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Original Paper

Characterization and transgenic study of *CONSTANS-LIKE8* (*COL8*) gene in *Arabidopsis thaliana*: expression of *35S:COL8* delays flowering under long-day conditions

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Abstract CONSTANS (CO) is a regulator of photoperiodic flowering that activates the transcription of a florigen gene, *FLOWERING LOCUS T* (*FT*), in Arabidopsis. CO belongs to the CO/CONSTANS-LIKE (COL) protein family, which consists of 17 proteins and is subclassified into three groups. Here, we characterized the gene for one of the group II members, *COL8*. The *COL8* mRNA accumulated in seeds, leaves, flowers, and siliques. Transgenic Arabidopsis plants with a beta-glucuronidase (GUS) reporter gene driven by a 3-kb *COL8* promoter displayed strong GUS activity in leaves. Both transiently and stably produced fluorescence-tagged COL8 proteins were localized in the nucleus. Transgenic Arabidopsis plants possessing *COL8* cDNA driven by a cauliflower mosaic virus (CaMV) 35S promoter did not show any altered circadian rhythm under constant light conditions, but showed a late-flowering phenotype under long-day conditions. In these transgenic plants, *CO* mRNA did not decrease from zeitgeber time (zt) 12 to zt 16, whereas *FT* mRNA decreased from zt 8 to zt 24. The possible mechanisms for the late-flowering phenotype of the *COL8* plants driven by the CaMV 35S promoter are discussed.

Key words: *Arabidopsis thaliana*, CONSTANS, CONSTANS-LIKE 8, flowering time.

For plants to survive, it is essential for them to integrate signals from their external environment and respond to these signals. Flowering induction is one of the most important processes in the lives of seed plants. In Arabidopsis (*Arabidopsis thaliana* L.), induction of the flowering process is precisely regulated by four signaling pathways: the daylength (photoperiod) signal pathway, the autonomous pathway, the vernalization pathway, and the gibberellin pathway (Koornneef et al. 1998; Mouradov et al. 2002; Piñeiro and Coupland 1998; Simpson and Dean 2002). The signals from the pathways converge on the transcriptional regulation of floral morphogenesis integrator genes such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* that activate the transcription of genes necessary for flowering, such as *APETALA1* and *LEAFY* (Moon et al. 2003; Piñeiro et al. 2003; Takada and Goto 2003). FT has been identified as the long-sought "florigen" that moves from leaves to the meristem and promotes flowering (Abe et al. 2005; Corbesier et al. 2007; Kardailsky et al. 1999; Kobayashi et al. 1999; Wigge et al. 2005). *FT* transcription is regulated by the integration of various activators and repressors. The key activator in the photoperiod signal pathway is CONSTANS (CO).

Levels of expression of *CO* mRNA and production of CO protein are regulated by the circadian clock and day length in Arabidopsis. *CO* mRNA transcription is high around sunset or dusk and low at mid-day (Suárez-López et al. 2001; Yanofsky and Kay 2002). Because CO protein is stabilized by light (Valverde et al. 2004), CO protein is abundant and promotes *FT* expression under long-day (LD) conditions, leading to induction of flowering (Súarez-López et al. 2001; Yanofsky and Kay 2002). Arabidopsis plants carrying loss-of-function mutations in *CO* have a late-flowering phenotype under LD conditions but flower normally under short-day (SD) conditions (Koornneef et al. 1991; Putterill et al. 1995). In contrast, *CO* overexpression in Arabidopsis causes early flowering under both LD and SD conditions (Onouchi et al. 2000; Putterill et al. 1995; Simon et al. 1996; Suárez-López et al. 2001).

CO belongs to the CO/COL protein family, which has

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17 members in Arabidopsis (Robson et al. 2001). Each CO/COL family protein possesses two highly conserved domains: one or two B-box zinc-finger domains near the amino (N)-terminus and a CCT domain near the carboxyl terminus (Putterill et al. 1995; Robson et al. 2001; Strayer et al. 2000). Members of the CO/COL protein family are subclassified into three groups. CO and COL1 to COL5 belong to group I, whose members have two B-boxes. COL6 to COL8 and COL16 belong to group II, whose members have only one B-box. COL9 to COL15 belong to group III, whose members have one CO-like B-box and one diverged zinc-finger domain (Griffiths et al. 2003; Robson et al. 2001).

In addition to the CO protein, several proteins in the CO/COL family have been studied and expression of the genes that encode them has been reported. Ectopic overexpression of *COL1* or *COL2* in transgenic Arabidopsis plants had little effect on flowering time, whereas transgenic plants that overexpressed *COL1* showed a shorter circadian rhythm (Ledger et al. 2001). A loss-of-function *col3* mutant had longer hypocotyls under red light and SD conditions, and flowered earlier under both LD and SD conditions (Datta et al. 2006). In *COL5*-overexpressing plants, flowering time was earlier under SD conditions, whereas in plants with a silenced endogenous *COL5*, flowering time was not altered by either SD or LD conditions (Hassidim et al. 2009). Overexpression of *COL9* in transgenic plants resulted in delayed flowering, whereas co-suppression lines and a knockout line of *COL9 s*howed earlier flowering under LD conditions (Cheng and Wang 2005).

Even though several members of the CO/COL family have been studied, the characteristics and functions of more than half of the members of this family remain to be elucidated. One such uninvestigated member is *COL8* (*At1g49130*). Here, we characterize *COL8* and report that expression of *35S:COL8* in Arabidopsis delays flowering under LD conditions.

Materials and methods

Plant materials and growth conditions

All plant materials were the Columbia (Col) or Landsberg *erecta* (L*er*) accessions of *A. thaliana*. Seeds of the *COL8* T-DNA knockout line *col8* (GT7084; L*er* background) were obtained from the Martienssen laboratory at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, USA). To determine flowering time, seeds were sown on vermiculite in pots supplemented with 1 : 1000 Hyponex nutrient solution (Hyponex Japan, Osaka, Japan). Pots were cold-treated at 4°C for 3 days in the dark (D), and were then transferred to LD conditions [16 h light (L) and 8 h D] and incubated at 22°C under white illumination (90 to 100 μmol m^{-2} s^{-1}).

Construction of the COL8 transgenic plants

To generate a *COL8* overexpression construct, we used the PCR primers COL8/F and COL8/R to amplify the *COL8* gene. (All primer sequences used in this study are listed in the Supporting Information in Table S1.) Both primers have a *Bam*HI site. cDNAs prepared from the mRNAs of the wild-type plants (Col) were used as PCR templates. The PCR fragment was subcloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA, USA), sequenced entirely to verify the sequence, and then digested with *Bam*HI and subcloned into the *Bam*HI site of the pBE2113-*Not*I binary vector (Liu et al. 1998; Mitsuhara et al. 1996). The pBE2113 vector contained the cauliflower mosaic virus (CaMV) *35S* promoter, and the resulting vector was named *35S:COL8* and *35:COL8-AS*. The *35S:COL8-AS* constructs contained the *COL8* gene in the opposite orientation (antisense orientation). To generate a COL8-green fluorescent protein (GFP) construct, the *COL8* gene fragment without a stop codon was amplified with the PCR primers COL8/F and COL8-GFP/R. The COL8-GFP/R primer has a *Bam*HI site. The GFP coding region was amplified using the primers SmaI/GFP/F and SacI/GFP/R. SmaI/GFP/F contains *Sma*I and *Kpn*I sites, and SacI/GFP/R contains a *Sac*I site. The PCR products were subcloned into pCR-Blunt II-TOPO, and sequenced entirely to verify their sequences. The GFP fragment was digested with *Sma*I and *Sac*I and subcloned into pBE2113-*Not*I; the resulting vector was named pBE/GFP. The *COL8* fragment was digested with *Bam*HI and subcloned into the *Bam*HI site of the pBE/GFP vector, and the resultant construct was sequenced entirely to verify the sequence. For the COL8:beta-glucuronidase (GUS) construct, COL8/genome/F and COL8/pro/R primers were used to amplify the 3-kbp upstream region of the *COL8* gene. The PCR product was subcloned into the pENTR/D-TOPO entry vector (Invitrogen) in accordance with the manufacturer's instructions. After sequencing of the entire insert, the 3-kbp *COL8* promoter region was transferred into the pGWB203 binary vector (Nakagawa et al. 2007) by an LR reaction using the Gateway cloning system (Invitrogen), and the resultant construct was sequenced entirely to verify the sequence.

All constructs were introduced into *Agrobacterium tumefaciens* LBA4404 by means of tri-parental mating (Figurski and Helinski 1979) and transformed into *A. thaliana* (Col) by the floral-dip method (Clough and Bent 1998).

RNA analysis

To examine the tissue specificity of *COL8* expression, we performed RT-PCR analysis as described previously (Ogura et al. 2008). For real-time quantitative RT-PCR analysis of *CO* and *FT* expression, seeds were surface sterilized and plated on germination medium (Valvekens et al. 1988) supplemented with 0.8% agar (Nacalai Tesque, Inc., Kyoto, Japan), incubated at 4°C in the dark for 3 days to break dormancy, and then grown under LD conditions at 22°C under white illumination (90 to 100 μmol m^{-2} s^{-1}). We harvested 10-day-old seedlings every 4 h over a 24-h period. Total RNA was extracted from whole seedlings or plant tissues using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) in accordance with the manufacturer's instructions. Equal amounts of total RNA (500 ng) were used to prepare cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The quantitative RT-PCR analysis was performed with a Power SYBR Green PCR

Master Mix (Applied Biosystems). The *ACTIN2* (*ACT2*) or *UBIQUITIN10* (*UBQ10*) gene was used as an internal control. The primers used in these experiments are also listed in the Supporting Information in Table S1.

Histochemical GUS analysis

To visualize GUS activity, we stained COL8:GUS transgenic plants that harbored the *COL8pro:GUS* reporter construct. The transgenic plants were treated in 90% acetone on ice for 15 min, and were then washed with 100 mM sodium phosphate buffer (pH 7.0). Plant tissues were then submerged in GUS staining solution [100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.1% Triton X-100, 0.5 mg ml^{-1} X-Gluc (Nacalai Tesque)] under vacuum for 15 min. The samples were then incubated overnight at 37°C in the GUS staining solution. After GUS staining, samples were washed with 70% ethanol and then treated with ethanol–acetic acid solution (85.7% ethanol plus 14.3% acetic acid, v/v) to extract chlorophyll. After being washed again in the ethanol–acetic acid solution, the samples were stored in 70% ethanol.

Subcellular localization analysis

To study transient expression, we subcloned full-length cDNA sequences of *COL8* into expression vectors carrying the CaMV 35S promoter paired with yellow fluorescent protein (YFP) (pAVA554). Full-length cDNA of *COL8* was amplified by means of PCR so that we could attach *Bam*HI sites at both ends, excised using *Bam*HI, and then subcloned into the *Bgl*II sites of pAVA554. The resulting plasmid was verified by sequencing. A control plasmid for nuclear localization, 35S:NLS-tdTomato, was previously described (Biswas et al. 2007). We prepared protoplasts from Arabidopsis leaves and performed transfection of the protoplasts as previously described (Abdel-Ghany et al. 2005) with some modifications. Two grams fresh weight of leaf tissue was suspended in 30 mL of a buffer containing 1% (w/v) cellulase Onozuka R-10, 0.25% Macerozyme R-10 (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan), 8 mM $CaCl_2$, 0.5 M mannitol, and 5 mM MES, pH 5.6, vacuum infiltrated for 1 min, and incubated for 3 h at 25°C with gentle shaking. The clear digest was filtered through a 37- to 70-μm nylon mesh, and the protoplasts were harvested by centrifugation for 2 min at 560 rpm and washed twice in 10 mL of W5 wash solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 0.5 M mannitol, adjusted to pH 5.8 with KOH). The pellet was suspended in 2 mL of mannitol/Mg solution (15 mM $MgCl_2$, 0.4 M mannitol, and 0.1% MES, pH 5.6). Protoplasts were counted using a hemocytometer, and their concentration was adjusted to 3×10^6 cells mL^{-1} with mannitol/Mg solution. Fifty micrograms of plasmid DNA, 100 μg of salmon sperm DNA, and 300 μL of polyethylene glycol solution (40% polyethylene glycol 4000, 0.4 M mannitol, and 0.1 M $CaCl_2$) were added to 300 μL of the protoplast solution, very gently mixed, and left for 30 min on ice. The solution was very slowly diluted with 10 mL of W5 solution and then pelleted by centrifugation for 2 min at 560 rpm. Protoplasts were further resuspended in 2 mL of protoplast culture medium composed of MS medium supplemented with 0.4 M glucose, 0.4 M mannitol, pH adjusted to 5.8 with KOH, and left at 22°C for 16 h under continuous dark. Then we observed the fluorescence signals using an IX71 microscope connected to a Disc Scan Unit system (Olympus, Tokyo, Japan). YFP and tdTomato signals were detected with standard YFP and WIY filters (Olympus), respectively. The images were analyzed using the MetaMorph Imaging Software (Universal Imaging Company, Downingtown, PA, USA). To observe the subcellular localization of the GFP-tagged COL8 and GFP in Arabidopsis plants, we used the roots of 16-day-old *35S:COL8-GFP* overexpressor (COL8-GFP-ox) and *35S:GFP* overexpressor (GFP-ox) transgenic plants, respectively. GFP signals were detected with a BX51 microscope equipped with a fluorescent unit and a standard GFP filter (Olympus).

Bioluminescence monitoring

To analyze the circadian rhythms of the transgenic plants, we crossed *COL8*-overexpresser (COL8-ox) lines with two luciferase (LUC) reporter lines, one of which harbored a *CAB2:LUC* reporter construct and the other of which harbored a *CCR2:LUC* reporter construct (Millar et al. 1992; Strayer et al. 2000). The resultant F_1 seeds were sown on GM agar medium supplemented with 1% sucrose and 1 mM luciferin (Dojindo, Kumamoto, Japan). Plates were cold-treated at 4°C for 3 days, and then transferred to 12 L (20 μmol m^{-2} s^{-1})/12D conditions at 22°C for 5 days. Bioluminescence was monitored under constant white illumination (50 μmol m^{-2} s^{-1}) with a multi-channel bioluminescence-monitoring apparatus (Okamoto et al. 2007). Period lengths of the bioluminescence rhythm were estimated using the RAP software (Okamoto et al. 2005).

Results

Organ-specific expression and promoter activity of COL8

We searched for expression patterns of *COL8* in an online database using the eFP browser (Winter et al. 2007); however, no information was available, because no *COL8* sequence was present on the ABI Affymetrix chip that had been used for the construction of the database. Instead, tiling-array data on the predicted expression of *COL8* suggested that *COL8* would be expressed in the stem, leaf, whole flower, silique, and shoot apex inflorescence, but would be expressed weakly in the root and shoot apices during the vegetative stage (Laubinger et al. 2008). To characterize *COL8* expression, we analyzed transcription of *COL8* by using RT-PCR (Figure 1A). *COL8* was expressed clearly in most of the observed organs, although expression in the root and dry seed was weak. These results were consistent with the expression information presented in the tiling array data of Laubinger et al. (2008). To elucidate *COL8* promoter activity, we fused a 3-kb *GUS* reporter gene downstream of the *COL8* promoter, and transformed the construct into wild-type plants to construct COL8:GUS transgenic plants. The GUS activity of the transgenic plants was then observed (Figure 1B-E). Similar to the results of the RT-PCR, we found that the *COL8* promoter was expressed in most of

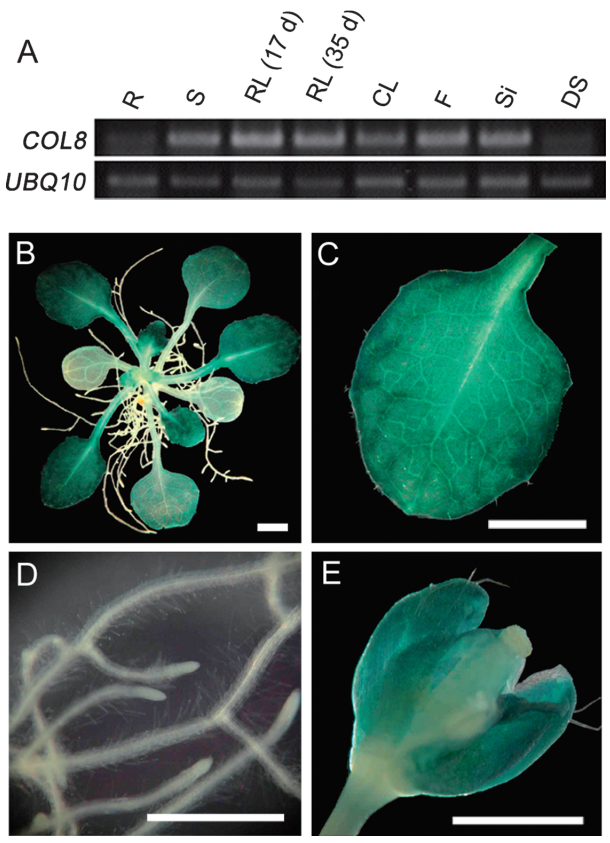

Figure 1. Expression patterns of *COL8* mRNA and *COL8* promoter. (A) RT-PCR of *COL8* mRNA. RT-PCR was performed using RNA extracted from the root (R), stem (S), rosette leaf (RL), cauline leaf (CL), flower (F), silique (Si), and dry seed (DS) tissues of wild-type Arabidopsis plants. RT-PCR of *ubiquitin 10* (*UBQ10*) is shown as a control. (B-E) GUS staining of COL8:GUS transgenic plants: (B) whole plant, (C) rosette leaf, (D) root, and (E) flower. Scale bars are 2 mm in (B) and (C) and 1 mm in (D) and (E).

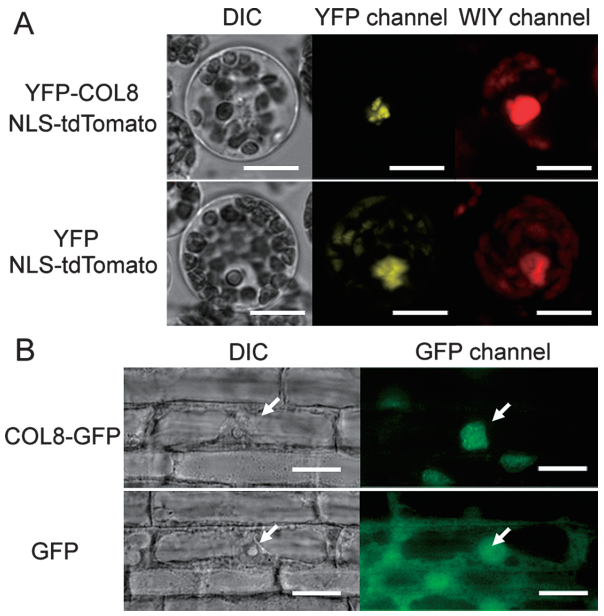

Figure 2. Subcellular localization of YFP-COL8 and COL8-GFP fusion proteins. (A) YFP or YFP-COL8 were transiently co-produced with NLS-tdTomato in protoplasts from arabidopis leaves. The panels show differential interference contrast (DIC) YFP, and tdTomato images of representative cells. The tdTomato signal was captured in the WIY channel. The nucleus of each cell was visualized by co-production of tdTomato fused with the SV40 nuclear localization signal peptide (NLS-tdTomato). Scale bars are 10 μm. (B) Roots of 16-day-old *35S:COL8-GFP* (COL8-GFP) and *35S:GFP* (indicated as GFP) transgenic Arabidopsis. Arrows indicate nuclei. Scale bars are 50 μm in the left images, and 25 μm in the right images.

the observed tissues except the roots. The weak GUS staining in the roots was consistent with the tiling array and RT-PCR results.

Fluorescent protein–tagged COL8 proteins are localized in the nucleus

Based on information from the WoLF PSORT database (Horton et al. 2007), we predicted that the COL8 protein would be localized in the nucleus. To test this prediction, we analyzed the localization of transiently produced YFP-tagged COL8 in the leaf protoplasts and stably produced GFP-tagged COL8 in the roots of the transgenic plants. In both cases, fluorescent protein–associated signals were detected in the nucleus, confirming the nuclear localization of the COL8 protein (Figure 2).

COL8-ox plants possess a normal circadian rhythm

COL1 overexpression shortens the period of the circadian rhythm (Ledger et al. 2001). To determine whether overexpression of *COL8* affected the circadian rhythm in the same way as *COL1* overexpression, we generated *35S:COL8* (COL8-ox) transgenic plants. Expression of *COL8* in the vector control (VC) plants and in three lines of COL8-ox plants was analyzed by RT-PCR (Figure 3A), and we confirmed higher expression of *COL8* in the COL8-ox lines than in the VC. Using these lines, we analyzed expression of *CAB2* and *CCR2* by detecting the luminescence from the LUC reporter protein. The amplitude of the circadian rhythms of LUC bioluminescence intensity derived from both the *CAB2* and *CCR2* promoters did not change in the COL8-ox plants compared with those in VC plants (Figure 3B). The period lengths of the *CAB2* and *CCR2* bioluminescence intensity in the COL8-ox lines did not differ significantly from that of the VC plants ($p<0.05$; Student's *t*-test; Table 1).

COL8-ox transgenic plants flower late under LD conditions

To determine the influence of *COL8* overexpression on flowering time, we measured the changes in flowering time in the wild-type plants and in three independent COL8-ox transgenic lines under LD conditions (Figure 4A). The number of days required for bolting and the

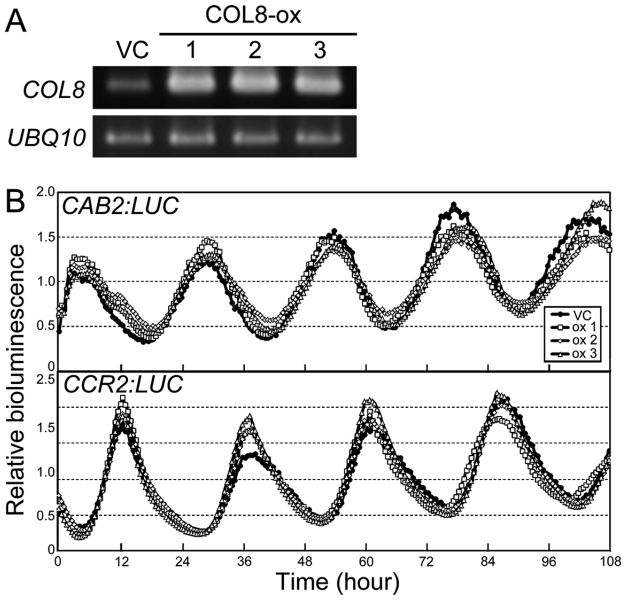

Figure 3. Circadian rhythm in *COL8*-overexpressing lines (COL8-ox). (A) Expression of *COL8* mRNA in the vector control (VC) and in three independent transgenic *COL8* overexpressor (COL8-ox) lines was confirmed by means of RT-PCR (30 cycles). The RT-PCR results for *ubiquitin 10* (*UBQ10*) mRNA are shown as a control in each line (20 cycles). (B) *CAB2* and *CCR2* transcript patterns in the VC and COL8-ox lines were determined by using the bioluminescence from *CAB2:LUC* and *CCR2:LUC*, respectively. Five-day-old plants grown under SD (12L:12D) were observed under constant white illumination.

Table 1. Period lengths of the oscillations of LUC bioluminescence intensity derived from the *CAB2* and *CCR2* promoters in transgenic plants.

Line	Promoter	Period (hours)
VC	*CAB2*	24.79 ± 0.72
COL8-ox1	*CAB2*	24.62 ± 0.59
COL8-ox2	*CAB2*	25.26 ± 0.76
COL8-ox3	*CAB2*	25.35 ± 0.68
VC	*CCR2*	24.94 ± 0.56
COL8-ox1	*CCR2*	24.49 ± 0.56
COL8-ox2	*CCR2*	24.82 ± 0.57
COL8-ox3	*CCR2*	24.68 ± 0.34

Oscillation time was measured in the vector control (VC) and three *COL8* overexpressor (COL8-ox) transgenic lines. Data represent the average oscillation durations (in hours) ±SD during the 108-h observation period (n=4). Period length did not differ significantly ($P>0.05$, Student's *t*-test).

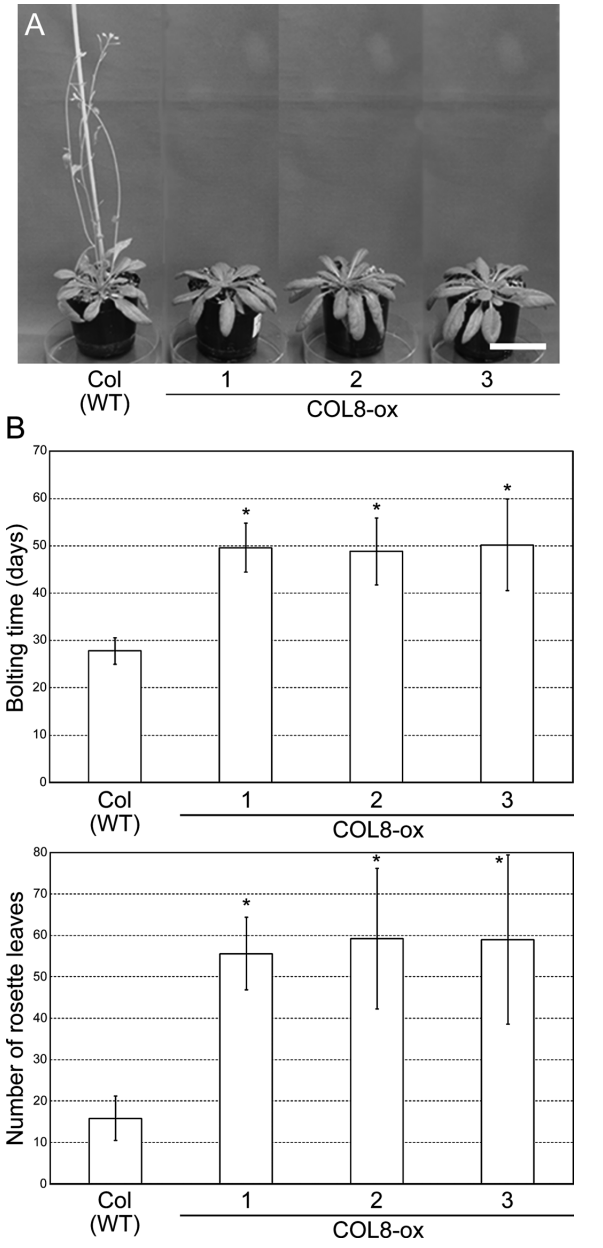

Figure 4. Delay of bolting time in *COL8* overexpressor (COL8-ox) transgenic plants under LD conditions. (A) Phenotypes of 41-day-old wild-type (WT, Col) plants and of three independent transgenic COL8-ox lines. (B) Number of days until bolting and number of rosette leaves at the time of bolting were determined in the WT and the COL8-ox transgenic plants. Error bars represent the standard deviation (n=5). Bars labeled with an * differ significantly from the wild-type ($P<0.05$, Student's *t*-test).

number of rosette leaves at bolting were recorded (Figure 4B). The average number of days required for bolting was 27.8 in the wild-type plants, versus 49.6, 49.8, and 50.2, respectively, in the COL8-ox 1, COL8-ox 2, and COL8-ox 3 plants. The corresponding average number of rosette leaves at the time of bolting was 15.8, 55.6, 59.2, and 59.0, respectively. The bolting time was significantly delayed and the number of leaves at the time of bolting increased significantly in the COL8-ox lines compared with the wild-type (both using Student's *t*-test, $P<0.05$), but the three overexpressor lines did not differ significantly in either parameter. In contrast, under short-day (10L:14D) conditions the bolting time did not differ significantly among the COL8-ox lines or between these lines and the wild-type (Supporting Information, Figure S1). Next, we measured the bolting time and the number of rosette leaves at the time of bolting in *COL8* knockout (*col8*) mutant plants and in the *COL8* antisense (COL8-AS) transgenic plants (see Supporting Information, Figure S2A-C). In both of these lines, the number of

days required for bolting and the number of leaves at the time of bolting did not differ significantly from those in the control (wild-type) plants under LD conditions.

Functional fluorescence-tagged COL8 protein is localized in the nucleus

As with the other members of the CO/COL family that have been analyzed to determine their subcellular localization (Cheng and Wang 2005; Datta et al. 2006; Robson et al. 2001), fluorescence-tagged COL8 is localized in the nucleus (Figure 2). To verify the activity of the COL8-GFP fusion protein in the COL8-GFP-ox transgenic plants, we examined the bolting time and number of rosette leaves at the time of bolting (Figure S3). The number of days required for bolting and the number of rosette leaves at the time of bolting differed significantly ($P<0.05$; Student's *t*-test) between the COL8-GFP-ox transgenic lines and the two control lines (WT and GFP-ox), as occurred with the COL8-ox transgenic plants, indicating that the COL8-GFP fusion protein was functional.

Expression of FT is reduced in the COL8-ox plants

Flowering time regulatory signals from the photoperiod pathway converge on *FT*. Therefore, to find an explanation for the delayed flowering time in the COL8-ox transgenic plants under LD conditions, we examined the expression levels of *FT* and the mRNA of its regulatory factor, *CO*. Under LD conditions, *CO* mRNA transcription is relatively low from zeitgeber time (zt) 0 to zt 8 and high from zt 12 to zt 20 in both the COL8-ox plants and the wild-type plants (Figure 5A). *FT* had a minor peak at the beginning of the light period and a major peak towards the end of the light period in the wild-type plants (Figure 5B). In contrast, *FT* mRNA accumulation was clearly reduced from zt 8 to zt 24 in the COL8-ox plants compared with that in the wild-type, although the first minor *FT* expression peaks (at zt 4) were similar in both types of plant. Thus, the late-flowering phenotype of the COL8-ox plants under LD conditions seems to be due to decreased expression of the florigen gene *FT*.

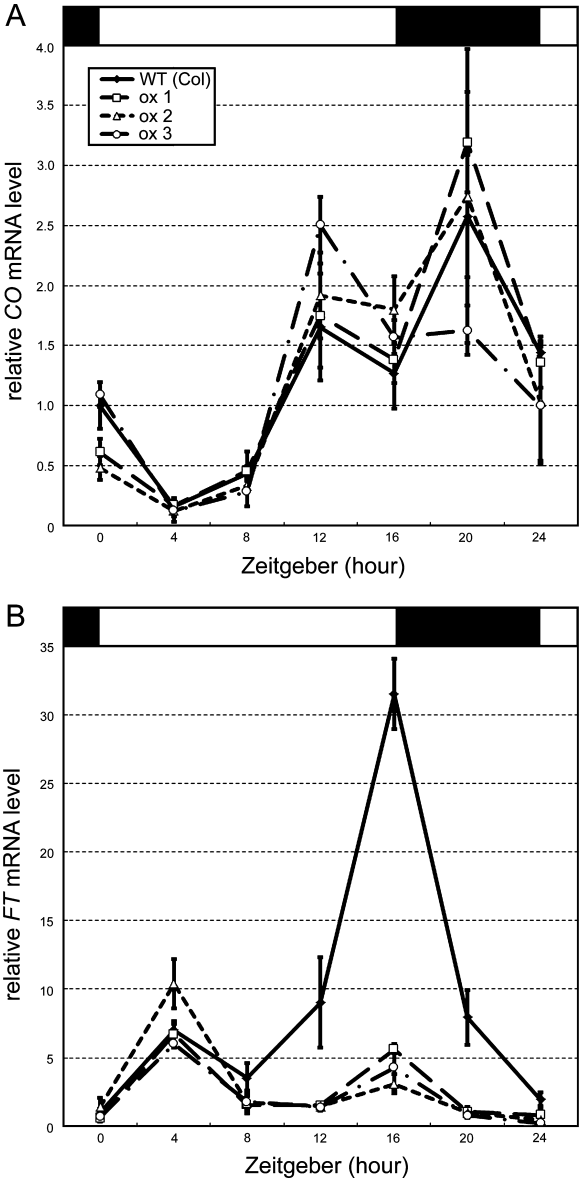

Figure 5. Relative *CO* and *FT* mRNA levels in the *COL8* overexpressor (COL8-ox) plants. Relative levels of (A) *CO* mRNA and (B) *FT* mRNA in the Col wild-type (WT) plants and in three independent lines of COL8-ox transgenic plants. Values were normalized against *ACTIN2* mRNA and are shown as relative intensities. Plants were grown under LD (16L:8D) conditions for 10 days. Black bars represent dark periods; light bars represent light periods. Error bars represent the standard deviation (n=3).

Discussion

Unlike the case with *CO* or *COL5*, whose overexpression causes early flowering in Arabidopsis (Hassidim et al. 2009; Onouchi et al. 2000), overexpression of *COL8* delayed flowering under LD conditions (Figure 4B). Flowering time in *COL9*-overexpressing plants is also later than in wild-type plants under LD conditions (Cheng and Wang 2005). However, in *COL9*-over-expressing plants, the level of expression of *CO* is reduced compared with that in the wild-type (Cheng and Wang 2005), whereas in COL8-ox plants, the expression level of *CO* did not decrease between zt 12 and zt 16; high levels of *CO* at this time are important for *FT* activation (Figure 5A).

In the COL8-ox plants, *FT* mRNA levels were low between zt 8 and zt 24 (Figure 5B). There are several possible scenarios for this *FT* repression in the COL8-ox lines. COL8 might function as a transcriptional repressor of *FT*, and overexpression of *COL8* would therefore have resulted in increased repression of *FT* transcription. Another possibility is that native COL8 may not function

as a flowering time regulator, whereas the ectopically overproduced protein mimics or interferes with the function of another flowering regulator protein (e.g., one similar to CO), thus delaying flowering time.

To clarify the function of COL8, we determined bolting time and rosette leaf number at the time of bolting in *col8* and COL8-AS plants; in both lines, they were not significantly different from wild-type (Figure S2C). This lack of change in the phenotypes of the *col8* and COL8-AS plants compared with wild-type might have occurred because (1) there were sufficient quantities of other proteins that had functions homologous to that of COL8 to compensate for the lack of COL8's flowering function; or (2) COL8 might not function in flowering time regulation; or (3) COL8 might regulate flowering time, but the altered phenotype was not observable under our experimental conditions.

Although overexpression of *COL8* caused late flowering under LD conditions, we could not conclude whether the late flowering in the COL8-ox plants was a true function of COL8. However, since overexpression of *COL8* confers a dominant effect, *COL8* might be a useful tool for controlling time of harvest a diverse range of photoperiodic crop plants.

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