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

CURRENT STATUS: UNDER REVIEW

Chenyang Liu
Qingdao Agricultural University

Qianqian Zhang
Qingdao Agricultural University

Hong Zhu
Qingdao Agricultural University

Chunmei Cai
Qingdao Agricultural University

Shuai Li
Qingdao Agricultural University

li2014shuai@qau.edu.cn Corresponding Author
ORCiD: https://orcid.org/0000-0002-6905-2349

10.21203/rs.3.rs-24842/v1

SUBJECT AREAS
- Plant Physiology and Morphology
- Plant Molecular Biology and Genetics

KEYWORDS
- Mungbean, Flowering time, CONSTANS, VrCOL2, Genome wide
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, VrCOL2/VrCOL5 and VrCOL6/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, VrCOLs showed distinct expression patterns in different tissues. 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 FT and TSF.

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. 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, CO (BBX1) and its homologs are well-studied in many plant species [11, 20, 23–25]. CO is expressed in a rhythmic manner and coordinates light pathway and circadian clock signal inputs in A. thaliana [26–29]. Thus, CO plays an important role in the regulation of flowering time via the photoperiod-dependent pathway. A. thaliana co 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, CO overexpression plants show early flowering time under both LD and SD conditions [11, 20]. The CO protein binds to cis-acting elements in the promoter region of the flowering activator FLOWERING LOCUS T (FT) to active FT expression. Moreover, CO is regulated by many flowering factors, such as GI (GIGANTEA), CDF1 (CYCKLING DOF FACTOR 1) and FKF1 (FLAVIN BINDING, KELCH REPEAT, F-BOX1) [30–31]. Hd1 (Heading date 1), the CO ortholog in rice, accelerates flowering under SD but delays flowering time under LD conditions through the regulation of the FT orthologs Hd3a (Heading date 3a) and RFT1 (RICE FLOWERING LOCUS T1)
The soybean CO orthologs GmCOL1, GmCOL2, GmCOL3 and GmCOL4 can complement the late flowering phenotype of A. thaliana co 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]. The sequencing of its genome provides genetic resources for the investigation of gene functions in mungbean [38]. Flowering time is an important factor that influences mungbean production, and the investigation of mungbean flowering time genes can therefore provide essential information for further modification of mungbean cultivars to increase yield. 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 A. thaliana CO and COL 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 34.64–50.39%, and twelve of the fourteen VrCOL genes had lower than 50% GC content (Table 1).

Table 1

| Gene ID     | Genomic length/bp | CDS/bp | No. of AA | pI | Mol.Wt /Da | Chr | Strand | GC% | Gene names |
|-------------|-------------------|--------|-----------|----|------------|-----|--------|-----|------------|
| XP_014511   | 3495              | 1239   | 412       | 4.95 | 46524.78   | 1   | +      | 36.89| VrCOL1     |
| XP_014509   | 2195              | 1071   | 356       | 5.27 | 39685.26   | 1   | -      | 40.79| VrCOL2     |
| XP_014496   | 4536              | 1035   | 344       | 6.47 | 38345.14   | 3   | -      | 45.16| VrCOL3     |
| XP_014497   | 3750              | 1119   | 372       | 7.0  | 41848.90   | 4   | +      | 39.46| VrCOL4     |
| XP_014498   | 2481              | 1074   | 357       | 5.82 | 39253.50   | 4   | -      | 42.93| VrCOL5     |
| XP_014523   | 14007             | 1230   | 409       | 4.86 | 44487.59   | 5   | +      | 40.33| VrCOL6     |
| XP_014502   | 1506              | 933    | 310       | 7.01 | 33756.82   | 5   | -      | 50.13| VrCOL7     |
| XP_022637   | 4494              | 1329   | 442       | 6.47 | 48806.90   | 5   | +      | 42.28| VrCOL8     |
| XP_014505   | 8864              | 1236   | 411       | 5.21 | 45045.34   | 6   | +      | 34.64| VrCOL9     |
| XP_014510   | 1778              | 1119   | 372       | 6.11 | 40396.31   | 7   | -      | 50.39| VrCOL10    |
| XP_014505   | 2175              | 1110   | 369       | 5.64 | 41395.12   | 7   | +      | 40.37| VrCOL11    |
| XP_022641   | 3819              | 954    | 317       | 6.82 | 35966.39   | 8   | -      | 40.02| VrCOL12    |
| XP_014512   | 2060              | 1254   | 417       | 5.28 | 46964.34   | 8   | -      | 43.83| VrCOL13    |
| XP_014523   | 1960              | 1113   | 370       | 9.22 | 42031.83   | 7   | +      | 40.1  | VrCOL14    |

Chromosomal distribution of the 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. XP_014523547 was discarded due to a lack of related chromosome information.

Seven of the fourteen VrCOL genes were located on the positive strand, and the VrCOL genes were named from VrCOL1 to VrCOL14 based on their chromosomal locations (Table 1). Seven of the eleven mungbean chromosomes contained VrCOL genes, with the exception of chromosomes 2, 9, 10, and 11 (Fig. 1, 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 (VrCOL3, VrCOL4 and VrCOL5) were located on the relatively short chromosomes 3 and 4 (Fig. 1).

Classification and phylogenetic analysis of VrCOL proteins
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 (RYX2KX3RX2KX2RYX2RX2RX2RXR) 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. 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 VrCOL8, which showed a closer relationship with class II members (Fig. 3).

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 and 14 mungbean CO and COL proteins [11, 35]. The proteins from each group were clustered together in the phylogenetic tree (Fig. 4). VrCOL2 and VrCOL5 showed close relationships to A. thaliana CO and soybean GmCOL1a, GmCOL1b, GmCOL2a, and GmCOL2b, all of which have documented roles in the regulation of flowering time [11, 20, 35]. This result suggests that VrCOL2 and VrCOL5 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 the NCBI and analyzed them using the GSDS program [39]. 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 VrCOL8, which contained 6 exons and 6 introns and had a close relationship with group II
members (Fig. 5). 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. 5). 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 CCT and BBX1 domains, respectively (Fig. 5, 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 VrCOL8) (Fig. 5).

Duplication analysis of VrCOL genes

Mungbean has experienced one round of whole-genome duplication that produced many duplicated gene pairs [38, 40]. 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, including the duplicated gene pairs VrCOL2/VrCOL5 and VrCOL6/VrCOL9 (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 VrCOL2 and VrCOL5 showed similar exon-intron organization and similar motifs, as did VrCOL6 and VrCOL9 (Fig. 5), 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 [41]. 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 VrCOL9 (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 VrCOL9 promoter (nine elements), indicating that VrCOL9 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 (Additional file 3), suggesting potential functions of these genes in related signaling pathways.

Table 2

| Gene name  | Development related elements | Environmental stress related elements | Hormone-responsive elements | Light-responsive elements | Promoter related elements | Site-binding related elements | Others |
|------------|------------------------------|---------------------------------------|-----------------------------|---------------------------|---------------------------|-------------------------------|--------|
| VrCOL1     | 1                            | 3                                     | 3                           | 6                         | 2                         | 0                             | 17     |
| VrCOL2     | 1                            | 3                                     | 4                           | 6                         | 2                         | 1                             | 18     |
| VrCOL3     | 0                            | 2                                     | 5                           | 6                         | 3                         | 0                             | 20     |
| VrCOL4     | 4                            | 1                                     | 4                           | 6                         | 2                         | 2                             | 15     |
| VrCOL5     | 0                            | 3                                     | 4                           | 11                        | 2                         | 0                             | 18     |
| VrCOL6     | 0                            | 1                                     | 5                           | 7                         | 2                         | 0                             | 14     |
| VrCOL7     | 2                            | 1                                     | 4                           | 8                         | 2                         | 0                             | 19     |
| VrCOL8     | 1                            | 2                                     | 4                           | 6                         | 2                         | 1                             | 14     |
| VrCOL9     | 1                            | 9                                     | 4                           | 8                         | 2                         | 0                             | 13     |
| VrCOL10    | 1                            | 0                                     | 4                           | 11                        | 2                         | 2                             | 17     |
| VrCOL11    | 1                            | 0                                     | 4                           | 11                        | 2                         | 2                             | 17     |
| VrCOL12    | 0                            | 3                                     | 4                           | 7                         | 2                         | 1                             | 16     |
| VrCOL13    | 1                            | 1                                     | 5                           | 8                         | 2                         | 2                             | 17     |
| VrCOL14    | 0                            | 0                                     | 4                           | 7                         | 2                         | 0                             | 14     |

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, VrCOL7 was highly expressed in all the tested tissues, whereas VrCOL2 and VrCOL8 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, VrCOL11 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 [42–43]. To investigate the conservation and diversity of duplicated genes, we also analyzed their tissue-specific
expression patterns. VrCOL2 and VrCOL5 differed in their expression levels across all the tissues we examined, indicating that they had undergone functional divergence. VrCOL6 and VrCOL9 showed similar expression levels in roots and nodule roots, but they exhibited different expression levels in other tissues (Fig. 7).

Diurnal rhythm of VrCOL2 expression

In A. thaliana, the expressions of CO, COL1 and COL2 are regulated by the circadian clock and show diurnal oscillations [11, 44]. VrCOL2 displayed a close phylogenetic relationship with CO, COL1 and COL2 (Fig. 4). We therefore investigated whether VrCOL2 exhibited diurnal expression rhythms in mungbean leaves under LD and SD conditions. The expression of VrCOL2 showed daily oscillations under SD conditions, reaching a peak at ZT 16. However, under LD conditions, VrCOL2 showed no daily oscillations (Fig. 8). In addition, the expression of VrCOL2 was higher under SD than under LD conditions at ZT 16, suggesting that VrCOL2 is a daily oscillation gene and works under a photoperiod-dependent pathway.

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

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 4). The VrCOL2 transgenic A. thaliana lines showed high levels of VrCOL2 expression (Additional file 5). 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.

FT and TSF accelerate flowering and are regulated by CO in A. thaliana [11, 20], and we therefore investigated the expression of FT and TSF in wild-type and VrCOL2 transgenic plants under LD and SD conditions. FT and TSF showed similar expression levels in VrCOL2 transgenic and wild-type plants under LD conditions. By contrast, FT and TSF showed higher expression levels in VrCOL2 transgenic plants than in wild-type plants under SD conditions (Fig. 10). 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. 4) [35, 45], and their genome sizes are 125 Mb [46], 1100 Mb [47], 500 Mb [48] and 579 Mb [38], 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 [38, 47]. As a result, the COL gene number in mungbean is approximately half that of soybean. Seven of the eleven (63.6%) mungbean chromosomes (Fig. 1), seven of the eight (87.5%) Medicago chromosomes and sixteen of the twenty (80.0%) soybean chromosomes contained COL genes [35, 45], 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 VrCOL8, which contained one BBX and one CCT domain and showed a close relationship with group II members (Fig. 3–4). VrCOL8 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. 5), suggesting that VrCOL8 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 [42]. Two duplicated gene pairs, VrCOL2/VrCOL5 and VrCOL6/VrCOL9 (Fig. 6), were found among the mungbean VrCOLs. The duplicated genes showed close relationships in the phylogenetic
tree and contained similar motifs (Fig. 3–5), 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, VrCOL6 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, Additional file 3). VrCOL6 and VrCOL9 showed similar expression levels in roots and nodule roots, but their expression differed in flowers, pods, leaves, seeds, stems and shoot apices (Fig. 7). 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 VrCOL11 and VrCOL12) 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 VrCOL2, VrCOL8 and VrCOL13 (Fig. 7, Table 2, Additional file 3). 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, 44]. For example, VrCOL2 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). VrCOL8 and VrCOL13 may exhibit higher expression levels at other time points or under other photoperiod conditions.

CO and CO-homologous genes, such as OsHd1, play critical roles in flowering time regulation [11, 20]. VrCOL2 showed close relationships with A. thaliana CO and soybean GmCOL1a, GmCOL1b, GmCOL2a and GmCOL2b, and accelerated flowering under SD but not LD conditions in transgenic A. thaliana
A. thaliana CO regulates FT and TSF to accelerate flowering [26–29], and the expression of FT and TSF increased in VrCOL2 transgenic A. thaliana lines under SD but not LD conditions (Fig. 10), indicating that VrCOL2 regulates downstream genes via photoperiod-dependent pathways. In addition, CO protein accumulation is also regulated by circadian clock. CO mRNA abundance is highly expressed from late afternoon to the dawn, but CO protein only accumulates in the late afternoon under long day conditions [27, 49–51]. 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 A. thaliana CO 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 [52–53] 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, VrCOL2 and VrCOL5 form a duplicated gene pair and show a close relationship with one another (Figs. 3 and 6), indicating that VrCOL5 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 A. thaliana CO 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 FT and TSF, but not LD conditions.

Methods
Plant materials and growth conditions
The reference genome variety VC1973A (named Zhonglu in China) supplied by Suk-Ha Lee at Seoul National University, Seoul, Korea, was used for all experiments [38]. 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 transcriptome analysis, and all samples were stored at -80 °C before RNA extraction. 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) [38] to search for mungbean VrCOL proteins. The presence of conserved BBX and CCT domains in candidate genes were confirmed using the Pfam database [54] and InterPro program with default parameters [55].

Phylogenetic relationship analysis
The amino acid sequences of CO and COL proteins from A. thaliana, soybean, Medicago and mungbean were aligned using ClustalW2 [56], and the resulting alignment was used to construct a phylogenetic tree in MEGA 7.0 using the Neighbor-Joining method with default parameters [57]. 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, 58]. The duplicated gene pairs were identified with amino acid sequences more than 60% similarity, and visualized using Circos software [59].

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) [39] to analyze their gene structures. 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 [55]. The sequence logos of the conserved BBX1, BBX2 and CCT domains were analyzed using the WebLogo platform [60]. 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 [61]. The cis-acting elements in each VrCOL promoter, 2 kb upstream of the initiation codon, were predicted by PlantCARE [41].

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 [62], and successful transformation was confirmed by PCR.

All primers are listed in Additional file 6.

RNA extraction and transcription analysis
RNA isolation and quantitative real-time PCR (qRT-PCR) analysis were carried out as described in Li et al. [40]. Gene expression levels were normalized to an Actin gene from mungbean (Vr1d03g00210). Each sample was analyzed using three biological replicates. All primers are listed in Additional file 6. For RNA-seq analysis, total RNA was extracted from different tissues of mungbean variety VC1973A, including flowers, pods, leaves, seeds, nodule roots, stems, roots and shoot apices, and the RNA purity and RNA integrity were checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA), respectively. The library preparation and RNA-seq were performed by Novogene in Beijing, China, using standard Illumina protocols. In details, the libraries were carried out using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) according to the manufacturer’s instructions. And then the libraries were sequenced on an Illumina Nova platform. After removing low quality reads and reads containing adapter and poly-N from raw data, the clean data was mapped to mungbean genome database in NCBI. The RNA-seq analysis was performed as described by Gao et al. [63]. The gene expression level of each gene was calculated by FPKM values based on the gene length and reads count mapped to that gene, and the FPKM values of each gene were listed in Additional file 7. Each sample was analyzed using two biological replicates. The RNA-seq results were visualized using a heatmap generated with MeV 4.9.0 (Multiple Experiment Viewer) using FPKM values from the RNA-seq database [64].

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 radiate; Gm, Glycine max; pl, Isoelectric point; MW, Molecular weight; Hd1, Heading date 1; Hd3a, Heading date 3a; NCBI, National Center for Biotechnology Information; qRT-PCR, quantitative real-time PCR.

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.

Funding
This research was funded by the National Natural Science Foundation of China (grant 31971898), and the Qingdao Agricultural University Scientific Research Foundation (grant 6631119010). The funders have no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

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.

Acknowledgements
We thank Suk-Ha Lee at Seoul National University, Seoul, Korea, for supplying mungbean VC1973A seeds.

References
1. Wickland DP, Hanzawa Y. The FLOWERING LOCUS T/TERMINAL FLOWER 1 gene family: functional evolution and molecular mechanisms. Mol Plant. 2015;8(7):983-97.
2. Beinecke FA, Grundmann L, Wiedmann DR, Schmidt FJ, Caesar AS, Zimmermann M, Lahme M, Twyman RM, Prуfer D, Noll GA. The FT/FD-dependent initiation of flowering under long-day conditions in the day-neutral species Nicotiana tabacum originates from the facultative short-day ancestor Nicotiana tomentosiformis. Plant J. 2018;96(2):329-42.
3. Eom H, Park SJ, Kim MK, Kim H, Kang H, Lee I. TAF15b, involved in the autonomous pathway for flowering, represses transcription of FLOWERING LOCUS C. Plant J. 2018;93(1):79-91.
4. Xu S, Chong K. Remembering winter through vernalisation. Nat Plants. 2018;4(12):997–1009.

5. Boss PK, Bastow RM, Mylne JS, Dean C. Multiple pathways in the decision to flower: enabling, promoting, and resetting. Plant Cell. 2004;16(Suppl):18–31.

6. Jack T. Molecular and genetic mechanisms of floral control. Plant Cell. 2004;16(Suppl):1–17.

7. Baurle I, Dean C. The timing of developmental transitions in plants. Cell. 2006;125(4):655–64.

8. Ronald J, Davis SJ. Focusing on the nuclear and subnuclear dynamics of light and circadian signaling. Plant Cell Environ. 2019;42:2871–84.

9. Taylor CM, Kamphuis LG, Zhang W, Garg G, Berger JD, Mousavi-Derazmahalleh M, Bayer PE, Edwards D, Singh KB, Cowling WA, et al. INDEL variation in the regulatory region of the major flowering time gene *LanFTc1* is associated with vernalization response and flowering time in narrow-leafed lupin (*Lupinus angustifolius* L.). Plant Cell Environ. 2019;42:174–87.

10. Zhang W, Yuan J, Cheng T, Tang MJ, Sun K, Song SL, Xu FJ, Dai CC. Flowering-mediated root-fungus symbiosis loss is related to jasmonate-dependent root soluble sugar deprivation. Plant Cell Environ. 2019;42(12):3208–26.

11. Gangappa SN, Botto JF. The *BBX* family of plant transcription factors. Trends Plant Sci. 2014;19(7):460–70.

12. del-Olmo I, Poza-Viejo L, Piñeiro M, Jarillo JA, Crevillén P. High ambient temperature leads to reduced *FT* expression and delayed flowering in *Brassica rapa* via a mechanism associated with H2AZ dynamics. Plant J. 2019;100:343–56.

13. Jin H, Tang X, Xing M, Zhu H, Sui J, Cai CM, Li S. Molecular and transcriptional characterization of phosphatidyl ethanolamine-binding proteins in wild peanuts.
"Arachis duranensis" and "Arachis ipaensis." BMC Plant Biol. 2019;19:484.

14. Jing Y, Guo Q, Zha P, Lin R. The chromatin-remodeling factor PICKLE interacts with CONSTANS to promote flowering in Arabidopsis. Plant Cell Environ. 2019;42:2495-507.

15. Lee C, Kim S, Jin S, Susila H, Youn G, Nasim Z, Alavilli H, Chung K, Yoo SJ, Ahn JH. Genetic interactions reveal the antagonistic roles of FT/TSF and TFL1 in the determination of inflorescence meristem identity in Arabidopsis. Plant J. 2019;99:452-64.

16. Nam J, dePamphilis CW, Ma H, Nei M. Antiquity and evolution of the MADS-box gene family controlling flower development in plants. Mol Biol Evol. 2019;20:1435-47.

17. Ning Y, Chen Q, Lin R, Li Y, Li L, Chen S, He X. The HDA19 histone deacetylase complex is involved in the regulation of flowering time in a photoperiod-dependent manner. Plant J. 2019;98:448-64.

18. Parenicova L, de Folter S, Kieffer M, Horner DS, Favalli C, Busscher J, Cook HE, Ingram RM, Kater MM, Davies B, et al. Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: new openings to the MADS world. Plant Cell. 2019;15:1538-51.

19. Jin H, Xing M, Cai C, Li S. B-box proteins in Arachis duranensis: genome-wide characterization and expression profiles analysis. Agronomy. 2020;10:23.

20. Khanna R, Kronmiller B, Maszle DR, Coupland G, Holm M, Mizuno T, Wu SH. The Arabidopsis B-box zinc finger family. Plant Cell. 2019;21(11):3416-20.

21. Robson F, Costa MMR, Hepworth SR, Vizir I, Pineiro M, Reeves PH, Putterill J, Coupland G. Functional importance of conserved domains in the flowering-time gene CONSTANS demonstrated by analysis of mutant alleles and transgenic plants. Plant J. 2001;28:619-31.
22. Yan H, Marquardt K, Indorf M, Jutt D, Kircher S, Neuhaus G, Rodríguez-Franco M. Nuclear localization and interaction with COP1 are required for STO/BBX24 function during photomorphogenesis. Plant Physiol. 2011;156:1772–82.

23. Luo X, Gao Z, Wang Y, Chen Z, Zhang W, Huang J, Yu H, He Y. The NUCLEAR FACTOR-CONSTANS complex antagonizes polycomb repression to de-repress FLOWERING LOCUS T expression in response to inductive long days in Arabidopsis. Plant J. 2018;95:17–29.

24. Luccioni L, Krzymuski M, Sánchez-Lamas M, Karayekov E, Cerdán PD, Casal JJ. CONSTANS delays Arabidopsis flowering under short days. Plant J. 2019;97:923–32.

25. Serrano-Bueno G, Said FE, de los Reyes P, Lucas-Reina El, Ortiz-Marchena MI, Romero JM, Valverde F. CONSTANS-FKBP12 interaction contributes to modulate photoperiodic flowering in Arabidopsis. Plant J. 2020;101:1287–302.

26. Putterill J, Robson F, Lee K, Coupland G. Chromosome walking with YAC clones in Arabidopsis: isolation of 1700 kb of contiguous DNA on chromosome 5, including a 300 kb region containing the flowering-time gene CO. Molec Gen Genet. 1993;239:145–57.

27. Putterill J, Robson F, Lee K, Simon R, Coupland G. The CONSTANS gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. Cell. 1995;80(6):847–57.

28. Andres F, Coupland G. The genetic basis of flowering responses to seasonal cues. Nat Rev Genet. 2012;13(9):627–39.

29. Song YH, Ito S, Imaizumi T. Flowering time regulation: photoperiod-and temperature-sensing in leaves. Trends Plant Sci. 2013;18(10):575–83.

30. Imaizumi T, Schultz TF, Harmon FG, Ho LA, Kay SA. FKF1 F-Box protein mediates cyclic degradation of a repressor of CONSTANS in Arabidopsis. Science.
31. Sawa M, Nusinow DA, Kay SA, Imaizumi T. FKF1 and GIGANTEA complex formation is required for day-length measurement in Arabidopsis. Science. 2007;318(5848):261-5.

32. Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, et al. Hdl, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene CONSTANS. Plant Cell. 2000;12(12):2473–83.

33. Komiya R, Ikegami A, Tamaki S, Yokoi S, Shimamoto K. Hdl3a and RFT1 are essential for flowering in rice. Development. 2008;135(4):767–74.

34. Komiya R, Yokoi S, Shimamoto K. A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice. Development. 2009;136(20):3443–50.

35. Wu F, Price BW, Haider W, Seufferheld G, Nelson R, Hanzawa Y. Functional and evolutionary characterization of the CONSTANS gene family in short-day photoperiodic flowering in soybean. PLoS One. 2014;9(1):e85754.

36. Keatinge JDH, Easdown WJ, Yang RY, Chadha ML, Shanmugasundaram S. Overcoming chronic malnutrition in a future warming world: the key importance of mungbean and vegetable soybean. Euphytica. 2011;180:129–41.

37. Fuller DQ. Contrasting patterns in crop domestication and domestication rates: recent archaeobotanical insights from the old world. Ann Bot. 2007;100:903–24.

38. Kang YJ, Kim SK, Kim MY, Lestari P, Kim KH, Ha BK, Jun TH, Hwang WJ, Lee T, Lee J, et al. Genome sequence of mungbean and insights into evolution within Vigna species. Nat Commun. 2014;5:5443.

39. Hu B, Jin J, Guo A, Zhang H, Luo J, Gao G. GDS 2.0: an upgraded gene feature visualization server. Bioinformatics. 2015;31(8):1296–7.
40. Li S, Wang R, Jin H, Ding Y, Cai C. Molecular characterization and expression profile analysis of heat shock transcription factors in mungbean. Front Genet. 2019;9:736.

41. Lescot M, Dehais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P, Rombauts S. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 2002;30:325–7.

42. Kondrashov FA, Rogozin IB, Wolf YI, Koonin EV. Selection in the evolution of gene duplications. Genome Biol. 2002;3:RESEARCH0008.

43. Wang Z, Zhou Z, Liu Y, Liu T, Li Q, Ji Y, Li C, Fang C, Wang M, Wu M, et al. Functional evolution of phosphatidyl ethanolamine binding proteins in soybean and Arabidopsis. Plant Cell. 2015;27:323–36.

44. Suárez-López P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. Nature. 2001;410:1116–20.

45. Wong ACS, Hecht VFG, Picard K, Diwadkar P, Laurie RE, Wen J, Mysore K, Macknight RC, Weller JL. Isolation and functional analysis of CONSTANS-LIKE genes suggests that a central role for CONSTANS in flowering time control is not evolutionarily conserved in Medicago truncatula. Front Plant Sci. 2014;5:486.

46. Initiative AG. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature. 2000;408:796–815.

47. Schmutz J, Cannon S, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, et al. Genome sequence of the palaeopolyploid soybean. Nature. 2010;463:178–83.

48. Young N, Debellé F, Oldroyd G, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H, et al. The Medicago genome provides insight into the evolution of rhizobial symbioses. Nature. 2011;480:520–4.
49. Shim JS, Imaizumi T. Circadian clock and photoperiodic response in *Arabidopsis*: from seasonal flowering to redox homeostasis. Biochemistry. 2015;54:157–70.

50. Shim JS, Kubota A, Imaizumi T. Circadian clock and photoperiodic flowering in *Arabidopsis*: **CONSTANS** is a hub for signal integration. Plant Physiol. 2017;173(1):5–15.

51. Song YH, Shim JS, Kinmonth-Schultz HA, Imaizumi T. Photoperiodic flowering: time measurement mechanisms in leaves. Annu Rev Plant Biol. 2015;66:441–64.

52. Imrie BC. Mung bean. In: Hyde K, editor. The New Rural Industries: A Handbook for Farmers and Investors. Canberra: Rural Industries Research and Development Corporation); 1996. pp. 355–60.

53. Kim SK, Nair RM, Lee J, Lee SH. Genomic resources in mungbean for future breeding programs. Front Plant Sci. 2015;6:626.

54. El-Gebali S, Mistry J, Bateman A, Eddy S, Luciani A, Potter S, Qureshi M, Richardson L, SalazarG, Smart A, et al. The Pfam protein families database in 2019. Nucleic Acids Res. 2019;47:427–32.

55. Finn R, Attwood T, Babbitt P, Bateman A, Bork P, Bridge A, Chang H, Dosztányi Z, El-Gebali S, Fraser M, et al. InterPro in 2017–beyond protein family and domain annotations. Nucleic Acids Res. 2017;45:190–9.

56. Oliver T, Schmidt B, Nathan D, Clemens R, Maskell D. Using reconfigurable hardware to accelerate multiple sequence alignment with clustalW. Bioinformatics. 2005;21:3431–2.

57. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4.

58. Fischer S, Brunk BP, Chen F, Gao X, Harb OS, Iodice JB, Shanmugam D, Roos DS, Stoeckert CJ. Using OrthoMCL to assign proteins to OrthoMCL-DB groups or to cluster
proteomes into new ortholog groups. Curr Protoc Bioinformatics. 2011;35:1–19.

59. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, Jones SJ, Marra MA. Circos: An information aesthetic for comparative genomics. Genome Res. 2009;19:1639–45.

60. Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res. 2004;14(6):1188–90.

61. Bailey T, Boden M, Buske F, Frith M, Grant C, Clementi L, Ren J, Li W, Noble W. MEME suite: Tools for motif discovery and searching. Nucleic Acids Res. 2009;37:W202–8.

62. Bent A. Arabidopsis thaliana floral dip transformation method. Methods Mol Biol. 2006;343:87–103.

63. Gao J, Bi W, Li H, Wu J, Yu X, Liu D, Wang X. WRKY transcription factors associated with NPR1-Mediated acquired resistance in Barley are potential resources to improve wheat resistance to Puccinia triticina. Front Plant Sci. 2018; 9:1486.

64. Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, et al. TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 2003;34(2):374–8.

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

Additional file 5: 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 6: Primers used in this study.

Additional file 7: RNA-seq analysis of mungbean gene expression in different tissues.

Roots, nodule roots, shoot apices, stems, leaves, flowers, pods and seeds were used for analysis.

Figures
Figure 1

Chromosomal locations of the VrCOL genes. Chromosome number and length 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

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 and mungbean were used to construct the phylogenetic tree in MEGA7.0.
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 6

Duplication analysis of VrCOL proteins. The duplicated gene pairs are connected by lines.
Relative expression levels of VrCOL genes in different tissues. The expression levels were visualized using a heatmap generated with MeV 4.9.0 using FPKM values from the RNA-seq database.
Relative expression of VrCOL2 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 VrCOL2 was normalized to an ACTIN gene from mungbean.
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). ***P < 0.001, ** P < 0.01, bars = 4 cm.
The expression of FT and TSF in VrCOL2 transgenic lines and wild-type plants under LD (a, c) and SD conditions (b, d). 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.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.
Additionalfile7.xlsx
