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Molecular Cloning and Expression Analysis of a Novel *CONSTANS*-like Gene from Potato

J. L. Guo, Q. Yang*, F. Liang, Y. J. Xing, and Z. Wang

College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China;
fax: 86-25-8439-5221; E-mail: qyang19@njau.edu.cn

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Abstract—A full-length cDNA of a *StCONSTANS*-like (*StCOL*) gene was cloned from potato (*Solanum tuberosum* L.) by RT-PCR and RACE. The predicted amino acid sequence of this cDNA has a high degree of identity with other homologous members of the CO or COL family. Analysis of mRNA levels for *StCOL* shows that it is highly expressed in leaves and becomes weaker during tuberization; moreover, is independent of gibberellin A_3 and sucrose.

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Key words: *StCONSTANS*-like, cloning, expression, potato

Many plants flower in response to seasonal fluctuations in day length. Long day length triggers flowering of Arabidopsis. In 1995, Coupland's group tested the flowering time of *co-2 gai* double mutants grown under long day length conditions. They reported that 13% of them did not flower, and that the rest flowered much later than either the *co-2* or *gai* parental lines [1]. To gain detailed insight into the molecular mechanism in late-flowering mutants, the group isolated a gene named *CONSTANS* (*CO*) from Arabidopsis, and found that transgenic lines carrying extra copies of the *CO* gene flowered slightly earlier than wild-type plants [1]. Up to now, a series of *CONSTANS* and *CONSTANS*-like (*COL*) genes have been identified in several plants [2-6]. The proteins encoded by these genes are mainly involved in the mediation of the photoperiodic induction of flowering. CO/COL proteins probably act as transcriptional activators, directly or indirectly activating the floral meristem identity gene *LEAFY* [7].

Tuberization and flowering are two distinct reproduction strategies. As well known, several factors including temperature, gibberellin A_3 (GA_3), photoperiod, sucrose concentration, etc. influence potato tuber formation; of these, photoperiod is an important factor influencing potato production, short day length being favorable for tuberization and advancing the early growth of tuber [8].

Previous studies have demonstrated a high similarity between plant tuberization and flowering in the photoperiod pathway. To investigate whether any common factors might control two photoperiodic evocation responses, Prat's group analyzed the expression of Arabidopsis *CO* gene in transgenic potato plants and found that AtCO inhibited tuberization by reducing the total number and weight of tubers [9]. However, it is still not clear how the gene regulates tuberization in a genetic way. Cloning the genes involved in photoperiod pathways may be put forward for investigating the role of the genes during tuberization in potato. In the present study, we isolated a *CONSTANS*-like gene from *Solanum tuberosum* and studied the expression pattern of the gene in different tissues and during tuberization.

MATERIALS AND METHODS

Favorita (*Solanum tuberosum* L.) is an important breed that is broadly planted in potato production. Plants were cultivated in a greenhouse under a regime of 8 h light (25°C) and 16 h dark (23°C).

Total RNA was extracted from 0.1 g of fresh leaves with total RNA isolation reagent (Tiangen, China) following the manufacturer's instructions.

To clone the conserved cDNA region of the *StCONSTANS*-like gene, a pair of primers, P1 (GTGTCTGGGTTTGTGAAGTGTG) and P2 (TCCTCTTCTCTCTGTACCTCAT), was designed according to

Abbreviations: GA_3) gibberellin A_3; MS medium) Murashige and Skoog medium.

* To whom correspondence should be addressed.

the conserved regions of *CO* genes from other plants using DNAssist 2.0 software.

First-strand cDNA was synthesized with M-MLV-reverse transcriptase from Promega (USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out according to the following program: 94°C for 4 min, followed by 30 cycles, 94°C for 40 sec, 55°C for 40 sec, 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR product was about 800 bp long.

To clone the 3′ end of the gene, the first strand cDNA synthesis from leaves was performed according to the manufacturer's instructions of rapid amplification of cDNA 3′ ends system (3′RACE). Primary amplification, using gene specific primer P3 (CATCATCAACAGCATCAACACG) and 3′ sites adaptor primer (CTGATCTAGAGGTACCGGATCC), was carried out according to the following program: 94°C for 4 min, and then 30 cycles of 94°C for 40 sec, 55°C for 40 sec, and 72°C for 1 min, with a final extension step at 72°C for 5 min. An aliquot of 1 μl (1 : 30 diluted) primary amplification products was used for 3′ end nested amplification under the same PCR condition using adaptor primer and gene specific primer P4 (ACACGGCCATTACAGCTCC). The PCR reaction yielded a product about 450 bp long.

To obtain 5′ end sequence, a primer P5 (ATGTTGAAGGAAGAGAGC) was designed according to 5′ end of the sequence (Acc. No. BT013260) from tomato. Primary amplification was carried out using P5 and gene specific primer P6 (CATCATCAGCAGCATCAGCATC) under the following PCR condition: 94°C for 4 min, followed by 30 cycles (94°C for 30 sec, 67°C for 40 sec, 72°C for 1 min) and by extension at 72°C for 5 min. An aliquot of 1 μl (1 : 20 diluted) primary amplification products was used for 5′ end nested amplification under the same PCR condition using primer P5 and gene specific primer P7 (GAACTACCGGAAACCGCTCATG). The PCR reaction yielded a product about 400 bp long.

All PCR products were separated on 1% agarose gels and target DNA bands were recovered by gel extraction and cloned into pMD18T vector (Takara Biotech, China), and finally transformed into competent cells of *E. coli* strain DH5α. White clones were checked by PCR, and the positive clones were sequenced (Invitrogen Biotech, China).

The full length of the gene was amplified with gene specific primers P8 (ATGGGAACGGAGAATTGGAGT) and P9 (GTTGATCGTGGAAGAGAGAG). PCR conditions were: 94°C for 4 min, followed by 30 cycles (94°C for 30 sec, 58°C for 40 sec, 72°C for 1 min) and with a final extension step at 72°C for 5 min. The PCR products were sequenced. Sequencing data accumulation, processing, and sequence alignment analysis were performed using DNAMAN. Blastp was performed on http://www.ncbi.nlm.nih.gov. The phylogenetic tree was constructed with DNAMAN.

For the organ expression studies, white fibrous roots, stems, leaves, apical buds, floral buds, flowers, initiation stolons, stolons, swollen stolons, small tubers, and mature tubers were harvested from potato plants cultivated in a greenhouse for 60 days under short day length. The materials were frozen immediately in liquid nitrogen and stored at −70°C. Total RNA was extracted as described above.

To study the effects of GA_3 and sucrose on *StCONSTANS*-like gene expression, stem cuttings were inoculated and cultured on MS (Murashige−Skoog) liquid medium containing 3% (m/v) sucrose for a month, and then microtubers were induced in MS liquid medium supplement with 3% (m/v) sucrose, 8% (m/v) sucrose, and 3% (m/v) sucrose plus GA_3 (1 mg/liter), respectively. The percentage of plants with tubers was periodically recorded during the following month in darkness, and stems were harvested weekly and frozen in liquid nitrogen and stored at −70°C. Total RNA was extracted as described above.

The potato *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an internal control in RT-PCR. The reaction for *GAPDH* using specific primers *GAPDH*-S (CAAGGACTGGAGAGGTGG) and *GAPDH*-A (TTCACTCGTTGTCGTACC) was performed 94°C for 4 min, and then 26 cycles of amplification using the following parameters: denaturation (40 sec at 94°C), annealing (40 sec at 54°C), extension (40 sec at 72°C). The corresponding amount of cDNA was used as template among samples with *StCONSTANS*-like specific primers P8 and P9, the reaction conditions being as described above but reduced to 26 cycles.

RESULTS AND DISCUSSION

PCR products cloned from potato were sequenced and the full length of the cDNA (Acc. No. DQ882684) contains an open reading frame of 1080 bp coding a protein of 360 amino acids, corresponding to a 39.21 kD polypeptide with an isoelectric point of 5.09.

The amino acid sequence analysis using DNAMAN showed StCOL has a high identity with AtCO (63.9%), AtCOL1 (62.1%), AtCOL2 (64.2%), and AtCOL3 (61.2%) from Arabidopsis (Fig. 1), especially in their N- and C-terminal regions; highly conserved amino region indicates B-box [2] and the C-terminal region indicates CCT domain, respectively. In addition, the 7-amino acid stretch (YGVVPSF) that is conserved in all CO or COL was also observed in this new protein, suggesting that it belongs to the CO or COL protein family (Fig. 1).

The number of CO/COLs varies in different species of plants, for example: four in *B. nigra* [10], 16 in *O. sativa*, nine in *H. vulgare*, and 17 in *A. thaliana* [7]. In Arabidopsis, CO belongs to a family of 17 members defined by two conserved domains of B-box and CCT [1]. Based on the variation of zinc finger region, the gene

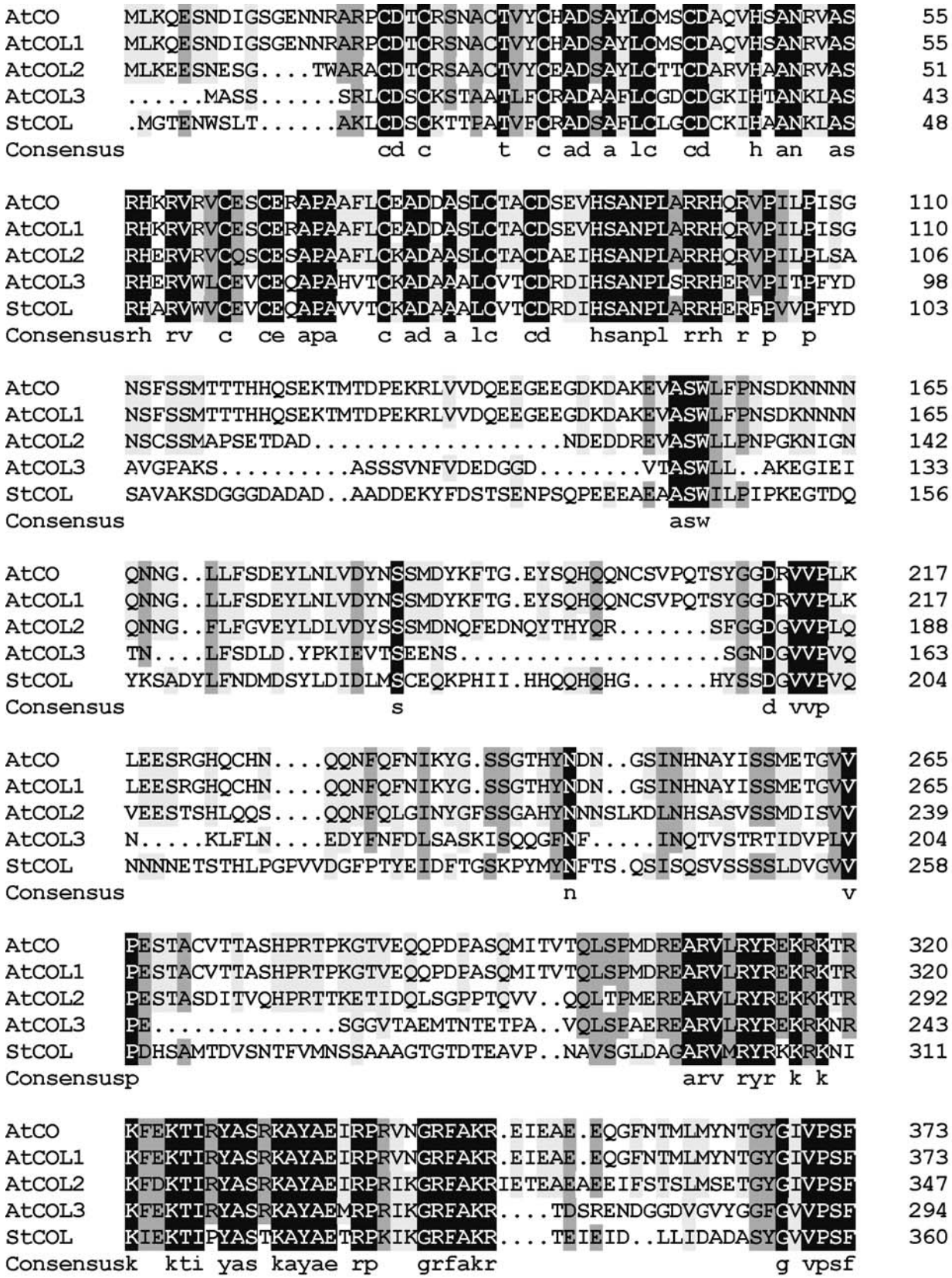

Fig. 1. Alignment of amino acid sequence of StCOL (Acc. No. ABH09237) from *S. tuberosum* with those of AtCO (Acc. No. NP_197088), AtCOL1 (Acc. No. NP_197089), AtCOL2 (Acc. No. NP_186887), AtCOL3 (Acc. No. NP_180052). The identical amino acids are shaded in black, and the conserved amino acids are shaded in gray.

family is divided into three subgroups: group I includes CO and COL1 to COL5 with two B-boxes; group II has COL6-COL8 and COL16 with one B-box; group III includes COL9 to COL15 with one B-box and a second

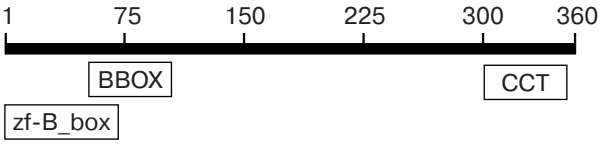

Fig. 2. Scheme of distribution of conserved B-box, CCT, and variable regions in the StCOL protein molecule. Numbers delineate addresses in amino acid sequences.

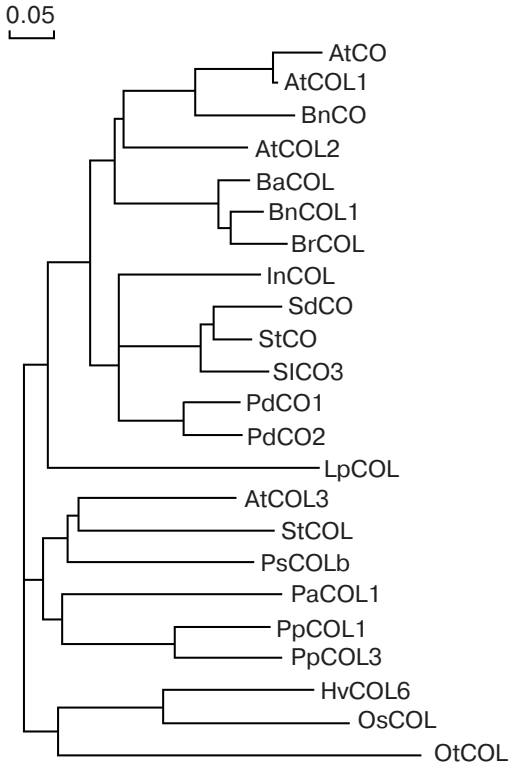

Fig. 3. Phylogenetic analysis of the CONSTANS or CONSTANS-like proteins from different plant species. The tree is displayed as a phylogram in which branch lengths are proportional to distance. The proteins are as follows: AtCO, AtCOL1, AtCOL2, AtCOL3 (Acc. No. NP_001031887, NP_197089, NP_186887, NP_180052) from *A. thaliana*; OtCOL (Acc. No. AAU14282) from *O. tauri*; PsCOLb (Acc. No. AAX47173) from *P. sativum*; PaCOL1 (Acc. No. CAK26129) from *P. abies*; PpCOL1, PpCOL3 (Acc. No. BAD89084, CAI64585) from *P. patens*; LpCOL (Acc. No. AAT42130) from *L. perenne*; InCOL (Acc. No. AAG24863) from *I. Nil*; PdCO1, PdCO2 (Acc. No. AAS00054, AAS00055) from *P. deltoids*; SlCO3 (Acc. No. AAS67379) from *S. lycopersicum*; SdCO (Acc. No. ABF56054) from *S. demissum*; StCO, StCOL (Acc. No. ABF56053, ABH09237) from *S. tuberosum*; BnCO (Acc. No. AAC27694) from *B. napus*; BaCOL (Acc. No. CAL29796) from *B. alboglabra*; BnCOL (Acc. No. AAN09813) from *B. nigra*; BrCOL (Acc. No. AAQ84234) from *B. rapa*; OsCOL (Acc. No. BAD27992) from *O. sativa*; HvCOL6 (Acc. No. AAL99266) from *H. vulgare*.

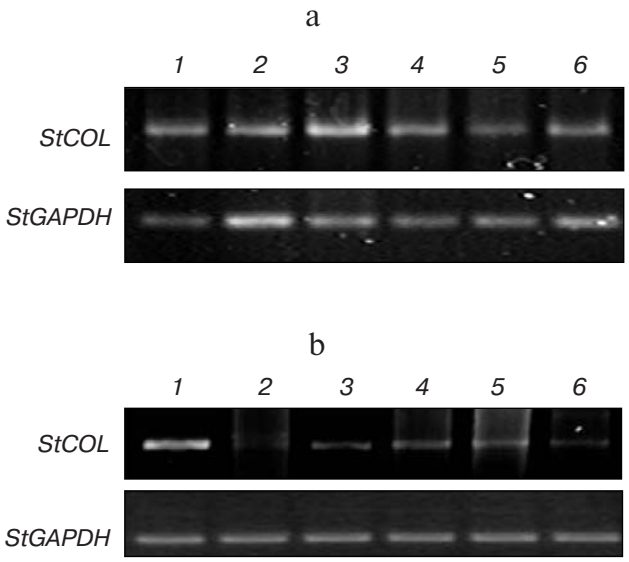

Fig. 4. a) Expression of *StCOL* in different tissues: *1*) roots; *2*) stems; *3*) leaves; *4*) apical buds; *5*) floral buds; *6*) flowers. b) Expression of *StCOL* during tuberization: *1*) stems; *2*) initial stolons; *3*) stolons; *4*) swelling stolons; *5*) small tubers; *6*) mature tubers.

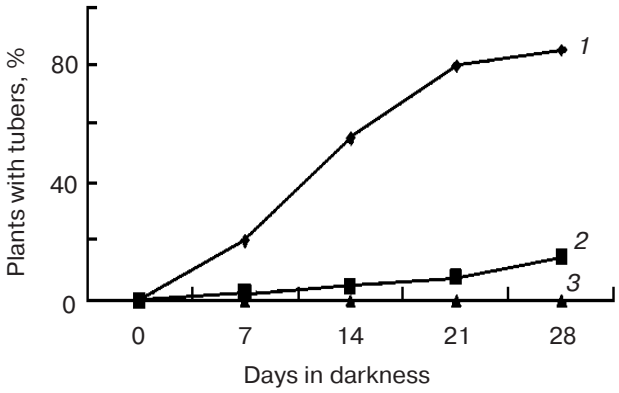

Fig. 5. Kinetics of tuber initiation in plants on various nutrient media in continuous darkness (8% sucrose (*1*), 3% sucrose (*2*), 3% sucrose plus 1 mg/liter GA_3 (*3*)).

diverged zinc finger [11]. In this study, NCBI Blastp analysis was carried out. As shown in Fig. 2, the Blastp result indicates that StCOL has two B-box domains and a CCT domain. It suggests that the new protein belongs to the first group.

On the other hand, we downloaded some other sequences of CO/COL proteins from different species from GenBank and constructed a phylogenetic tree (Fig. 3). The phylogenetic tree showed that all members could be divided into four divergent groups (Fig. 3) and that the new protein was clustered to PsCOLb and had a high homology with other COL sequences of higher plants including PpCOL1 and PpCOL3 from *P. patens* and

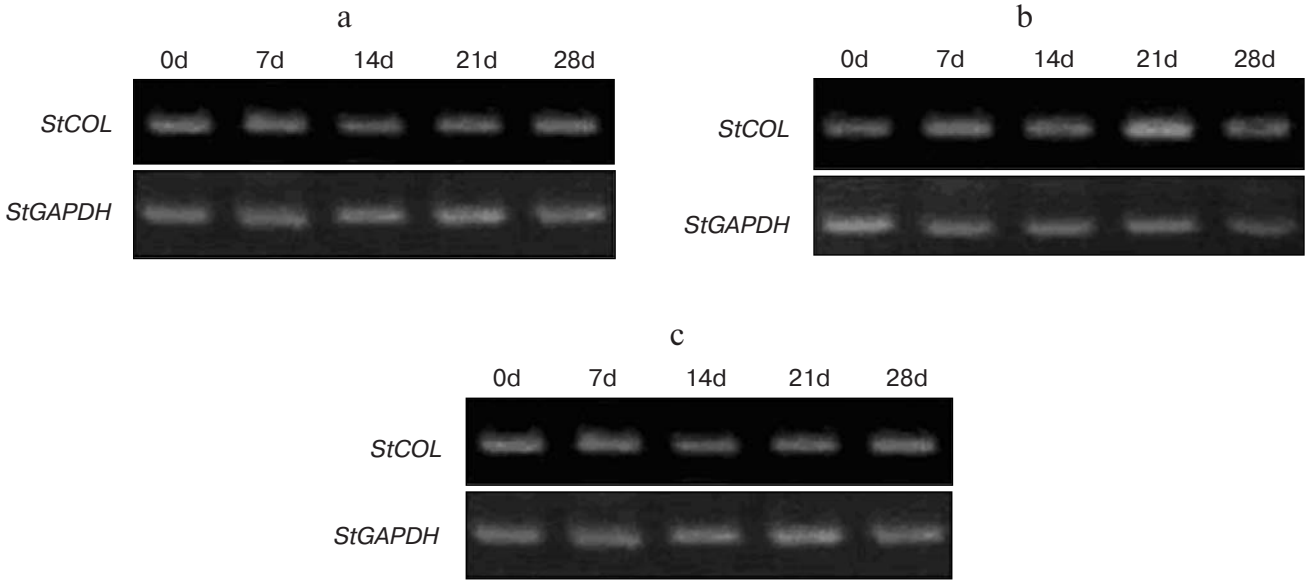

Fig. 6. Expression of *StCOL* in darkness on MS supplement with 3% sucrose, 8% sucrose, 3% sucrose plus 1 mg/liter GA_3 (a-c, respectively).

AtCOL3 from *A. thaliana*. So we refer to the predicated protein as StCOL.

To investigate the pattern of expression of the *StCOL* gene in potato plants, total RNA was extracted from different tissues. The level of transcript varied among the different tissues and high level of *StCOL* expression was observed in leaves. This result is consistent with the fact that leaf is an organ perceiving photoperiod, where the CO protein is responsible for sensing the day length signal. In this study we also observed *StCOL* expression in other organs, especially in roots. This suggested that StCOL may participate in some other common and basic processes of plant (Fig. 4a). A similar result was reported in *A. thaliana* [12].

For determining the function of *StCOL* during tuberization, we observed the expression of *StCOL* mRNA during tuber development (Fig. 4b). *StCOL* mRNA accumulation was observed to drop down and become low till mature tuber stage. This result suggests that a negative direct involvement of StCOL in preceding events may lead to inducing stolon and stolon-to-tuber transition during tuber formation.

Previous studies have shown the initiator role of sucrose in developing tubers and inhibition of GAs on tuber initiation [13-18]. In our study, three treatments including 3% sucrose, 8% sucrose, and 3% sucrose + 1 mg/liter GA_3 displayed different time-courses of tuber initiation (Fig. 5). By the end of the month, frequencies of tuber induction varied among the three treatment groups: nearly 85% of potato plants on MS supplement with 8% sucrose; only 15% of potato plants on MS supplement with 3% sucrose; no tuber was observed on MS supplement with GA_3.

The effects of sucrose and GA_3 on *StCOL* expression in stems were further examined. *StCOL* was expressed stably and was not influenced by either sucrose or GA_3 during the culture (Fig. 6). The result show that the inhibitor GA_3 does not cooperate with StCOL in blocking tuber formation, and sucrose activator does not antagonize *StCOL*. In addition, transcript levels of *StCOL* were observed in continuous darkness; this fact is consistent with the statement that *PnCO* and *AtCOL1* in darkness has been described before [3, 19], meaning that COL is involved in other processes besides participating in flowering by photoperiod pathways in higher plants.

In conclusion, *StCOL*, a *CO* homolog, was isolated from potato. The sequence of StCOL was highly conserved in two B-box domains and a CCT domain. *StCOL* transcription was not affected by GA_3 and sucrose. Further investigation of the function of the *CO* and *COL* family is likely to come from performing RNAi assay of *StCOL* and identifying interacting proteins during tuber formation.

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