean *VrCOL* members

The amino acid sequences of *A. thaliana* CO and COLs were used as blast queries against the National Center for Biotechnology Information (NCBI) and mungbean genome database (http://plantgenomics.snu.ac.kr/mediawiki-1.21.3/index.php/Main_Page) [45] to search for mungbean *VrCOL* proteins. The presence of conserved BBX and CCT domains in candidate genes were confirmed using the Pfam database [61] and InterPro program with default parameters [62].

Phylogenetic relationship analysis

The amino acid sequences of CO and COL proteins from *A. thaliana*, soybean, *Medicago*, mungbean, rice and maize were aligned using ClustalW2 [63], and the resulting alignment was used to construct a phylogenetic tree in MEGA 7.0 using the Neighbor-Joining method with default parameters [64]. In addition, *VrCOL* proteins were aligned separately in ClustalW2 and used to construct a phylogenetic tree in MEGA7.0 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 Jin et al. [19, 65]. The duplicated gene pairs were identified with amino acid sequences more than 60% similarity, and visualized using Circos software [66].

Analyses of exon-intron organizations, 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 Structure Display Server (GSDS) to analyze their gene structures [48]. 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 [62]. The sequence logos of the conserved BBX1, BBX2 and CCT domains were analyzed using the WebLogo platform [67]. The conserved motifs present in the *VrCOL* proteins were identified using MEME tools, and the parameters of the optimum motif widths were 11-50 amino acid residues [68]. The *cis*-acting elements in each *VrCOL* promoter, 2 kb upstream of the initiation codon, were predicted by PlantCARE [50].

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 *XhoI* and *XbaI* digestion site sequences. The resulting PCR fragment was digested by the restriction endonucleases *XhoI* and *XbaI* to generate cohesive ends. The pRTL2 vector was digested with *XhoI* and *XbaI* to generate a linearized plasmid. Then the *VrCOL2* and pRTL2 fragments were ligated using T4 DNA ligase (Promega). The constructed plasmid was verified by sequencing. It was then introduced into *A. thaliana* using the floral dip method [69], and successful transformation was confirmed by PCR. In addition, the *VrCOL2* overexpression construct was transformed into VC1973A to obtain transgenic root hairs using the method as described by Kereszt et al. [70]. Three-week old transgenic root hairs grown under LD and SD conditions were collected for gene expression analysis, respectively. Each sample was analyzed using three independent root hairs. All primers are listed in Additional file 9.

RNA extraction and transcription analysis

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis were carried out as described in Li et al. [49]. Gene expression levels were normalized to an *Actin* gene from mungbean (*Vradi03g00210*). Each sample was analyzed using three biological replicates. All primers are listed in Additional file 9.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' 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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Abbreviations

BBX, B-box; CCT, CONSTANS, CONSTANS-LIKE and TIMING OF CAB1; LD, Long day; SD, Short day; DNA, Deoxyribonucleic acid; RNA, Ribonucleic acid; CDS, Coding domain sequence; UTR, Untranslated Regions; AA, Amino acid; CO, CONSTANS; FT, FLOWERING LOCUS T ; COL, CO-like; GSDS, Gene Structure Display Server program; Vr, Vigna radiata; Gm, Glycine max; pI, Isoelectric point; MW, Molecular weight; OsHd1,

Heading date 1; OsHd3a, Heading date 3a; NCBI, National Center for Biotechnology Information; qRT-PCR, quantitative real-time PCR.

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Supplementary Files

Additional file 1: Sequence alignments of conserved B-Box1, B-Box2 and CCT domains of VrCOL proteins.

Additional file 2: Sequence logos of 17 distinct motifs in VrCOL proteins.

Additional file 3: *Cis*-acting elements identified in each *VrCOL* promoter regions.

Additional file 4: Relative expression levels of *VrCOL* duplicated genes in different tissues. The expression level of *VrCOL 1* in flowers was set as 1, and the others were adjusted accordingly. *** and ** are significantly different at $P < 0.001$ and $P < 0.01$, respectively, compared with the expression levels in its duplicated gene.

Additional file 5: The rosette leaf numbers of empty vector transgenic lines and wild-type plants grown under LD and SD conditions.

Additional file 6: Expression analysis of *VrCOL2* in *VrCOL2* transgenic lines and wild-type *A. thaliana* measured by qRT-PCR. The leaves of transgenic and wild-type plants grown under LD conditions were sampled 5 h after lights-on for qRT-PCR analysis. Gene expression levels were normalized to an *Actin* gene from *A. thaliana*. ND, not detected.

Additional file 7: Expression levels of two mungbean *FT* homologous genes in *VrCOL2* transgenic root hairs. (a) The positions of CORE (TGTG(N2-3)ATG motif) *cis*-acting elements in the promoter regions of mungbean *FT* homologous genes *XP_014496932*, *XP_014497364*. The red boxes in the promoter regions indicate CORE *cis*-acting elements. (b) The expression levels of *XP_014496932*, *XP_014497364* in *VrCOL2* transgenic root hairs under LD and SD conditions. The empty vector was transformed as a control. Tissues were sampled 5 hours after lights-on (ZT 5). The gene expression in each control plants was set as 1 and those in other samples were adjusted accordingly. ****P* < 0.001.

Additional file 8: Expression levels of *VrCOL2* in leaves at three growth stages, before flowering (S1), during flowering (S2), and after flowering (S3).

Additional file 9: Primers used in this study.

Tables

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	2481	1074	357	5.82	39253.5	42.93	4	-	<i>VrCOL1</i>
XP_014509740	2195	1071	356	5.27	39685.26	40.68	1	-	<i>VrCOL2</i>
XP_014502470	1506	933	310	7.01	33756.82	50.13	5	-	<i>VrCOL3</i>
XP_014510275	1778	1119	372	6.11	40396.31	50.39	7	-	<i>VrCOL4</i>
XP_014511026	3495	1239	412	4.95	46524.78	36.89	1	+	<i>VrCOL5</i>
XP_014505914	2175	1110	369	5.64	41395.12	40.37	7	+	<i>VrCOL6</i>
XP_014512653	2060	1254	417	5.28	46964.34	43.83	8	-	<i>VrCOL7a</i>
XP_014523547	1960	1113	370	9.22	42031.83	40.10	N/A	+	<i>VrCOL7b</i>
XP_014505158	8864	1236	411	5.21	45045.34	34.64	6	+	<i>VrCOL8</i>
XP_014523701	14007	1230	409	4.86	44487.59	40.33	5	+	<i>VrCOL9</i>
XP_022637309	4494	1329	442	6.47	48806.9	42.28	5	+	<i>VrCOL10</i>
XP_014496130	4536	1035	344	6.47	38345.14	45.16	3	-	<i>VrCOL11</i>
XP_022641108	3819	954	317	6.82	35966.39	40.02	8	-	<i>VrCOL12</i>
XP_014497035	3750	1119	372	7.00	41848.9	39.46	4	+	<i>VrCOL13</i>

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

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
<i>VrCOL1</i>	0	3	4	11	2	0	18
<i>VrCOL2</i>	1	3	4	6	2	1	18
<i>VrCOL3</i>	2	1	4	8	2	0	19
<i>VrCOL4</i>	1	0	4	11	2	2	17
<i>VrCOL5</i>	1	3	3	6	2	0	17
<i>VrCOL6</i>	1	0	4	11	2	2	17
<i>VrCOL7a</i>	1	1	5	8	2	2	17
<i>VrCOL7b</i>	0	0	4	7	2	0	14
<i>VrCOL8</i>	1	9	4	8	2	0	13
<i>VrCOL9</i>	0	1	5	7	2	0	14
<i>VrCOL10</i>	1	2	4	6	2	1	14
<i>VrCOL11</i>	0	2	5	6	3	0	20
<i>VrCOL12</i>	0	3	4	7	2	1	16
<i>VrCOL13</i>	4	1	4	6	2	2	15

Figures

Figure 1

Phylogenetic analysis of the evolutionary relationships among VrCOL proteins and COL proteins from other species. The amino acid sequences of COL proteins from *A. thaliana*, soybean, Medicago, mungbean, rice and maize were used to construct the phylogenetic tree in MEGA7.0. Different groups of VrCOL genes are indicated.

Figure 2

Sequence logos of the BBX1, BBX2 and CCT domains of VrCOL proteins. The conserved domains were analyzed using the WebLogo platform.

Figure 3

Phylogenetic relationship and conserved domain analyses of the VrCOL proteins. (a) VrCOL protein sequences were used to construct a phylogenetic tree. (b) The positions of conserved BBX1, BBX2 and CCT domains in the VrCOL proteins. The blue, purple and orange boxes indicate the BBX1, BBX2 and CCT domains, respectively. (c) Classifications and conserved domain positions of the VrCOL proteins.

Figure 4

Gene structures and conserved motifs of the VrCOL proteins. (a) Exon-intron organizations of VrCOL genes. The blue boxes, pink boxes and black lines indicate UTRs, exons and introns, respectively. (b) Conserved motifs of the VrCOL proteins. Different motifs are indicated by different colored boxes.

Figure 5

Chromosomal locations of the VrCOL genes. Chromosome number and length are indicated.

Figure 6

Duplication analysis of VrCOL proteins. The duplicated gene pairs are connected by lines.

Figure 7

Relative expression levels of VrCOL genes in different tissues analyzed by qRT-PCR. The expression level of VrCOL1 in flowers was set as 1, and the others were adjusted accordingly. ***, ** and * are significantly different at $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively, compared with the expression levels in the relative flowers.

Figure 8

Relative expression of VrCOLs in 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.

Figure 9

Overexpression of VrCOL2 accelerates flowering under SD conditions. Phenotypes of VrCOL2 transgenic lines and wild-type *A. thaliana* (Col) grown under LD (a) and SD conditions (b). The rosette leaf numbers of VrCOL2 transgenic lines and wild-type plants grown under LD (c) and SD conditions (d). The expression levels of *AtFT* and *AtTSF* in VrCOL2 transgenic lines and wild-type plants under LD (e, g) and SD conditions (f, h). *A. thaliana* leaves were sampled 5 hours after lights-on (ZT 5) from 3-week-old plants. The gene expression in wild type plants was set as 1 and those in other samples were adjusted accordingly. *** $P < 0.001$, ** $P < 0.01$, bars = 4 cm.

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