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ORIGINAL ARTICLE

A *CONSTANS-LIKE* gene of *Nelumbo nucifera* could promote potato tuberization

Dingding Cao[1,2] · Zhongyuan Lin[2] · Longyu Huang[3] · Rebecca Njeri Damaris[1] · Ming Li[1] · Pingfang Yang[1]

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Abstract

Main conclusion **CONSTANS-LIKE 5 of *Nelumbo nucifera* is capable of promoting potato tuberization through CONSTANS-FLOWERING LOCUS T and gibberellin signaling pathways with a probable association with lotus rhizome enlargement.**

Abstract Lotus (*Nelumbo nucifera*) is an aquatic plant that is affiliated to the Nelumbonaceace family. It is widely used as an ornamental, vegetable, and medicinal herb with its rhizome being a popular vegetable. To explore the molecular mechanism underlying its rhizome enlargement, we conducted a systematic analysis on the *CONSTANS-LIKE (COL)* gene family, with the results, indicating that this gene plays a role in regulating potato tuber expansion. These analyses included phylogenetic relationships, gene structure, and expressional patterns of lotus *COL* family genes. Based on these analyses, *NnCOL5* was selected for further study on its potential function in lotus rhizome formation. NnCOL5 was shown to be located in the nucleus, and its expression was positively associated with the enlargement of lotus rhizome. Besides, the overexpression of *NnCOL5* in potato led to increased tuber weight and starch content under short-day conditions without changing the number of tubers. Further analysis suggested that the observed tuber changes might be mediated by affecting the expression of genes in CO–FT and GA signaling pathways. These results provide valuable insight in understanding the functions of *COL* gene as well as the enlargement of lotus rhizome.

Keywords CONSTANS-LIKE 5 · Gibberellin · Lotus · Rhizome enlargement

Abbreviations

COL	CONSTANS-LIKE
FT	FLOWERING LOCUS T
LD	Long day
SD	Short day
SP5G	SELF-PRUNING 5G

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✉ Pingfang Yang
yangpf@hubu.edu.cn

[1] State Key Laboratory of Biocatalysis and Enzyme Engineering, School of Life Sciences, Hubei University, Wuhan 430062, China

[2] Institute of Oceanography, Minjiang University, Fuzhou 350108, China

[3] Institute of Cotton Research of Chinese Academy of Agriculture Science, Anyang 455000, China

Introduction

Phylogenetically, Nelumbonaceae is a small family with only one genus (*Nelumbo*) and two species, *Nelumbo nucifera* and *Nelumbo lutea*. *Nelumbo nucifera* is also known as Asian lotus or simply lotus. There are two ecotypes of lotus, temperate and tropical lotus where the rhizome of temperate lotus enlarges in the autumn, while that of tropical lotus keeps in the stolon form throughout its life cycle. Budding rhizomes are useful for lotus vegetative propagation as it is the main way of lotus propagation and production. The enlarged lotus rhizome is also consumed as a popular vegetable with moderate calories but abundance of vitamins (Borgi et al. 2007; Liu et al. 2010). Taken together, the rhizome enlargement is important not only for the plant to survive the winter time but also for agricultural production. Because of its importance, several transcriptomic and proteomic studies have been conducted focusing on the rhizome development (Cheng et al. 2013; Yang et al. 2015; Cao et al. 2019; Lin et al. 2019). However, the underlying molecular mechanisms

of this process, especially the rhizome enlargement, are still elusive.

Morphologically, the lotus rhizome is a type of modified subterranean stem that is similar to those of other plant species, such as bamboo, onion, and potato. Based on practical experiences, it has been documented that light and temperature are the main environmental factors affecting modified roots or stems development (Chailakhyan et al. 1981). A previous study has shown that potato (*Solanum tuberosum*) flowering and tuber formation are similarly induced (Chailakhyan et al. 1981). After perceiving a photoperiod signal, the 'florigen' or 'tuberigen' is produced in the leaves, and then transduced to the aerial shoot tip or tuber (Martínez-García et al. 2002) to induce a flowering transition or a tuberization transition, respectively (Jackson 1999; Lifschitz et al. 2006; Turck et al. 2008; Zeevaart 2008). Phytohormones, especially auxin, abscisic acid (ABA), and gibberellic acid (GA), play important roles in the formation of storage organs (Fernie and Willmitzer 2001; D'Alessandro et al. 2015). Auxin is a crucial regulator in plant growth, including root growth and tuber formation (Roumeliotis et al. 2012; D'Alessandro et al. 2015; Velasquez et al. 2016). Overexpression of GA 20-oxidase gene (*StGA20ox1*) increased the GA content, promoted potato plants stem elongation, and delayed tuber induction (Carrera et al. 2000), whereas exogenous ABA promoted tuberization and decreased stolon length (Xu et al. 1998).

It has been well elucidated that the CO-FT (CONSTANS/CONSTANS-LIKE; FLOWERING LOCUS T) pathway regulates the vegetative to reproductive growth transition, of which CO/COL regulates the flowering time together with FT in long-day (LD) conditions in Arabidopsis (Imaizumi et al. 2003; Bailey et al. 2009). The CO/COL family proteins can be divided into three groups based on its protein structure with group I containing 2 B-box domains and a CCT domain; group II containing only one B-box domains and a CCT domain; while group III contains one B-box domain, a second diverged zinc finger, and a CCT domain (Putterill et al. 1995; Griffiths et al. 2003). Overexpression of *AtCOL5* induces flowering in Arabidopsis under short-day (SD) conditions (Hassidim et al. 2009), with the CONSTANS CCT domain being crucial in photoperiod sensing (Brambilla and Fornara 2017). Although *AtCOL4* was reported to be involved in salt stress response (Min et al. 2015), it could also act as a modulator of flowering time along with FT and APETALA 1 (AP1) (Steinbach 2019). Proteins in this pathway including the FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1), GIGANTEA (GI), and CYCLING DOF FACTOR 1 (CDF1) could function through either regulating the expression of *CO* or interacting with CO (Sawa et al. 2007; Song et al. 2012).

In tuber forming plants, the CO-FT pathway is critical for the transition from flowering to tuber formation, of which overexpression of *AtCO* in potato delayed the tuber formation in SD conditions (Martínez-García et al. 2002). StCOL1 promotes the expression of *SP5G* (*SELF-PRUNING 5G,* an *FT* homolog in potato), a tuberization repressor in potato (Abelenda et al. 2016), whereas StCDF1 initiates tuberization through repression of *StCO* expression and activation of *StSP6A* (another *FT* homolog in potato) (Kloosterman et al. 2013). In the downstream of CO-FT pathway, StSP6A, together with a 14–3-3 protein, and an StFD1-like protein act as the tuber activation complex (Teo et al. 2017). In the circadian rhythm pathway, StBEL5 together with StSP6A protein participates in tuber activation signal (Banerjee et al. 2006; Hannapel et al. 2017). *StSP6A* is one of the target genes of StBEL5 and overexpression of *StBEL5* led to early and increased tuberization (Banerjee et al. 2006; Navarro et al. 2011; Sharma et al. 2016). The diurnal oscillation of *CO* gene expression might also affect the tuberization through the circadian rhythm pathway, although this varies in different plants (de Montaigu et al. 2015).

It seems that the enlargement of temperate lotus rhizome also experiences a transition from flowering, which is regulated by the CO–FT pathway. To characterize the CO/COL members and explore their potential functions in the enlargement of the rhizome in lotus, we conducted a systematic analysis of this gene family. A total of 13 *NnCOL* genes were characterized in lotus through the genome-wide screening. Based on the phylogenetic relationship, gene structure, and expression profiles analyses, *NnCOL5* was selected for functional characterization in the potato transgenic system, and it was shown to be involved in lotus rhizome enlargement.

Materials and methods

Plant materials and growth conditions

Solanum tuberosum cv E-Potato 3 (hereafter named as E3) was used in this study. E3 is the main crop variety with its microtuber formation being independent of day length when cultivated in the field, but can only tuberize under SD *in-vitro*. Plants were propagated *in-vitro* using single stem nodes on MS medium (Montaldi and Claver) containing 4% sucrose at 20 °C with a long-day photoperiod of 16 h light/8 h dark, and a light intensity of 1000 μmol m^{-2} s^{-1}. The experiment was conducted for three biological replicates with each replicate sampling 17 cultivated plantlets from 8 pot-grown plants. For the tuberization experiments, the concentration of sucrose in the MS medium was increased to 8%, and tubers were harvested after 3 months.

To check the effect of diurnal conditions on the expression of *NnCOL5*, potatoes were subjected to SD treatment (light treatment from ZT0 to ZT8) and LD treatment (light treatment from ZT0 to ZT16). ZT here was defined

as different time of light treatment monitored by zeitgeber. The samples were immediately frozen in liquid nitrogen and stored at − 70 °C until used.

Sequence retrieval and *COL* family member identification in the lotus genome

Gene and protein sequences of *Nelumbo nucifera* were downloaded from the NCBI database (Ming et al. 2013; Wu et al. 2015), and the *CO/COL* gene and protein data of Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and potato (*Solanum tuberosum*) were downloaded from the Arabidopsis information resource website (TAIR, https://www.arabidopsis.org/) (Berardini et al. 2015), the rice genome annotation project website (RGAP, http://rice.plantbiology.msu.edu/) (Kawahara et al. 2013), and Solanaceae Genomics Resource (SpudDB, http://solanaceae.plantbiology.msu.edu/) (Leisner et al. 2018), respectively. These Arabidopsis CONSTANS sequences were used for BLASTing (Altschul et al. 1990) against the *N. nucifera* genome sequence with the parameters of expected values ≤ 1E-3 and more than 80% coverage, and all of the putative CONSTANS proteins were aligned to Arabidopsis CONSTANS proteins to classify them into different groups. All the sequences of CO/COL from these species are listed in Suppl. Table S1.

Phylogenetic and gene structure analyses of the *COLs*

Multiple alignments of Arabidopsis, rice, potato, and lotus COL protein sequences were carried out using ClustalW with default parameters (Thompson et al. 2002). Unrooted phylogenetic trees of all COL proteins were generated with MEGA (V10.0) using the Neighbor-Joining (NJ) method with the following parameters: Poisson correction, the partial deletion with a site coverage cutoff of 70%, and 1000 bootstrap replicates (Kumar et al. 2018).

The gene structure was analyzed using the Gene Structure Display Server tool (http://gsds.cbi.pku.edu.cn/, v2.0) (Berardini et al. 2015). Conserved motifs in lotus COL proteins were identified using the motif finding tool MEME (Multiple EM for Motif Elicitation, V5.0.1) (Bailey et al. 2009). MEME searching was performed across lotus COL proteins sequencing using the following parameters: (1) optimum motif width was set to ≥ 10 and ≤ 200; (2) the maximum number of motifs was set to identify 5 motifs; (3) occurrences of a single motif distributed among the sequences with model: zero or one per sequence (-mod zoops).

Expression analysis of *NnCOLs* in lotus

The gene expression data of the *NnCOL* genes were extracted from a previous report (Yang et al. 2015). The RNA-seq data from the three rhizome developmental stages: the stolon, middle swelling, and later swelling stage in the cultivars 'ZO' (temperate lotus with enlarged rhizome) and 'RL' (tropical lotus with stolon) were used. Gene expression values of six libraries were presented as RPKM (reads per kilobase per million measure) normalized by a logarithmic base (log10). Primers used in this study are listed in Suppl. Table S2.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA of each sample was extracted using an RNA reagent (OminiPlant RNA Kit, CWBIO, Beijing, China). Primers were designed with the Primer 3.0 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). qRT-PCR reactions were performed in the CFX Connect (Bio-Rad) using the SYBR Green Master Mix (Bio-Rad, http://www.bio-rad.com/), and amplified with 1 μL of cDNA template, 5 μL of 2 × SYBR Green Master Mix, and10 μM of each primer, to a final volume of 10 μL. The amplification program consisted of one cycle of 95 °C for 10 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Fluorescent products were detected in the last step of each cycle. Melting curve analysis was performed at the end of 40 cycles to ensure proper amplification of target fragments. Each gene was performed in three technical replicates. The relative expression of each gene was normalized by comparison with the expression of potato *actin* (*PGSC0003DMT400010174*) and analyzed using the $2^{-\Delta\Delta CT}$ method.

Transformation of *Solanum tuberosum* and starch content measurement

The open-reading frame (ORF) sequence of *NnCOL5* was amplified from the plasmids harboring complete *NnCOL5* cDNA. After purification, it was cloned into the PRI101 vectors, in which the target genes were driven by CaMV35S promoter. Then, the recombinant vector was transformed into the *Agrobacterium tumefaciens* strain GV3101. The transformed *A. tumefaciens* strain was used to infect the CIS-sensitive potato variety E3 plants as described previously (Huaijun et al. 2005). The overexpression plants were named as OE plants and were compared with E3 (CK) plants in the following section. Regenerated shoots were rooted on MS medium-containing 50 μg/mL kanamycin and 400 μg/mL carbenicillin. The transgenic plants together with untransformed controls were grown at 20–25 °C in 24-cm-diameter

plastic pots in the greenhouse with 16 h of light per day for LD treatment and in the incubator (PQX-300, Ningbodongnan CO. Ltd) with 8 h of light per day for SD treatment. Starch content measurement was conducted according to the anthrone colorimetric method (You 2013).

RNA-seq profiling and gene enrichment analysis

Differential expression analysis of genes between leaves of OE and CK (two biological replicates per group) was performed using theDESeq2 R package. To control the false discovery rate, the resulting *P* values were adjusted using the Benjamini and Hochberg's method. Genes found by DESeq2 with an adjusted *P* value < 0.05 were defined as differentially expressed genes (DEGs). We used the cluster Profiler R package to implement Gene Ontology (GO) enrichment analysis and to test the statistical enrichment in KEGG pathways of DEGs. GO terms with corrected *P* value < 0.05 were considered as significantly enriched.

Subcellular localization analysis of NnCOL5

To check the subcellular localization of NnCOL5, the framework of plasmid pCAMBIA1302 was used by adding EGFP (Enhanced Green Fluorescent Protein fragment), and then using homologous recombination to insert the cDNA of *NnCOL5* into the expression vector *35S::NnCOL5s-GFP* under the control of Caulifower mosaic virus 35S promoter. Then, the plasmid was transformed into the epidermal cells of tobacco (*Nicotiana tabacum*) using a modified Agrobacterium infection method (Sparkes et al. 2006). GFP-dependent fluorescence was detected at 48 h after transfection with a confocal laser scanning microscope TCS SP8 (Leica, Wetzlar, Germany).

Results

Analyses of the *COL* gene family and their expressional patterns during rhizome development in lotus

To characterize the *COL* genes in lotus, we screened the lotus genome by conducting blastn and blastp against Arabidopsis *COL* sequences. A total of 13 *COL* genes were characterized in the lotus genome, which was slightly lower than those in *Arabidopsis thaliana*, *Oryza sativa*, and *Solanum tuberosum* (Suppl. Table S1). Phylogenetic analysis of the *COL* genes from these four species categorized them into three groups, group I, II, and III (Fig. 1a). There are 4, 2 and 7 genes belonging to group I, II and III in lotus. Gene structure and motif analyses showed that the length of COL family proteins ranges from 259 to 523 amino acids (aa), and contains three major motifs (Fig. 1b). Motif 1 is a CCT domain, motif 2 is a B-BOX domain, and motif 3 has no valid domain hit. To analyze the gene expressional patterns in lotus rhizome during its development, we extracted RNA-seq data from a previous study (Yang et al. 2015) where 10 of the *COL* family genes were detected during lotus rhizome development in both, tropical and temperate lotus. Among them, gene *NNU_14974-RA*, belonging to group I, has an opposite expressional pattern in temperate lotus and tropical lotus during rhizome development (Fig. 1c, d). Its corresponding protein contains a truncated B-BOX domain (Fig. 1b). This gene is closest to *AtCOL5* in the genetic distance (Fig. 1a), based on which we named it as *NnCOL5*.

Expression analysis and subcellular localization of *NnCOL5*

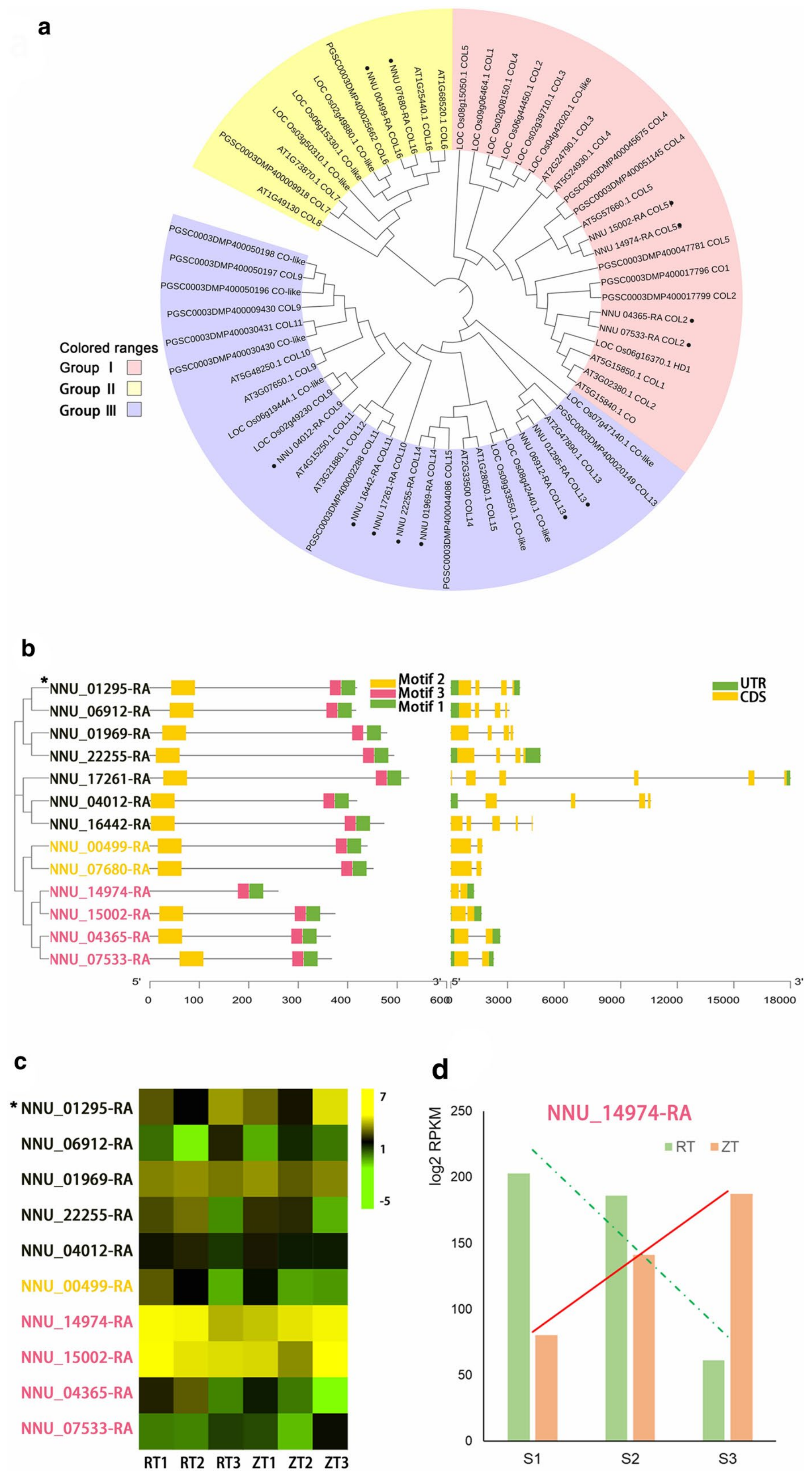
As reported, *AtCOL5* could induce Arabidopsis flowering under SD by sensing photoperiodism (Hassidim et al. 2009). We, therefore, selected *NnCOL5* for further study to explore if it could also mediate the transition between flowering and vegetative growth, especially the rhizome enlargement. RNA-seq data showed that the expression of *NnCOL5* was positively associated with rhizome enlargement (Fig. 1c, d). To confirm this relationship, qRT-PCR was applied to analyze the expression level of the *NnCOL5* in different tissues, including rhizome, leaf, and leaf stalk at four lotus rhizome developmental stages, S1 (stolon stage), S2 (initiation stage), S3 (rhizome middle stage), and S4 (rhizome late stage) (Fig. 2a). The expression of *NnCOL5* showed a gradual increase from S1 to S3 in both rhizome and leaf stalk, and a sharp increase from S2 to S3 in leaf, which then decreased at S4 (Fig. 2b). *NnCOL5* expression peaked at stage S3 and then decreased at S4 (Fig. 2b), which indicated that COL5 might contribute to the process of lotus rhizome development.

CO functions as a transcription factor in both Arabidopsis and potato. To confirm its transcriptional factor features, we investigated the subcellular localization of NnCOL5. The *NnCOL5*–GFP fusion protein driven by the 35S promoter construct was transformed into tobacco leaves for transient expression analysis. Confocal microscopic observations showed that the signal of GFP was totally overlapping with the DAPI staining signal, which demonstrated the nucleus localization of NnCOL5 protein (Fig. 2c).

Overexpression of *NnCOL5* promotes tuberization and starch accumulation in potato

Since the expression of *NnCOL5* was positively related to the enlargement of lotus rhizome, we suspected that it might regulate its enlargement. To verify this hypothesis, we conducted a transgenic analysis in the potato system, since there

Fig. 1 Phylogenetic, gene structure, and expression analyses of lotus *COL* gene family. **a** Phylogenetic analysis of *COL* genes from Arabidopsis, rice, potato, and lotus. Stars indicate *COL5* genes of Arabidopsis and lotus. **b** Gene and protein structure of 13 *COL* genes in lotus. **c** Expressional level of 13 *NnCOL* genes during underground stem formation of tropical lotus (RT1, RT2, and RT3) and temperate lotus (ZT1, ZT2, and ZT3). Star indicates *NnCOL5*. **d** The expression of *NnCOL5* in stolon stage, middle stage, and rhizome stage of typical species of tropical lotus (RT) and temperate lotus (ZT). The red, yellow, and black fonts in **b** and **c** stand for the members of group I, II, and III, respectively



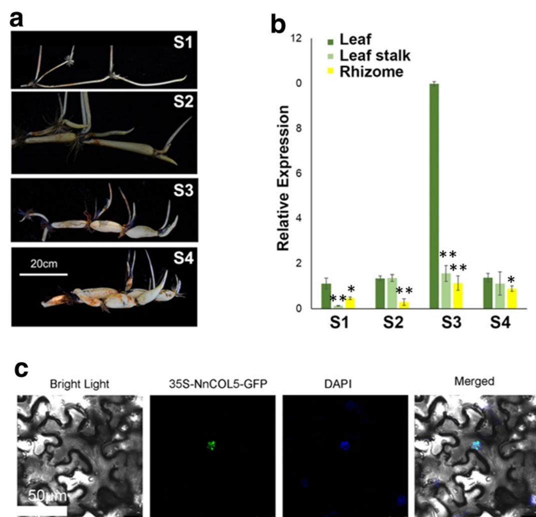


Fig. 2 Expression of *NnCOL5* and its protein subcellular localization analyses. **a** The lotus rhizome at four different developmental stages. Bar = 20 cm. **b** The temporal and spatial expression pattern of *NnCOL5* during the development of the lotus rhizome ($n=3$; *, $P \leq 0.05$; **, $P \leq 0.01$). **c** The subcellular localization of NnCOL5 protein in tobacco leaves through a transient expression system. The NnCOL5 was fused with GFP and driven by the 35S promoter, and the nuclear signal was visualized through DAPI staining. The first image from the left side is the region observed under the bright light; the second one is the same region showing the green fluorescent signal; the third one is the DAPI staining signal; and the fourth one shows the overlapping of GFP and DAPI signals. Bar = 50 μm

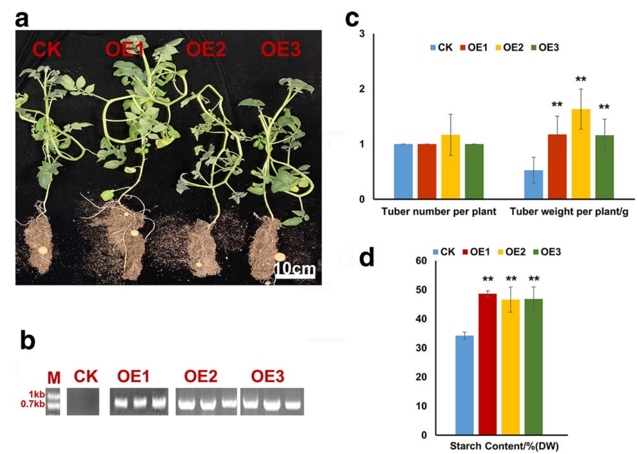


Fig. 3 The genotyping and phenotyping of *NnCOL5* overexpression potato. **a** The plants and tubers of potato variety E3 (CK) and three *NnCOL5* overexpression lines of the E3 cultivar (OE1-3) under short-day condition. **b** RT-PCR verified the overexpression of *NnCOL5* in the transgenic lines of E3. M, marker; CK, control E3 line; OE1-3, three *NnCOL5* overexpression lines of E3. **c** The tuber number and tuber weight (fresh weight/ g) per plant in CK plants and OE plants. **d** The starch content (dry weight/%) of CK and OE plants under short day ($n=3$; *, $P \leq 0.05$; **, $P \leq 0.01$)

is no such system established in lotus. After we constructed 35S::*NnCOL5* vector and employing the Agrobacterium infection method, *NnCOL5* was overexpressed in the potato variety E3 (named as OE plants hereafter). The transgenic lines were analyzed for tuberization after 8 weeks under SD condition (Fig. 3a–b). Compared with E3 control plants (CK), OE plants showed no significant changes in tuber number, but an increase in tuber weight (Fig. 3c). Potatoes are rich in carbohydrates with the main constituent being starch. Accumulation of starch occurs at the late stage of tuberization. To investigate the starch change in OE plants, we checked the starch content of tubers in both CK and OE plants. The starch content increased significantly under SD conditions in the tubers of OE plants (Fig. 3d), which was consistent with that of tuber weight.

Overexpression of *NnCOL5* influenced the CO–FT pathway genes in potato

To explore how the overexpression of *NnCOL5* promote tuber expansion and starch accumulation in OE potato, transcriptomes were compared between leaves of CK and OE plants under SD through RNA-Seq. After filtering, a total of 219 Mb clean data were obtained from the 238 Mb raw data. Among them, about 80% could be mapped onto the potato genome. Correlation analysis showed that there was a very good repeat between different replicates of the same sample (Suppl. Fig. S1a). After comparative analysis, the results showed that there were 5186 differentially expressed genes (DEGs, fold change > 2, P value < 0.05) out of the 26,320 detected genes, with 2534 being up-regulated and 2652 being down-regulated in the OE plants (Suppl. Table S1; Suppl. Fig. S1b). To evaluate the validity of RNA-Seq results, 15 genes were randomly selected and verified with qRT-PCR analysis, which showed that the two sets of data were generally consistent to each other (Suppl. Table S3).

We then performed GO and KEGG enrichment analyses. The pathways analysis showed that the top three pathways enriched in the up-regulated genes were "Ribosome", "DNA replication", and "Ribosome biogenesis in eukaryotes", whereas "Plant hormone signal transduction", "MAPK signaling pathway", and "Circadian rhythm" were the top three in the down-regulated genes (Fig. 4). There were 93 DEGs belonging to the "Plant hormone signal transduction" pathway, among which 83 were down-regulated in the transgenic plant.

Specifically, we analyzed the expressional data of genes in the CO-FT pathway. A total of 12 *COL* genes were detected in our RNA-seq data, among which five were differently regulated, including *COL3*, *COL4*, *COL13*, and two isoforms of *COL16*. All of them were down-regulated by the overexpression of *NnCOL5* (Fig. 5). The *FT*, *EARLY FLOWERING 3* (*ELF3*), and *ELF4* genes were also detected to be down-regulated, whereas, *FLOWERING*

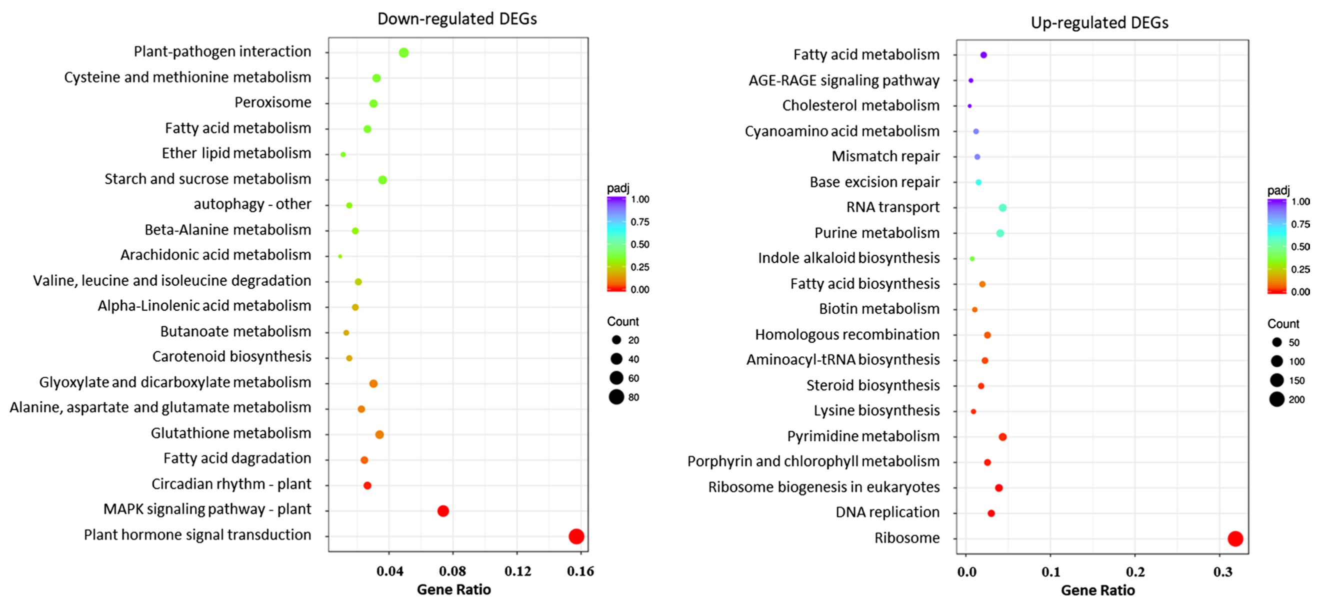


Fig. 4 KEGG pathway enrichment analysis on the down- and up-regulated genes from RNA-seq analysis, respectively

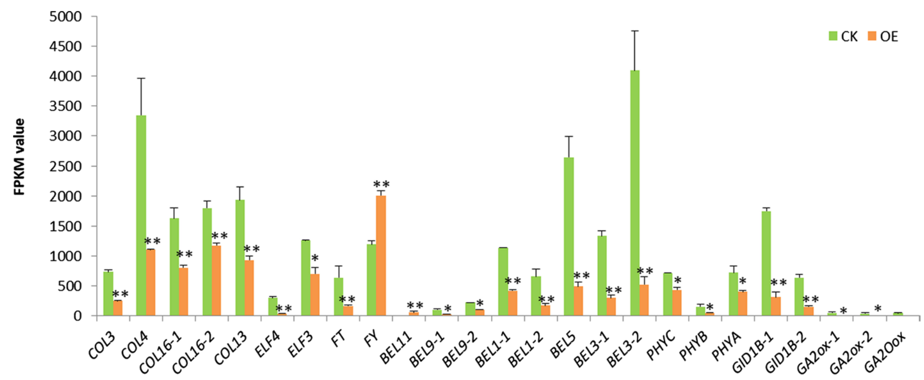


Fig. 5 Expression levels of the genes in the CO–FT pathway. Y-axis shows the FPKM value from RNA-seq data. Data for each gene are from three replicates of RNA-seq ($n=3$; *, $P \leq 0.05$; **, $P \leq 0.01$)

LOCUS Y (*FY*) was slightly up-regulated (Fig. 5). *Phytochrome A* (*PhyA*), *PhyB,* and *PhyC* were all down-regulated as well, with *PhyB* being the most down-regulated. Seven *Bell* (*BEL*) genes, including two isoforms of *BEL1*, *BEL3* and *BEL9*, and *BEL5*, were down-regulated, whereas *BEL11* was up-regulated (Fig. 5). We also found that two GA receptor-encoding genes and three GA metabolic genes were down-regulated (Fig. 5).

We did not detect any changes in the expression of *StSP5G* and *StSP6A* genes through RNA-seq, which were reported as being directly or indirectly regulated by StCOL1, respectively (Abelenda et al. 2016). We then analyzed the expression of these two genes through qRT-PCR in the CK and OE lines every 3 h for a whole day. Analysis of *StSP5G* expression was conducted under LD conditions, while analysis of *StSP6A* expression was under SD conditions. The expression of *StSP5G* at 2:00 AM was much higher in OE than CK plants, while it was lower at 5:00 AM. There was no difference between the CK and OE lines for *StSP6A* gene during the whole day (Fig. 6).

Discussion

Some plants are capable of altering their organs into abnormal storage organ, which has both ecological and economic significance. To explore the underlying mechanism of storage organ formation, numerous studies have been conducted in many land plants, including potato (*Solanum tuberosum*), radish (*Raphanus sativus* L.), and yam (*Dioscorea opposita* Thunb.), with potato being widely studied (Jackson 1999; Hannapel et al. 2017). Lotus rhizome is a typical abnormal storage organ. However, there are still very few studies on lotus rhizome development. Based on our previous study (Lin et al. 2019), several candidate genes were obtained, which might play roles in

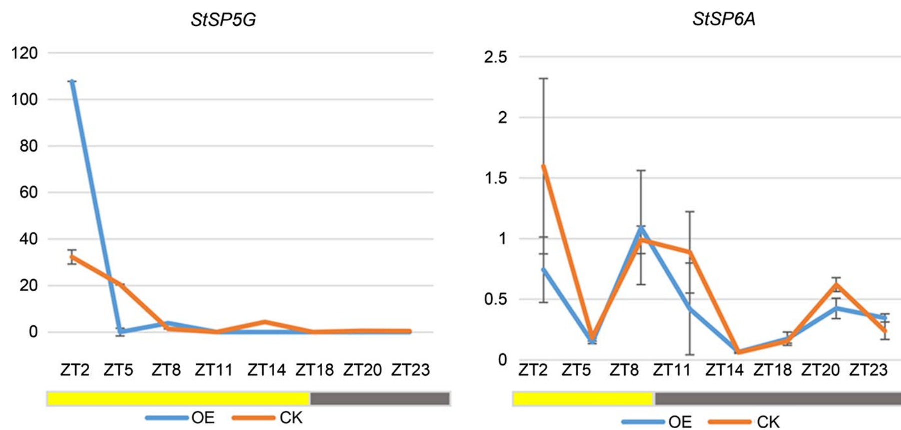


Fig. 6 The diurnal expression of *StSP5G* and *StSP6A* in OE plants under short-day (8 h of light/16 h of dark) and long-day condition (16 h of light/8 h of dark). Blue lines and orange lines represent gene expression in OE and CK plants, respectively. The y-axis represents the relative expression of genes, and the x-axis represents the zeitgeber time (ZT). The bar under the x-axis indicates light and dark treatments, with yellow bars representing light time and black bars representing dark time. Data are mean values ± SE from three replicates

lotus underground stem development. We focused on the *COL* gene family and characterized the potential function of one of its members *NnCOL5* in promoting the formation of underground stems.

The evolution of *COL* gene family and their functions

The *CO* gene was initially identified in Arabidopsis as an important regulator of flowering in the photoperiodic pathway, which is among the four pathways regulating the time of flowering in plants (Putterill et al. 1995; Mouradov et al. 2002; Griffiths et al. 2003). It belongs to a transcription factor family that is defined by two conserved domains: a zinc finger region named as B-box near the amino terminus and a CCT domain consisting of 43 amino acids near the carboxy terminus (Putterill et al. 1995; Griffiths et al. 2003). By combining data from the previous studies (Putterill et al. 1995; Griffiths et al. 2003) and that of this study, this gene family could be grouped into three clades in all plants, despite the varying number of *COL*s among different plant species. Phylogenetic analysis among different plant species, including Arabidopsis, rice, barley, maize, sorghum, potato, and lotus showed high bootstrap, conserved domain, and assembled gene structures among members within the same clade even from different plant species (Griffiths et al. 2003; Song et al. 2018). These results implied that *COL* genes in the same clade from different plant species are orthologous genes with a common ancestor, and the three clades appeared prior to the divergence of monocots and dicots lineages (Song et al. 2018). *NnCOL5* was named due to its phylogenetic relationship with *AtCOL5*. However, these two genes are phylogenetic closer to *StCOL4* than *StCOL5*. Although results from maize suggested limited functional divergence of *COL* genes in this species (Song et al. 2018), there exists an evolutionary trend, with a preferential one-B-box structure in most *COLs* in cereals as a result of gene duplication and selection variation in response to photoperiodism. Based on phylogenetic analysis, there were several duplicated genes within each clade. How these duplicated genes diverged in their functions seems to be important and could be utilized in exploring the mechanism underlying the control of flowering time and rhizome formation.

It has been well elucidated that the *CO* gene is mainly involved in controlling the time of flowering through the photoperiodic pathway in Arabidopsis, a facultative long-day plant (Putterill et al. 1995; Griffiths et al. 2003). It acts as a promoter of flowering under LD condition at the downstream of circadian and upstream of *FT* (Mouradov et al. 2002). Photoreceptors PHYA and PHYB both partially take part in this pathway by influencing *CO* at the post-transcriptional level (Yanovsky et al. 2000; Mouradov et al. 2002). Among angiosperms, the CO-FT pathway is highly conserved (Zobell et al. 2005), indicating that the function of *CO*/*COL* in flowering induction is conserved as well. However, some *COL*s have been found to be ABA and stress-responsive in Arabidopsis (Min et al. 2015). The homologs AtCOL1 and AtCOL2 in poplar are involved in metabolic processes (Hsu et al. 2012). These indicate that *CO/COL* genes from different plant species may obtain new functions or different members may have diversified their functions during evolution.

NnCOL5 functions in regulating underground stem expansion

Homologs of *CO* and *FT* have also identified potato and verified as being involved in tuberization (Rodriguez-Falcon et al. 2006; Abelenda et al. 2016). The expression of *NnCOL5* was positively associated with the enlargement of lotus rhizome. In addition, overexpression of *NnCOL5* in potato increased tuber weight and starch content (Fig. 3c, d). Taken together, this gene seems to promote the enlargement of the underground stem in both potato and lotus. This is in conflict with the functions of *StCOL1*, which suppresses tuberization in LD condition (Abelenda et al. 2016). The expression profile of *StSP5G* and *StSP6A* remained unchanged in the *NnCOL5* overexpression OE plants (Fig. 6), which indicated that *NnCOL5* might function differently from *StCOL1*. RNA-seq data from the transgenic potato showed that the expression of many genes in CO-FT pathway decreased in the OE plant (Fig. 5). The expression of an *FT* gene was down-regulated by the overexpression of *NnCOL5*. This implies the possibility of inhibition of the transition from vegetative to reproductive growth. This observation is consistent with the phenomenon in lotus rhizome enlargement, which occurs after the end of flowering and seed setting. Among all the phytohormones, GA stimulates stolon formation and growth, and inhibits tuberization (Rodriguez-Falcon et al. 2006). Down-regulation of both GA receptor and *StGA20ox* indicated that overexpression of *NnCOL5* might promote tuberization also by attenuating GA signaling, which is consistent with our previous RNA-seq data in lotus rhizome enlargement. Interestingly, among all the DEGs, the histone encoding genes, DNA replication, and cell cycle-related genes, and nearly all the detected GATA transcription factor encoding genes were up-regulated by the overexpression of *NnCOL5*. Altogether, it seems that NnCOL5 is capable of repressing the expression of CO-FT and GA signaling pathway genes, and promotes the expression of growth-related genes, ultimately resulting in the expansion of underground stems.

Author contribution statement PY, DC and, LH designed the experiments. DC, RND, and ZL conducted the experiments. DC analyzed the data, and wrote the manuscript. PY and RND revised the manuscript. All the authors have read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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