Studies on Circadian Oscillation of Expression of Clock-Controlled Gene *AtC401*, an *Arabidopsis* Homolog of *PnC401* Which Related to Photoperiodic Induction of Flowering

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Abbreviations

ATP: adenosine triphosphate

bp: base pair (s)

cDNA: complementary DNA

CT: circadian time

DD: continuous dark

DMSO: dimethylsulfoxide

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

FFT-NLLS: fast Fourier transform-nonlinear least squares

h: hour (s)

kbp: kilo base pair (s)

LED: light-emitting diode

LL: continuous light

luc: firefly luciferase

MES: 2-morpholinoethanesulfonic acid, monohydrate

min.: minute (s)

mRNA: messenger RNA

NAA: 1-naphthaleneacetic acid

nt: nucleotide (s)

ORF: open reading frame

PCR: polymerase chain reaction

PPR: pentatricopeptide repeat

PRC: phase response curve

RACE: rapid amplification of cDNA ends

RNA: ribonucleic acid

rpm: round per minute

rRNA: ribosomal RNA

SDS: sodium dodecyl sulfate

sec.: second (s)

SSC: 150 mM Tris-HCl (pH 8.0), 1 mM EDTA

SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 10 mM EDTA (pH 7.7)

TPR: tetratricopeptide repeat

UTR: untranslated region

UV: ultraviolet

Abstract

Photoperiodic induction of flowering in higher plants is closely related to the circadian rhythm. *AtC401* was isolated as an *Arabidopsis* homolog of *Pharbitis nil C401* (*PnC401*) that is a gene which encodes a leaf protein closely related to the photoperiodic induction of flowering and shows a circadian rhythm at the transcriptional level. The *AtC401* gene spans 5.6 kbp and contains 12 exons. Comparison of the sequence and genomic organization of *AtC401* and *PnC401* revealed that each has two exons at the 3' –end, which encode a highly conserved domain consisting of 12 repeats of the PPR motif. Phylogenetic analysis with at least 450 *Arabidopsis* proteins containing PPR motifs revealed that AtC401 and related proteins form a distinct sub-family. Moreover, the position of the intron between the two exons that encode the PPR domain was conserved exactly in other *C401*-related genes. These results suggested that the conserved domain of AtC401 has a function similar to that of PnC401.

The expression pattern of *AtC401* mRNA showed the oscillation as expected from the results of *PnC401* of *Pharbitis*. To study the transcriptional regulation of *AtC401* gene, I fused a firefly *luciferase* reporter gene (*luc+*) to the *Arabidopsis* promoter sequence and transformed into *Arabidopsis* plants and cultured cells. The chronobiological analysis using the reporter plants concluded that the rhythmic expression of *AtC401* satisfied the criteria of circadian rhythms: persistence under constant conditions, temperature compensation of period, and phase-resetting by light signals. Sequence comparison between *AtC401* and *PnC401* found no common transcriptional cis-acting motifs in the promoter regions. Though there are several known transcriptional cis-acting motifs for circadian regulation in 5' upstream of *AtC401* gene, the promoter analysis using the reporter transgenic plants revealed that

these motifs were not required for the circadian expression of *AtC401* gene. The promoter analysis using the transgenic *Arabidopsis* plants and the transgenic cultured cell lines revealed that the *AtC401* promoter required the 5' UTR sequence for both circadian regulation and general transcription. Moreover the canonical TATA box was not required for *AtC401* transcription. These results suggest that the novel transcription machinery regulates the circadian transcription of *AtC401* gene. As far as I know, this is the first report of TATA-less promoter for circadian transcription in eukaryote.

Introduction

The circadian clock is an endogenous oscillator with a period of about 24 h that can be synchronized to external environmental cues (Bünning, 1973). The circadian clock regulates many biochemical, physiological, and behavioral processes in organisms that exhibit circadian rhythms. In higher plants, many physiological processes, such as leaf movement, stem elongation, stomata opening, photosynthesis, and gene transcription, exhibit circadian rhythms (Harmer *et al*., 2000; McClung, 2001; Schaffer *et al.*, 2001). In photoperiodic plant species, flowering is regulated by the absolute duration of light and dark during a cycle of approximately 24 h (Garner and Allard, 1920; Vince-Prue, 1975), and the induction of flowering is thought to depend upon a biological clock (Evans, 1971). Recent studies established molecular-genetic interactions between the control of the circadian-clock function and flowering time in *Arabidopsis* (Somers, 1999; Liu *et al.*, 2001; Simpson and Dean, 2002).

Molecular analyses of the circadian clock in animals and cyanobacteria provided the model of the oscillator as an autoregulatory transcriptional and translational negative-feedback loop (Dunlap, 1999). In *Arabidopsis*, mutations that affect the function of the circadian clock have been isolated by using luciferase reporter expressions under the control of the promoters of clock-controlled genes or by identifying mutants that exhibit altered flowering time (Mouradov *et al.*, 2002; Eriksson and Millar, 2003; Yanovsky and Kay, 2003). Analyses of the mutations and genes affected by these mutations revealed candidate genes, including *CCA1*, *LHY*, and *TOC1*/*APRR1*, for the central oscillator of circadian rhythm in *Arabidopsis*.

Pharbitis nil Choisy cv. Violet, an absolute short-day plant, is ideal for the study of early events in the photoperiodic induction of flowering because young seedlings grown in continuous light can be induced to flower quantitatively by exposure

to a single dark period of 16 h (Vince-Prue and Gressel, 1985). In *Pharbitis*, as in other plants, the regulation of processes related to photoperiodically induced flowering probably occurs in leaves through changes in gene expression. The results obtained with chemical inhibitors of gene expression and from biochemical analyses of macromolecules suggest that changes in gene expression in leaves might generate a state that leads to the induction of flowering (Vince-Prue and Gressel, 1985; O'Neill, 1992). In my laboratory, two genes, *PnGLP* and *PnC401*, whose transcript levels increase preferentially in the cotyledons and leaves of *Pharbitis nil* during floral-inductive dark periods and oscillate during extended dark periods have previously identified (Ono *et al.*, 1993; Ono *et al.*, 1996; Sage-Ono *et al.*, 1998). Moreover, treatments that inhibited the induction of flowering caused a reduction in *PnC401* mRNA levels (Sage-Ono, *et al.*, 1998). Those results proposed that the clock-controlled expression of these genes is related to the photoperiodic sensitivity of *Pharbitis*.

The deduced amino acid sequence of the PnC401 protein has no similarity to any proteins with known functions. Although there is abundant information on physiology and classical genetics in *Pharbitis*, much less is known about its molecular biology. Therefore, finding a homolog of *PnC401* in *Arabidopsis* would facilitate the elucidation of PnC401 function. In addition, it might also help to explain the difference in photoperiodic sensitivity between an absolute short-day plant, like *Pharbitis*, and a quantitative long-day plant, like *Arabidopsis*.

An *Arabidopsis* homologue, designated *AtC401*, which represent a single-copy gene was previously in my laboratory (Sage-Ono *et al*., 1998). Isolation of the full-length cDNA of *AtC401* revealed that it encodes a serine/threonine protein

kinase (SNF1)-related protein kinase and a domain very similar to PnC401, named C401 domain (Sage-Ono, 1998). An analysis of the protein kinase domain demonstrated protein kinase activity (Sage-Ono, *personal communication*), but the C401 domain has not yet been analyzed.

In this thesis, I revealed that the homologous domains were consisted of the pentatricopeptide repeat (PPR). The pentatricopeptide repeat (PPR) is a degenerate 35-amino-acid sequence that is present in tandem arrays of 2 to 26 motif repeats; it has been identified in a large number of plant proteins and a few other eukaryote proteins (Small and Peeters, 2000). There are more than 450 members of the PPR protein family in *Arabidopsis thaliana*. The structure of the PPR motif is predicted to resemble to that of the tetratricopeptide repeat (TPR) motif that is found in a wide array of proteins that interact with other proteins (Small and Peeters, 2000). It has been suggested that a specific single-strand RNA is the probable ligand of a particular PPR superhelix (Small and Peeters, 2000), but the functions of most PPR-containing proteins are largely unknown. Here, I identify a novel PPR protein sub-family of leaf-specific, circadian-regulated proteins that may be related to the photoperiodic induction of flowering.

The expression pattern of *AtC401* mRNA showed the oscillation as expected from the results of *PnC401* of *Pharbitis* (Sage-Ono, 1998). It is interesting that C401 mRNA oscillated in not only *Pharbitis* but also *Arabidopsis*. However, striking differences on regulation of oscillation was observed between *AtC401* and *PnC401*. *AtC401* mRNA was oscillating in continuous light condition that was the inhibitory condition of the expression of *PnC401* (Sage-Ono, 1998). It was suggested that the contrastive transcriptional regulation of genes for photoperiodic induction of flowering

between *Arabidopsis* and rice plants originates the difference in photoperiodism between a long-day plant *Arabidopsis* and a short-day plant, rice (Hayama *et al*., 2003). In my laboratory, it was inferred that the differences of expression pattern of *C401* genes also originates from the difference between a long-day plant *Arabidopsis* and a short-day plant *Pharbitis*, and have an influence on the sensitivity of day-length in photoperiodism (Sage-Ono, 1998).

In this thesis, I focused on the circadian oscillation of the expression of *C401* gene. Although there is abundant information on the physiology and classical genetics of *Pharbitis*, much less is known about its molecular biology. In this thesis, I isolated *AtC401* gene from *Arabidopsis*, a well-known model plant for molecular biology, because several transcriptional cis-acting motifs for circadian regulation had been reported in *Arabidopsis* (Anderson *et al*., 1994; Wang *et al*., 1997; Piechulla *et al*., 1998; Harmer *et al*., 2000). The molecular biological and the chronobiological analyses revealed that *AtC401* is a novel circadian clock-controlled gene that was regulated by very unique transcription regulatory mechanism. In this study, I will represent the results and discuss on the novel mechanism of the transcriptional regulation of circadian clock-controlled gene.

Materials and Methods

Plant materials and growth conditions of *Arabidopsis* plants

Arabidopsis thaliana ecotype Col-0 was mainly used in this thesis. The CAB2-luc+ transgenic line (C24 background) was kindly provided from Dr. S.A. Kay (The Scripps Research Institute, La Jolla, CA, USA). Seeds were surface-sterilized and sown on an agar plate containing half-strength Murashige-Skoog's medium (Murashige and Skoog, 1962) containing 0.8% (w/v) agar, with or without sucrose. Seeds were cold-treated at 4° C for 3 days in the dark before germination. Plants were grown in a chamber with white light (13.6 W m^{-2}) at 22° C. Light/dark conditions were 12 h light/12 h dark. When plants were cultured on soil, seeds were sown on fertilized granulated soil (Kureha Chemical Industry Co., Ltd, Tokyo, Japan). The growth conditions were same as those when plants were cultured on agar plate.

Plasmid construction and transformation of *Arabidopsis* plants

The genome DNA fragments were amplified by PCR with a pair of primers described in Table 2. The 101-luc+ Ti-plasmid vector was a modified pBI101 vector (Clontech, Polo Alto, CA, USA), in which the *β-glucuronidase* gene was replaced by the *luc*+ gene (Promega, Madison, WI, USA). The genome DNA fragment was digested by the restriction enzymes indicate in Table 2, and cloned in the 101-luc+ vector. For the linker-scanning construction, a pair of DNA fragment was fused with the *Eco*RI site, and inserted between the *Sal*I (or *Hin*d III) and *Xba* I sites of the 101-luc+ vector. To generate transgenic plants, the reporter constructs were transformed into the Col-0 ecotype of *Arabidopsis* by *Agrobacterium*-mediated floral-dip method (Clough and Bent, 1998).

Screening of an *Arabidopsis* genomic DNA library

The genomic clone containing *AtC401* was isolated from an *Arabidopsis* genomic library in the λEMBL3 SP6/T7 vector (Clontech). The 888-bp cDNA fragment amplified with a pair of primers (5'-CAT TCC ATT TTC AAC ACA CT-3' and 5'-GAT TCA AGA TTT GCT CCG CT-3') was used as a probe. About 150,000 recombinants were screened, and 24 positive plaques were isolated. The positive clones were plaque-purified, and the DNA was isolated. The genomic inserts were amplified with Long-Distance Insert Screening Amplimer Sets (Clontech) and a gene-specific primer (5'-AGC CAA GGA GGA CAA CTA AAA TCA GCA G-3'), then subcloned into the pCRII vector (Invitrogen, Carlsbad, CA). The subcloned fragment was sequenced with the automoated DNA sequencer (model 310; Applied Biosystems, Foster, CA, USA) by dideoxy sequencing method (Sanger et al., 1977) using a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Alignment and phylogenetic tree inference

Amino acid sequence alignments were performed using the CLUSTALW program available at the website of the DNA Data Bank of Japan (http://spiral.genes.nig.ac.jp/homology/) (Thompson *et al*., 1994). The Arabidopsis Genome Initiative (AGI) numbers of *Arabidopsis* PPR proteins were found in the *Arabidopsis thaliana* database of The Institute of Genome Research (TIGR) (http://www.tigr.org/tdb/e2k1/ath1/), and amino acid sequences were obtained with the Bulk Downloads program from The *Arabidopsis* Information Resource (TAIR) website (http://www.arabidopsis.org/tools/bulk/). The phylogenetic tree was inferred by the neighbor-joining method and plotted using Tree View software (Page, 1996).

Primer extension analysis

An antisense oligonucleotide (5'-GGA CAA GAA AGC TCT CGG CGA CA-3') was end-labeled with [γ-^{32}P]ATP (Ci/mmol) using T4 polynucleotide kinase (TAKARA, Kyoto, Japan). A 5-µg aliquot of total RNA from whole *Arabidopsis* plants harvested at CT0 was mixed with the labeled primer (2 x 10^6 cpm) in 11.75 µl of 7% DMSO. The mixture was incubated at 70° C for 10 min., quickly chilled on ice, and 4 µl of 5x First-Strand buffer, 2 µl of DTT, and 1.25 µl of 2 mM of each dNTP mix were added. After 5 min. of pre-heating, the primer was extended with 200 U of SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) at 42° C for 1 h. The primer extension was ethanol precipitated, electrophoresed on a 6% polyacrylamide/8 M urea gel, and sized against a sequencing reaction.

Bioluminescence assay and period estimation

Each transgenic seed was sown in a well of a 96-well white plate (Nalge Nunc International, Naperville, IL) containing 150 µl agar-medium (half-strength Murashige-Skoog's medium containing 0.8% (w/v) agar, with or without sucrose). Seedlings were entrained for 5 days at 22° C with cycles of 12 h light/12 h dark before transfer to experimental conditions. Each seedling was provided with 50 µl of 1 mM D-luciferin (Promega) aqueous solution before transfer. The bioluminescence of each seedling in LL was counted automatically at equal intervals using a TopCount NXT microplate luminescence and scintillation counter (Perkin-Elmer Biosystems). Light was supplied by a pair of red LED panels (100 µmol m^{-2} sec^{-1}; Tokyo Rikakikai, Tokyo,

Japan) positioned on either side of the plate-stacker. The bioluminescence in DD was monitored using the AQUACOSMOS/VIM system (Hamamatsu Photonics, Hamamatsu, Japan). Period estimates and fit curves were made using the fast Fourier transform-nonlinear least squares method (FFT-NLLS) (Plautz *et al*., 1997).

For the temperature compensation analyses, seedlings were transferred into the image box (A4178-11, Hamamatsu Photonics) which was placed in the temperature-controlled chamber, after entrainment. Bioluminescence of 24-48 seedlings was counted every 2 h for 20 min. using the AQUACOSMOS/VIM system. The temperature coefficient was calculated as described by Tsuchiya *et al*. (2003).

For bioluminescence imaging, transgenic seedlings were grown as described above, except on a 96-well clear plate. Each seedling was provided with 50 µl of 1 mM D-luciferin aqueous solution on the day before imaging. Transgenic plants were put in a dark box on the night before imaging and examined for bioluminescence in the next morning (CT0). Bioluminescence images were taken for 15 min. with a VIM camera (Hamamatsu Photonics, Hamamatsu, Japan).

Northern blot analysis

Total RNA was extracted from the aerial part of ten seedlings harvested at the indicated time. Total RNA (10 µg) was fractionated by electrophoresis on a formaldehyde-agarose gel and the bands of RNA were transferred to a nylon membrane filter (Biodyne B; Nihon Pall Ltd., Tokyo, Japan). The 224-bp partial AtC401 cDNA fragment amplified by PCR with a pair of primers (5'-ACA AGC TTA TCT GCT AGC AA-3' and 5'-GCT CTA GAT CCG CCA TAC TCC ATC GTG T-3') was used as the probe for *AtC401*, and the 1.3-kbp partial cDNA fragment amplified by PCR with a pair

of primers (5'-GGA AGA CGC CAA AAA CAT AA-3' and 5'-AGC CAC CTG ATA GCC TTT GT-3') was used as the probe for *luc*+. The RNA on the filter was allowed to hybridize with ^{32}P-labled *AtC401* or *luc*+ probe in a hybridization solution that contained 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS, and 150 μg ml^{-1} herring sperm DNA at 42° C for 20 h. The filter was first washed with 2× SSC at room temperature and then with 2× SSC and 0.1% SDS at 42° C. For visualization of the bands on the filter, I used a bio-imaging analyzer with an imaging plate (BAS5000; Fuji Photo Film Co., Ltd., Tokyo, Japan).

Phase response curve

Seedlings were grown as described above. After the entrainment, seedlings were set on the TopCount NTX which was placed in the dark room and started the monitoring of bioluminescence. Beginning after 1 full day in DD, individual plates of seedlings were exposed to 10-min red light pulse at 4 h intervals for 28 h. Light pulses were supplied by red LED panel (μmol m^{-2} sec^{-1}; Tokyo Rikakikai). The period and phase of the rhythms after the light pulses were estimated using the FFT-NLLS and converted to circadian time. Phase shifts were calculated as described by Covington *et al*. (2001).

Culture and bioluminescence assay of the *Arabidopsis* cell line, T87

The *Arabidopsis* established cell line, T87, was provided from RIKEN BioResource Center (BRC; Tsukuba, Japan). The T87 cells were suspended in 100 ml of JPL medium described by Axelos *et al*. (1992) in 300 ml flasks, and cultured at 22º C in continuous white light (13.6 W m^{-2}) on a gyratory shaker (100 rpm). The

transformation of T87 cells were performed by the *Agrobacterium*-mediated method (Nakamichi, *personal communication*). The transformed T87 cells were cultured on the CIM that is a modified Gamborg B5 medium (Gamborg *et al*., 1968) containing 0.6% (w/v) agar, 0.5 g l^{-1} MES, 3% (w/v) sucrose and 1 μM NAA, and light condition was LL.

For the bioluminescence measurement, a little piece of cells was transferred onto a squire plate filled with CIM containing 0.1 mM luciferin, and entrained for more than 2 days to 12 h light/ 12 h dark condition. After the entrainment, the cells were transferred into the image box (A4178-11, Hamamatsu Photonics) and performed the photon-counting every 2 h. The average rate of change of bioluminescence (ΔL) was calculated by the following equality:

$$\Delta L_t = L_{(t+2)}/L_{(t-2)}$$

In this equality, ΔL_t indicates the average rate of change of bioluminescence at time t, and L_t indicates the bioluminescence at time t.

Results

1. The genomic structural analysis of AtC401 *gene*

1-1. AtC401 protein contains a PPR domain

The *AtC401* cDNA consists of 2,905 bp and contains a 2,493-bp open reading frame that encodes a putative polypeptide of 831 amino acids (Fig. 1). The amino terminal region of AtC401 has a kinase domain, and the carboxy terminal region of AtC401, designated the C401 domain, shares 61% amino acid identity (81% similarity) with PnC401. The C401 domain contains no strong similarities to proteins with known functions. Protein motif searches revealed that the C401 domain contains 12 tandem repeats of the pentatricopeptide repeat (PPR) motif. PPR motifs consist of 35 degenerate amino acids, often arranged in tandem arrays of 2 to 26 repeats (Fig. 1B).

Database searches for other proteins containing PnC401 and AtC401 consensus sequences yielded an *Arabidopsis* protein, AT5G25630, and a rice protein, 001-114-A12. Each protein contains 12 tandem PPR repeats, but neither has been characterized (Fig. 1C). Although some amino acid residues are conserved in the PPR motif, PPR proteins display very low homologies with each other (Small and Peeters, 2000). Despite the fact that the *Arabidopsis* genome includes more than 450 genes encoding PPR sequences, a multi-alignment analysis of *Arabidopsis* PPR proteins revealed that AtC401 and AT5G25630 are more similar to PnC401 and the rice 001-114-A12 than to other *Arabidopsis* PPR proteins (Fig. 2). I found another an *Arabidopsis* PPR protein, AT5G38150, and its rice homologs which annotated as similar to PnC401. However, the phylogenetic tree revealed that these were evolutionally distinct from the PnC401 group. These results indicate that proteins containing the C401 domain form a distinct sub-family of the huge PPR protein family. Though the

function of the C401 domain is unknown, the predicted structure of the C401 domain implies that it interacts with other factors, possibly proteins or single RNA strands (Small and Peeters, 2000).

1-2. Genomic organization of *AtC401* gene

The *AtC401* genomic clone was isolated by screening an *Arabidopsis* genomic library with probes derived from the *AtC401* cDNA. The *AtC401* gene spans 5.6 kb and has 12 exons, ranging in length from 54 bp (exon 7) to 878 bp (exon 12) (Fig. 3, Table 1). The ATG translation initiation codon is located in exon 2, and the TAG termination codon is in exon 12. Exons 2 through 10 encode a protein kinase domain (Fig. 3). The C401 domain is encoded by two exons near the 3'-end, exons 11 and 12 (Fig. 3), as it is in all four *C401* genes identified to date.

The sequences of the exon/intron boundaries of *AtC401* are summarized in Table 1. All introns contain the conserved GT and AG sequences at the 5'- and 3'-splice sites, respectively. Intron sizes range from 77 bp (intron 7) to 574 bp (intron 9) (Table 1). The intron insertion site between the two exons encoding the C401 domain is exactly conserved between *AtC401* and *PnC401* (Fig. 3). Moreover the intron insertion site was also conserved in the genomic sequences of AT5G25630 and rice 001-114-A12 (Fig. 3). These results indicate that the C401 domains are conserved in the genomic structure among *Arabidopsis*, *Pharbitis* and rice plants.

1-3. Mapping of the transcriptional initiation site of the *AtC401* gene

The 5'-RACE analysis revealed that the *AtC401* transcript contains at least 726 bp upstream of the first ATG. To identify the transcription start site of *AtC401*, I

performed a primer extension assay using total RNA isolated from *Arabidopsis* whole plants harvested at CT0 and an antisense primer designed from the 5'-RACE product. The primer extension analysis yielded a major product corresponding to 49-nt upstream from the 5'-end of the 5'-RACE product (Fig. 4). The most upstream nucleotide position found in the 5'-end of the *AtC401* cDNA initiation site is indicated in Fig. 5. The sequence in the vicinity of this position agrees with the functional initiator consensus sequence, YYANT/AYY (Y = pyrimidine), with the initiation position expected within the consensus (Fig. 5) (Javahery *et al*., 1994; Smale *et al*., 1998).

1-4. Promoter activity of the 5'-flanking region

The mapping of the transcription initiation site revealed that *AtC401* is located only 156 nt away from the 3'-end of the neighboring gene, AT5G21170 (Fig. 5). An analysis of the 5'-flanking sequence revealed a TATA-like, TA-rich motif localized at position -36 nt relative to the transcriptional initiation site (+1). Several consensus motifs for circadian transcriptional regulation were mapped to the promoter region (Fig. 5). To examine the promoter activity of the 5'-flanking region of *AtC401*, I transformed three reporter constructs that contained a firefly luciferase reporter gene (luc+) (Promega) under the control of a successive deleted 5'-flanking fragments of *AtC401* into *Arabidopsis* plants (Fig. 6A).

To detect the circadian oscillation of bioluminescence from the higher plants, I established two kinds of luminescence measurement systems. The reporter analyses using these systems revealed that the bioluminescence of the reporter transgenic seedlings showed free-running rhythm during LL. Fig. 6B-D shows the bioluminescence record of all transgenic lines in T2-generation under continuous light

conditions. Although the luminescence intensity were decrease as the promoter fragment shorten, all of three constructs showed oscillation in bioluminescence levels with about 24 h period during continuous light condition (Table 3). The Northern blot analysis using the RNA extracted from the d3-luc+ transgenic plants indicated that the bioluminescence oscillations refer the transcriptional oscillations of the reporter gene (Fig. 7). The mRNA levels of the reporter gene almost agreed with mRNA levels of endogenous *AtC401* (Fig. 7). Furthermore, the bioluminescence of transformants almost agreed with endogenous *AtC401* expression in organ specificity (Sage-Ono, 1998) (Fig. 8). The reporter analysis indicated that the -174 to +73 nt fragment of the 5'-flanking region is sufficient to oscillation in transcriptional level during the constant condition.

2. Chronobiological characterization of the transcriptional rhythm of AtC401

2-1. The *AtC401* expression shows the free-running rhythm during not only LL but also DD

The circadian rhythm is an endogenous rhythm with about 24 h period and autonomously controlled by endogenous circadian oscillator as the biological clock. For the circadian rhythms as chronobiological definition, it is necessary to clarify that (i) its autonomous oscillation continues in constant condition and (ii) its circadian period is temperature-independent. Moreover, the endogenous oscillator requires (iii) responding to the environmental signals, such as light or temperature, for entrainment to environmental day/night cycle.

In the pervious section, I revealed that the transgenic *Arabidopsis* plants containing a luciferase reporter gene under the control of the -174 to +73 nt fragment of the 5'-flanking region of *AtC401* gene showed clear free-running rhythm with about 24 h period in the bioluminescence levels during LL (Fig. 6). Using the d3-luc+ transgenic plants, I verified whether the rhythm of *AtC401* satisfy these criteria of circadian rhythms. Following chronobiological analyses were performed using the homogeneously purified transgenic line (Fig. 9).

The free running rhythm of *AtC401* expression during continuous light (LL) was shown using the Northern blot analysis and the reporter analysis (Fig. 6, Fig. 7 and Fig. 9). However, the reporter expression levels in transformants cultured on sucrose-free medium decreased in continuous dark (DD) (Sage-Ono, 1998) (Fig. 13E). Then I examined the reporter assay using d3-luc+ transgenic seedlings cultured on sucrose-containing medium, and the bioluminescence oscillations were observed during DD (Fig. 10A). Sucrose addition induced not only dose-dependent increase of the levels of the reporter expression, but also recovery of the circadian oscillation of the reporter expression in DD (Fig. 10A). The effect of sucrose on the oscillation during DD could not be observed in the bioluminescence of the transgenic seedlings containing the CAB2-luc+ (Fig. 10B). The experiment performed during LL shows that sucrose addition induced dose-dependence of the bioluminescence levels of both d3-luc+ and CAB2-luc+, but had little effect on the waveform of the rhythms (Fig. 11). Period estimation of these free-running rhythms in LL revealed that 3% sucrose extended the rhythm periods of the bioluminescence of the d3-luc+ transgenic seedlings to about 1.5 h (Fig. 12A and C), but that there was little effect on those of the CAB2-luc+ transgenic seedlings (Fig. 12B and D). The d3-luc+ transgenic seedlings grown on medium with

3% sucrose showed almost the same period in both LL and DD (Fig. 12C and E). The Northern blot analysis revealed that the sucrose supplement also affects the mRNA levels of endogenous *AtC401* gene (Fig. 13). These result indicated that the *AtC401* expression shows the free-running rhythm during not only LL, but also DD with sucrose supplement.

2-2. Temperature compensation of the free-running period of *AtC401*

The circadian rhythm is commonly temperature compensated (Pittendrigh, 1954; Salisbury *et al*., 1968; Somers *et al*., 1998). To determine whether the free-running rhythm of *AtC401* expression is temperature compensated, I examined reporter expressions under various temperature conditions. After 5 day of entrainment to 12 h light/12 h dark cycle at 22° C, transgenic plants were transferred to DD at various temperature, 12° C, 16° C, 26° C or 30° C, and monitored the reporter expressions for more than 5 days. Though expression level increased with increasing temperature, the reporter expressions fluctuated rhythmically at all of those temperatures (Fig. 14). The calculated period length of those oscillations was almost constant (Fig. 15). The calculated temperature coefficient (the Q_{10} value) over the temperature range of 12-30° C is 0.97. Because these results fit in the previous report indicating that the free-running phase of CAB2-luc+ expression during LL was temperature compensated in *Arabidopsis* another ecotype, C24 (Somers *et al*., 1998), I suggest that the circadian clock of *Arabidopsis* plants also insensitivity to temperature during DD and the *AtC401* expression is controlled under the biological clock.

2-3. Phase response of free-running rhythm of *AtC401*

It is important to show that the endogenous circadian rhythm entrains to environmental rhythms, such as the light/dark cycle or fluctuations in temperature (Edmunds, 1988; Roenneberg and Foster, 1997). On the other hand, circadian rhythms do not change periods by entrainment to non-circadian photoperiods. An examination of the free-running rhythm of the reporter expression after entrainment to non-circadian photoperiods revealed that the free-running oscillation period of the reporter expression was constant at about 24 h (Fig. 16). The difference in the times between peaks of bioluminescence of d3-luc+ oscillations and of CAB2-luc+ oscillations were essentially the same (Fig. 16A-C). Because the difference between the phases of bioluminescence of d3-luc+ oscillations and CAB2-luc+ oscillations was constant, despite differences in entrainment conditions, these gene expressions must be regulated by the same epistatic factor(s).

Moreover I examined the phase responses of the reporter expression using light pulses under DD. The phase response curve (PRC) is shown in Fig. 17. The longest delay in the phase was observed at the middle of the subjective night; the longest advance in the phase was observed near dawn. The overall shape of this PRC was similar to that of a typical PRC and to those of PRC reported for other genes (Devlin, 2002; Covington *et al.*, 2001). The PRC indicates that the circadian expression of *AtC401* was accurately reset by the light signal.

3. Promoter analysis using transgenic Arabidopsis *plants*

3-1. *AtC401* promoter does not require CCA1-binding motif for circadian regulation

Because the d3-luc+ construct is sufficient to regulate circadian oscillation, the genomic fragment involved in this construct contains the cis-acting transcriptional regulatory element for circadian expression. Generally, it is guessed that the cis-acting factor is in 5' upstream region of the transcriptional initiation site of the gene. The sequence analysis revealed that there is a motif related to the consensus for binding site of an *Arabidopsis* Myb-related transcription factor, CIRCADIAN CLOCK ASSOCIATED1 (CCA1) in the 5' end of the d3 construct. Because CCA1 is known to closely relate to the *Arabidopsis* central oscillator for circadian rhythm, the motif was suspected to play a role in circadian regulation of *AtC401*. Then I made the d4-luc+ reporter construct in which the CCA1-binding-like motif was deleted from the d3-luc+ construct, and transformed to *Arabidopsis* plants (Fig. 18A). A homozygotic transgenic line (T3) was selected, and used for the experiment. The d4-luc+ transgenic seedlings show weaker bioluminescence than the d3-luc+ seedlings, but show significant circadian-oscillated bioluminescence during LL in spite of the absence of the CCA1-binding-like motif (Fig. 18B; Table 4).

3-2. Linker scanning analysis of the 5' upstream region of *AtC401* gene

To check whether the circadian regulatory cis-acting element presents in the upstream region of the d4 construct, a linker-scanning mutagenesis of the upstream region spanning from -80 to -32 was performed. Six linker scanning constructs in

which every 8 bp downstream of position -80 were replaced were constructed and transformed to *Arabidopsis* plants (Fig. 19A). The reporter analysis using these T2 seedlings showed that all kinds of linker-scanning mutagenesis caused no difference in the reporter expressions (Fig. 19B-G). It is very interesting that the mutation on the TATA-like motif found at about 35-bp upstream from the initiation site of *AtC401* also caused no difference in the reporter expression (Fig. 19G). Because *AtC401* promoter did not have the canonical TATA box or any other TATA-related motif coupled with the identified transcription initiation site (Fig. 5), it is suggested that *AtC401* gene did not require any TATA box for its expression.

3-3. The 5' transcribed region of *AtC401* is necessary for the transcription

To examine the promoter activity of the transcribed region of *AtC401*, the 3' deletion was performed. I constructed the d3A-luc+ reporter construct in which 71-bp of the 5' untranslated region (UTR) was deleted (Fig. 20A). No luminescence was detected from the plants transformed with d3A-luc+, though the same observation was performed (Fig. 20B). Because the deleted 71-bp of 5' UTR region was commonly contained in all reporter constructs, it is indicated that the essential element required for the transcription of *AtC401* presented in this region.

4. Promoter analysis using the transgenic Arabidopsis *cultured cell line, T87*

4-1. Circadian oscillation of *AtC401* transcription is observed in T87 cells

T87 is an established cell line from *Arabidopsis thaliana* (Axelos *et al*., 1992).

Nakamichi *et al*. (2003) have previously described that T87 cells show transcriptional oscillation of the *Arabidopsis* clock-related genes, including *TOC1/APRR1*, *CCA1* and *LHY1*, during DD. To examine the transcription of *AtC401* gene in T87 cells, the transgenic T87 cells containing the d3-luc+ reporter construct were generated. Because the culture in 96-well plate causes growth inhibition of T87 cells, the AQUA COSMOS system was used for the detection of the bioluminescence of transformed T87 cells. After the 3 days of entrainment to 12L/12D, the transgenic T87 cells showed bioluminescence oscillation during DD (Fig. 21B). Although the oscillations of the T87 cells were increased with time, it is rather difficult to detect the rhythm (Fig. 21A). Therefore, the average rate of change of bioluminescence was calculated and plotted. The calculated average rate of change clarified the oscillation of the d3-luc+ transgenic cells (Fig. 21B). The period of the oscillation was almost circadian, but it is longer than that of the d3-luc+ transgenic plants (Table 4). The similar results were shown by the observation of a number of genetically independent transgenic lines. These results indicate that *AtC401* gene expression also oscillates in T87 cells.

4-2. The 5' UTR is required for both the general transcription and the circadian oscillation of *AtC401*

Because the d3A-luc+ lost both circadian oscillation and general transcription, the essential element required for the transcription of *AtC401* might present in the 5' UTR. To examine the general transcription activity of the 5' UTR of *AtC401*, the d3B-luc+ reporter construct that has 20-bp longer 5' UTR than d3A-luc+ was made (Fig. 22). Although the cells transformed with d3A-luc+ showed almost no bioluminescence, the cells transformed with d3B-luc+ showed a little bioluminescence

(Fig. 23A). Any oscillation was not detected in bioluminescence of the d3B-luc+ cells (Fig. 23B). Those results indicate that the 20-bp of 5' UTR contained the element for the general transcription of *AtC401*.

On the other hand, I constructed d5-luc+ that contained 73-bp of 5' UTR and only 13-bp of 5' upstream region of *AtC401*, and transformed into T87 cells. The d5-luc+ transgenic cells showed the bioluminescence of strength ten times or more the d3B-luc+ cells, and the circadian oscillations were detected in its bioluminescence (Fig. 23A and C). The reporter construct containing two tandem array of the d5 fragment showed much clearly that the fragment is sufficient to the circadian regulation of *AtC401* (Fig. 24B) Those results indicated that the d5 fragment contained both the element for binding to the general transcription machinery and the cis-acting element(s) for circadian regulation. Because it is clear that there is no TATA box in this fragment, I conclude that *AtC401* requires no TATA box for its general transcription and circadian oscillation.

Discussion

PnC401 is a novel gene that shows good correlation to photoperiodic induction of flowering in *Pharbitis nil* (Sage-Ono *et al*., 1998). Interestingly *PnC401* showed circadian expression. It is important to clarify the mechanism regulating the circadian expression of *PnC401* for understanding the process of photoperiodic induction of flowering. However, it was difficult to use *Pharbitis nil* for studying the mechanism, because there was no available transformation protocol. Thus, I interested to use *Arabidopsis*, a model plant for molecular biology, for this study. At the start of my work, ORF of *AtC401* gene, a homolog of *PnC401*, was not found by the Arabidopsis genome project. In my laboratory, my colleague had already isolated cDNA of *AtC401*. Therefore, I had started to isolate genomic clone of *AtC401* and to characterize the *AtC401* gene.

At first, I had isolated the genomic clone of *AtC401*, an *Arabidopsis* homolog of *PnC401* to characterize the C401 proteins. Both AtC401 and PnC401 have consensus domain (C401 domain) consisting of 12 tandem repeats of the PPR motif (Fig. 1). The structure of the PPR motif is predicted to resemble to that of the tetratricopeptide repeat (TPR) motif, which contains two antiparallel α-helices. A tandem array of TPR motifs forms a superhelix with a central groove, which is likely involved in protein-protein interactions (Das *et al*., 1998). Small and Peeters (2000) suggested that PPR proteins might bind to RNA and be involved in regulating gene expression. Furthermore, genomic sequence analyses showed that the exon/intron organization of the region encoding the C401 domain is exactly conserved among *C401* genes (Fig. 3), suggesting that the C401 domain is evolutionarily conserved. In fact, the C401 domain of AtC401 has the highest identity with PnC401 among other *Arabidopsis* proteins. I speculate that the interactions between C401 domains and their

specific ligands, possibly protein or RNA, are important to the functions of C401 proteins.

AtC401 also has a protein kinase domain in the N-terminal region (Fig. 1A), and the protein kinase activity of this domain has been confirmed *in vitro* (Sage-Ono, *personal communication*). However, AtC401 is currently the only C401-related protein that is known to contain a kinase domain (Fig. 3). It is not known whether the kinase domain is necessary for the function of AtC401 protein. The finding of another C401 protein with a kinase domain might be important for appreciation of the kinase domain of AtC401 protein.

The analysis of organ specificity of *AtC401* expression using the luciferase reporter revealed that *AtC401* expression was observed only in aerial part of plants, especially the strongest expression was observed in the youngest leaves and inflorescences (Fig. 8). The organ specificity of the reporter expression agreed with the accumulation of endogenous *AtC401* mRNA (Sage-Ono, 1998). It had been known that leaves are organs responsible for the photoperiodic induction of flowering, and young leaves have higher flower-inducing activity (Vince-Prue, 1975). These results suggest the relation between *AtC401* expression and photoperiodism.

Despite the presence of the kinase domain, *AtC401* expression is regulated in the manner similar to that of *PnC401* expression (Sage-Ono *et al*., 1998). Several known motifs for circadian regulation were identified in upstream regions of *AtC401*, but I could not find any motifs common to the putative promoter regions of both *PnC401* and *AtC401*. In this thesis, it was revealed that *AtC401* promoter activity

oscillates with a circadian rhythm during both LL and DD (Fig. 10, Fig. 11 and Fig. 13). These results suggest that the circadian rhythm of *C401* gene expression is important for C401 protein function.

In this thesis, it was represented that the free-running rhythm of *AtC401* promoter*::luciferase* fusion gene (d3-luc+) expression satisfies the criteria of circadian rhythms: persistence under constant conditions, temperature compensations of period, entrainment manner, and phase-resetting by light signals (The second part of the result section). These results indicate that *AtC401* gene is chronobiologically established to an *Arabidopsis* clock-controlled gene. *Arabidopsis* has been predicted to contain approximately 2,000 of clock-controlled genes (Harmer *et al*., 2000; Schaffer *et al*., 2001). Though these genes have been shown to oscillate with circadian period at transcription level during LL, there are few genes chronobiologically established to have a circadian rhythm (Somers *et al*., 1998; Convington *et al*., 2001). In addition, my results suggested that sugar could be an essential component for the transcriptional regulation of some *Arabidopsis* clock-controlled genes in DD. I am currently performing promoter analyses to determine cis-acting element(s) related to the circadian regulation of *AtC401* expression. These results should reveal a novel mechanism for the transcriptional regulation of circadian clock-controlled genes in higher plants.

To clarify the mechanism of circadian regulation of *AtC401* expression, I performed that the promoter deletion analyses using the firefly *luciferase* reporter gene (*luc*+). The *luciferase* provides an ideal reporter, as luciferase enzyme activity is relatively unstable *in vivo* (Kay *et al.*, 1994). The luciferase enzyme emits green light, concomitant with the ATP- and O_2-dependent oxidation of beetle luciferin. Cells transformed with *luc*+ will luminesce when supplied with luciferin because ATP and O_2 are supplied by endogenous pools (Kay *et al*., 1994). In this thesis, I established the

nondestructive and automatic measurement systems for the low intensity of light produced by luciferase activity. These systems enable to perform the chronobiological analysis of circadian expression and the promoter analysis of *AtC401*.

Sequence analyses of the *AtC401* 5'-flanking region revealed the absence of a canonical TATA box coupled with the identified transcriptional initiation site (Fig. 4 and Fig. 5), and there have been no reports of a TATA-less promoter with circadian regulation. Database searches revealed that the 5' flanking sequence contains a consensus motif for circadian transcriptional regulation that is similar to the *Arabidopsis* CCA1-binding consensus sequence (CCA1b) (Wang *et al*., 1997) and a motif that is necessary for the circadian expression of the tomato *Lhc* gene (CIRCADIANLELHC) (Piechulla *et al*., 1998) (Fig. 5). The promoter analyses using *luciferase* reporter revealed that the 5' upstream region to the position -33 was not required for both the general transcription and the circadian regulation of *AtC401*, though it is generally guessed that the *cis*-acting factor is in the 5' upstream region from the transcriptional initiation site of the gene (Fig. 6, Fig. 18 and Fig. 19). These results suggest that a novel transcriptional mechanism involves in the circadian regulation of *AtC401*. However, the decrease of the level of the reporter expression by successive 5' deletion of *AtC401* promoter suggests that the 5' upstream region is important for the participation in the regulation of the level of *AtC401* expression (Fig. 6 and Fig. 18).

Moreover, it was suggested that the *AtC401* requires no canonical TATA box

for its transcription (Fig. 19G and Fig. 23). As far as I know, this is the first report showing TATA-less promoter with circadian regulation in eukaryote. The region spanning from -13 to +73 referred to the transcriptional initiation site of *AtC401* is sufficient for circadian regulation. This region contains an initiator consensus and four tandem repeat of the GAT/AAA motif (Fig. 5). It is suggested that about 20% of *Arabidopsis* gene promoter lack the TATA box (Nakamura *et al*., 2002), and the initiator motif is required for the transcription of these genes (Achard *et al*., 2003). From these results, I supposed a model of the *cis*-acting transcriptional regulation factors in the *AtC401* promoter: (1) the initiator motif is required for the binding of the novel general transcription machinery; (2) the tandemly repeated GAT/AAA motif is related to circadian regulation (Fig. 25). The results of the reporter analyses of d3A-luc+ and d3B-luc+ supports the model: the d3A fragment lacked the full-activity of initiator; the d3B-luc+ contained the complete initiator activity, but did not contain the complete circadian regulation (Fig. 22 and Fig. 23). It has been suggested that tandem repeat of the GATA motif in *Arabidopsis CAB2* promoter (CAB GATA FACTOR1, CGF1) is related to the regulation of circadian oscillation (Anderson *et al*., 1994; Anderson and Kay, 1995), but there is no information about this motif in the transcribed region. Although there is no information whether the CGF1 transcription factor could function with the transcription machinery bound on the initiator motif of *AtC401*, the CGF1 transcription factor might bind with the repeat motif of *AtC401*. Further experiments, including the screening of the binding protein on the initiation site or the repeat motif in *AtC401* promoter, are expected to reveal the novel circadian transcriptional regulation mechanism in higher plants.

Finally I tried to characterize AtC401 in photoperiodic induction of flowering

and circadian rhythm in *Arabidopsis*. The involvement of AtC401 in photoperiodic induction of flowering is supported by the late-flowering phenotype of *atc401* mutant (Sage-Ono, *personal communication*). The mutation on the genes that characterized as the members of the central oscillator, including CCA1, LHY and TOC1/APRR1, affect the period of endogenous circadian rhythm (Somers, 1999; Liu *et al.*, 2000; Simpson and Dean, 2002). Though the mutation causes no effect on the circadian expression of other clock-controlled genes, AtC401 might be an out-put factor of circadian clock (Sage-Ono, *personal communication*). The *AtC401* expression is not directly regulated by CCA1 (Fig. 17). This suggests that the novel mechanism might involve in transcriptional regulation of *AtC401* (Fig. 24). Because the C401 domain might form the super-helix, the interaction with other protein(s) might be important to the function of AtC401. The yeast two-hybrid screening using the C401 domain revealed that AtC401 can interact *in vivo* with *Arabidopsis* CONSTANS (CO) that strongly related to photoperiodic induction of flowering in *Arabidopsis* (Sage-Ono, personal communication). These results presented above indicate that *AtC401* is a novel clock-controlled gene related to photoperiodic induction of flowering, and suggest that the interaction between AtC401 protein and CO at the specific time might be important for regulation of the photoperiodic induction of flowering in *Arabidopsis* (Fig. 26).

I expected that the results presented in this thesis will contribute to the clarification of both the novel mechanism of the transcriptional regulation of circadian clock-regulated gene, and the relation between the circadian rhythm and photoperiodic induction of flowering.

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Tables and Figures

Table 1. Exon/intron organization and nucleotide sequence of exon/intron junctions of the *AtC401* gene.

Exon	Size(bp)	cDNA position	5' splice doner	Intron	Size(bp)	3' splice accepter
1	284	1-283	CAATTCGCAG**gt**gtgtattc	1	459	catgaaac**ag**CTTGTGAAGA
2	205	284-488	GGCTGAACAA**gt**aattcgct	2	172	gcttttac**ag**ATAAAGAGAG
3	63	489-551	GCTTTATGAG**gt**ctaattcc	3	134	atcattcc**ag**GTTTTGGCGA
4	73	552-623	TGACAAGATT**gt**gagttttc	4	171	acttgtga**ag**AAAAATGATG
5	107	624-731	AGACTTAAAG**gt**acaaggaa	5	108	ctcaatgc**ag**CCAGAAAATT
6	81	732-812	ACAAGCCGGG**gt**aactacgg	6	196	gttttggc**ag**GGAGATGGCC
7	54	813-866	TGCTCCTGAG**gt**actcttag	7	77	ttgtccca**ag**GTCTTAATGA
8	126	867-992	GTACAAAAAG**gt**aagaagtt	8	84	ttattttc**ag**ATCTCATCTG
9	90	993-1078	CCCTATGACT**gt**aagacatt	9	574	aacatgcc**ag**CGTATAACAA
10	123	1079-1201	AGATTCTGAA**gt**gagtcatt	10	180	tgcttcac**ag**GAAGGGAGAG
11	873	1202-2074	AGTTGGTGAG**gt**gcgcttta	11	86	aatataat**ag**GTGGTGGATC
12	878	2075-2905				

Nucleotides are numbered according to the cDNA sequence, starting with +1 for the first nucleotide of the transcriptional initiation site. Upper- and lowercase letters represent exonic and intronic sequences, respectively, and the intron residues adjoining the splice junction are shown in boldface.

Table 2. Primers for the plasmid construction.

Construction		Forward primer			Reverse primer		
d1-luc+		5'- AAC TGC AGC GGT GAA TCC AAA TAC ATC C -3'	(28 mer)	*Sal*I	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
d2-luc+		5'- AAC TGC AGG CTT CTC TCA TTG TAA CGA T -3'	(28 mer)	*Sal*I	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
d3-luc+		5'- AAC TGC AGG CTT CTC TCA TTG TAA CGA T -3'	(28 mer)	*Hin*dIII*	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
d3A-luc+		5'- AAC TGC AGG CTT CTC TCA TTG TAA CGA T -3'	(28 mer)	*Hin*dIII*	5'- AAA TTT TCA AGC TTA TAG ACG ACG TGG -3'	(27 mer)	*Hin*dIII
d3B-luc+		5'- AAC TGC AGG CTT CTC TCA TTG TAA CGA T -3'	(28 mer)	*Hin*dIII*	5'- CTT TAT TTC TCT TTA AGC TTT TTC TTA -3'	(27 mer)	*Hin*dIII
d4-luc+		5'- AAC TGC AGT GGT ATT GGA AAA GGC TAA C -3'	(28 mer)	*Sal*I	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
d5-luc+		5'- CCA CGT CGT CTA TAA GCT TGA AAA TTT -3'	(27 mer)	*Spe*I	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m1	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -3'	(28 mer)	*Sal*I	5'- GGA ATT CCA AGA TTA TTT TCA AAA ATT T -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCT ATT GGA AAA GGC TAA CCA G -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m2	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -3'	(28 mer)	*Sal*I	5'- GGA ATT CCC CAA AAA GAA AGA TTA TTT T -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCA AGG CTA ACC AGT TTG AAA G -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m3	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -4'	(28 mer)	*Sal*I	5'- GGA ATT CCT TCC AAT ACC AAA AAG AAA G -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCC CAG TTT GAA AGT GTA CTC A -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m4	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -4'	(28 mer)	*Sal*I	5'- GGA ATT CCC AAA CTG GTT AGC CTT TTC C -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCA AAG TGT ACT CAT AAT AAA T -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m5	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -5'	(28 mer)	*Sal*I	5'- GGA ATT CCT ACA CTT TCA AAC TGG TTA G -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCC TCA TAA TAA ATC CAC GTC G -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I
m6	A	5'- AAC TGC AGA ACC AAA CAC ATA AAT CCT G -5'	(28 mer)	*Sal*I	5'- GGA ATT CCA TTA TGA GTA CAC TTT CAA A -3'	(28 mer)	*Eco*RI
	B	5'- GGA ATT CCA AAT CCA CGT CGT CTA TTT G -3'	(28 mer)	*Eco*RI	5'- GCT CTA GAT CCG CCA TAC TCC ATC GTG T -3'	(28 mer)	*Xba*I

*The *Hin*dIII site in the genome DNA was used for the construction.

Table 3. Estimated period times of free-running rhythms of the transgenic seedlings in continuous light.

Construct	Amplitude	Period	Rel-Amp
d3-luc+	1378.0 ±208.4	23.94 ±0.2055	0.151
d4-luc+	320.5 ±4.442	24.13 ±0.1765	0.139
CAB2-luc+	183.0 ±1.884	24.22 ±0.1239	0.103

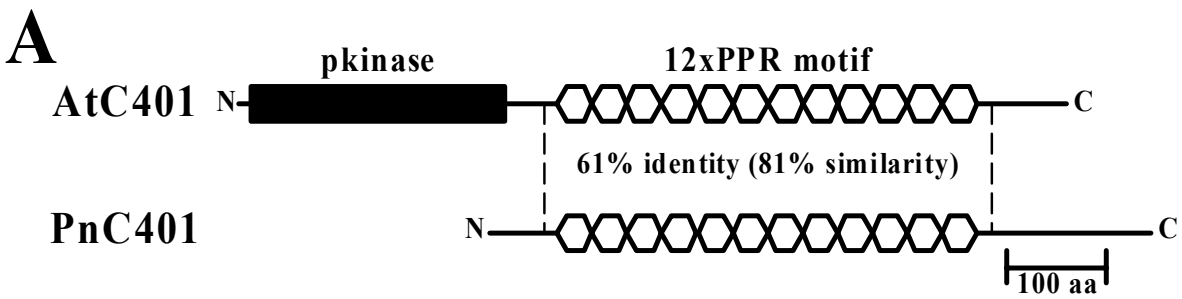
Arabidopsis plants were transformed with each construct, and their homozygotic T3 seedlings were used for the reporter analysis. Errors were shown 95% confidence intervals.

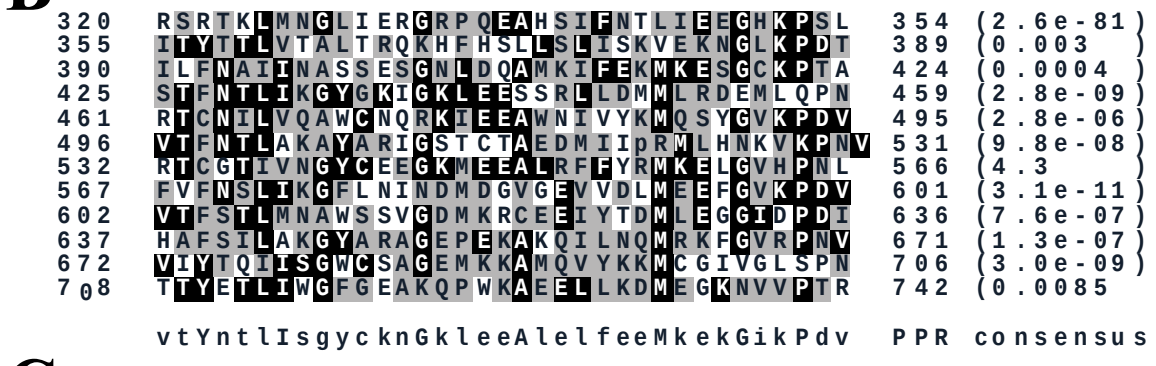
Table 4. Estimated period times of free-running rhythms of the transgenic T87 cells in continuous darkness.

Construct	Period	Rel-Amp
d3-luc+	31.18 ±1.641	0.193
d5*2-luc+	29.74 ±0.6193	0.214

Arabidopsis T87 cells were transformed with each construct, and used for the reporter analysis. Errors were shown 95% confidence intervals.

Figure 1. The predicted protein structure and amino acid sequence of AtC401 protein. (A) Schematic of predict protein structure of AtC401 and PnC401 proteins. Hexagons indicate PPR motifs and filled box indicates a protein kinase motif (pkinase). These motifs were identified with the Pfam database (http://www.sanger.ac.uk/cgi-bin/Pfam). N and C indicate the amino-terminal and carboxyl-terminal of proteins, respectively. (B) The alignment of the twelve PPR motifs of AtC401 protein. Residues identical to the consensus motif (showed at bottom) are shaded in black, and similar residues are shaded in gray. The number in the parenthesis indicates E-values for the consensus motif. (C) The alignment of the amino acid sequence of AtC401, PnC401, AT5G25630 and a rice 001-114-A12 protein. The alignment was carried out using the CLUSTAL W program. Identical residues are shaded in black. The PPR motifs were indicated by bars. The position of an intron insertion in two exons encoding the conserved region is indicated by open arrowhead.


A
pkinase
12xPPR motif
AtC401
N
C
61% identity (81% similarity)
PnC401
N
C
100 aa


B
320 RSRTKLMNGLIERGRPQEAHSIFNTLIEEGHKPSL 354 (2.6e-81)
355 ITYTTLVTALTRQKHFHSLLSLISKVEKNGLKPDT 389 (0.003)
390 ILFNAIINASSESGNLDQAMKIFEKMKESGCKPTA 424 (0.0004)
425 STFNTLIKGYGKIGKLEESSRLLDMMLRDEMLQPN 459 (2.8e-09)
461 RTCNILVQAWCNQRKIEEAWNIVYKMQSYGVKPDV 495 (2.8e-06)
496 VTFNTLAKAYARIGSTCTAEDMIIpRMLHNKVKPNV 531 (9.8e-08)
532 RTCGTIVNGYCEEGKMEEALRFFYRMKELGVHPNL 566 (4.3)
567 FVFNSLIKGFLNINDMDGVGEVVDLMEEFGVKPDV 601 (3.1e-11)
602 VTFSTLMNAWSSVGDMKRCEEIYTDMLEGGIDPDI 636 (7.6e-07)
637 HAFSILAKGYARAGEPEKAKQILNQMRKFGVRPNV 671 (1.3e-07)
672 VIYTQIISGWCSAGEMKKAMQVYKKMCGIVGLSPN 706 (3.0e-09)
708 TTYETLIWGFGEAKQPWKAEELLKDMEGKNVVPTR 742 (0.0085
vtYntlIsgyckn GkleeAlelfeeMkekGikPdv PPR consensus

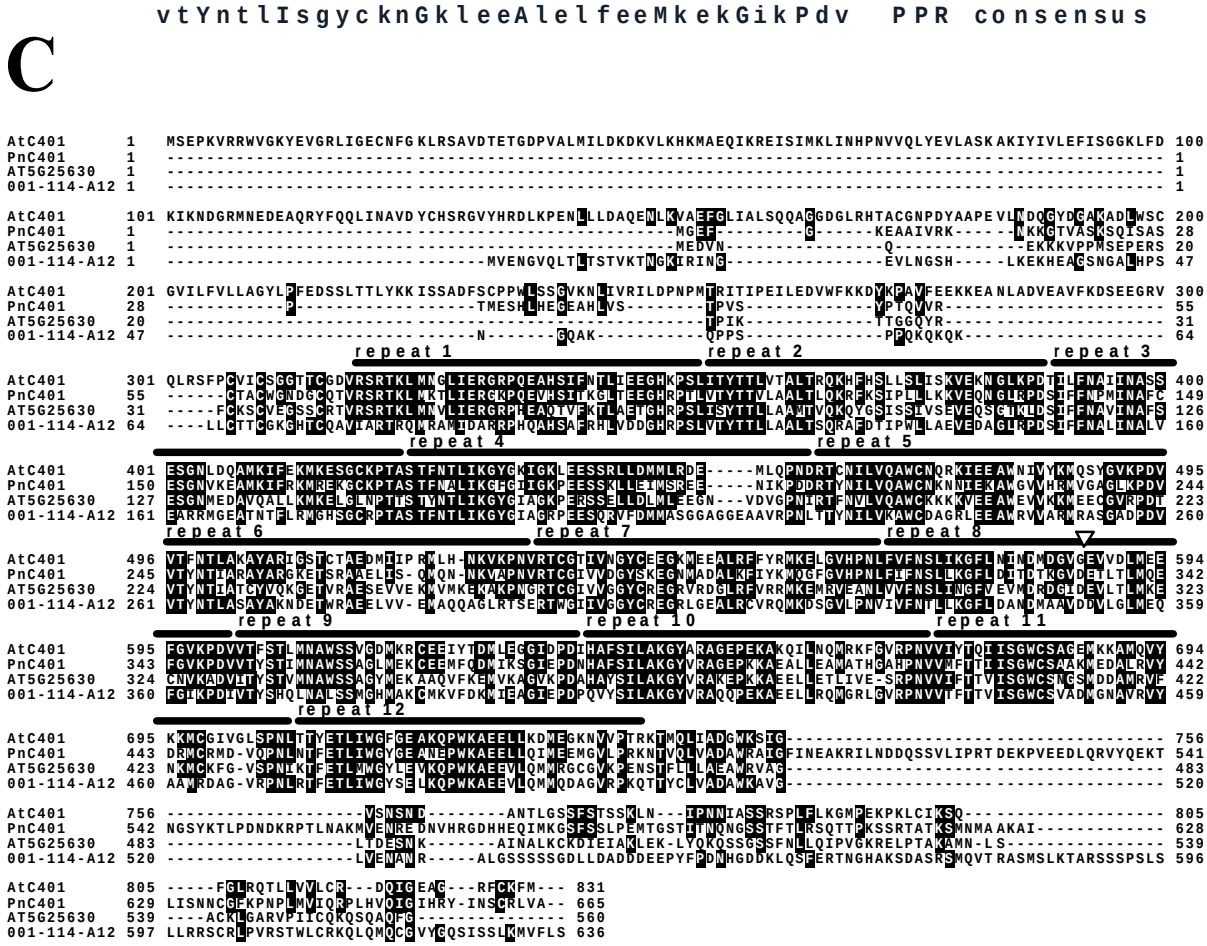

C
AtC401 1 MSEPKVRRWVGKYEVGRLIGECNFG KLRSAVDTETGDPVALMILDKDKVLKHKMAEQIKREISIMKLINHPNVVQLYEVLASK AKIYIVLEFISGGKLFD 100
PnC401 1 ---------- 1
AT5G25630 1 ---------- 1
001-114-A12 1 ---------- 1
AtC401 101 KIKNDGRMNEDEAQRYFQQLINAVD YCHSRGVYHRDLKPENLLLDAQENLKVAEFG LIALSQQAGGDGLRHTACGNPDYAAPEVLNDQGYDGAKADLWSC 200
PnC401 1 ---------- 28
AT5G25630 1 ---------- 20
001-114-A12 1 ---------- 47
AtC401 201 GVILFVLLAGYLPFEDSSLTTLYKK ISSADFSCPPWLSSGVKNLIVRILDPNPMTRITIPEILEDVWFKKDYKPAVFEEKKEANLADVEAVFKDSEEGRV 300
PnC401 28 ---------- 55
AT5G25630 20 ---------- 31
001-114-A12 47 ---------- 64
repeat 1 repeat 2 repeat 3
AtC401 301 QLRSFPCVICSGGTTCGDVRSRTKLMNGLIERGRPQEAHSIFNTLIEEGHKPSLITYTTLVTALTRQKHFHSLLSLISKVEKNGLKPDTILFNAIINASS 400
PnC401 55 ---------- 149
AT5G25630 31 ---------- 126
001-114-A12 64 ---------- 160
repeat 4 repeat 5
AtC401 401 ESGNLDQAMKIFEKMKESGCKPTASTFNTLIKGYGKIGKLEESSRLLDMMLRDE-----MLQPNDRTCNILVQAWCNQRKIEEAVNIVYKMQSYGVKPDV 495
PnC401 150 ---------- 244
AT5G25630 127 ---------- 223
001-114-A12 161 ---------- 260
repeat 6 repeat 7 repeat 8
AtC401 496 VTFNTLAKAYARIGSTCTAEDMIIPRMLH-NKVKPNVRTCGTIVNGYCEEGKMEEALRFFYRMKELGVHPNLFVFNSLIKGFLNINDMDGVGEVVDLMEE 594
PnC401 245 ---------- 342
AT5G25630 224 ---------- 323
001-114-A12 261 ---------- 359
repeat 9 repeat 10 repeat 11
AtC401 595 FGVKPDVVTFSTLMNAWSSVGDMKRCEEIYTDMLEGGIDPDIHAFSILAKGYARAGEPEKAKQILNQMRKFGVRPNVVIYTQIISGWCSAGEMKKAMQVY 694
PnC401 343 ---------- 442
AT5G25630 324 ---------- 422
001-114-A12 360 ---------- 459
repeat 12
AtC401 695 KKMCGIVGLSPNLTTYETLIWGFGEAKQPWKAEELLKDMEGKNVVPTRKTMQLIADGKKSIG---------- 756
PnC401 443 ---------- 541
AT5G25630 423 ---------- 483
001-114-A12 460 ---------- 520
AtC401 756 ----------VSNSNL--------ANTLGSSFSTSSKLN---IPNNIASSRSPLFLKGMPEKPKLCIKSQ---------- 805
PnC401 542 ---------- 628
AT5G25630 483 ---------- 539
001-114-A12 520 ---------- 596
AtC401 805 -----FGLRQTLLVLCR---DQIGEAG---RFCKFM--- 831
PnC401 629 ---------- 665
AT5G25630 539 ---------- 560
001-114-A12 597 ---------- 636

Figure 2. Phylogenetic tree showing the relationship between C401 proteins and other *Arabidopsis* PPR proteins. The data of amino acid sequences are obtained from PnC401-related proteins and randomly-selected 93 proteins of more than 450 Arabidopsis PPR proteins on TIGR database. On the basis of the alignment by the CLUSTAL W program, the tree was inferred by Neighbor-joining method. Filled box and white characters indicate the PnC401 subfamily. AT4G38150 subfamily is indicated by gray box. The scale bar represents 0.5 UNITS.

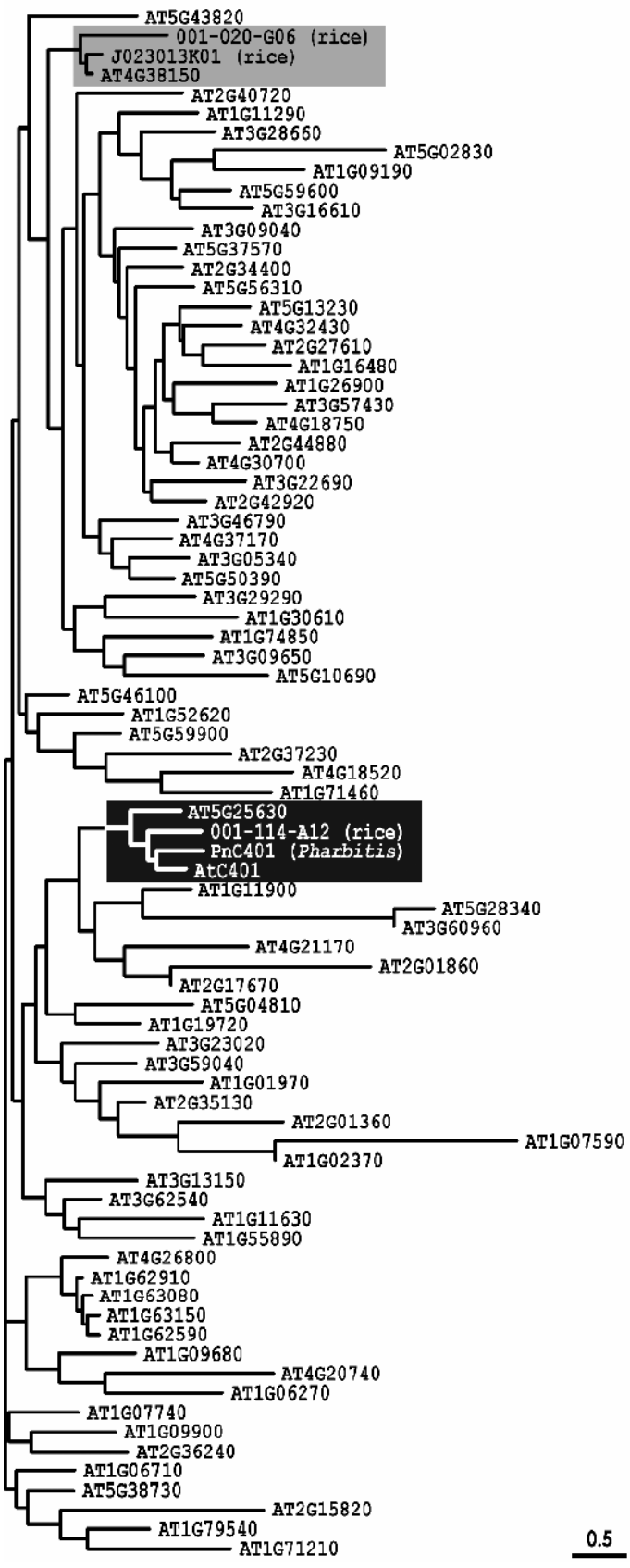

AT5G43820
001-020-G06 (rice)
J023013K01 (rice)
AT4G38150
AT2G40720
AT1G11290
AT3G28660
AT5G02830
AT1G09190
AT5G59600
AT3G16610
AT3G09040
AT5G37570
AT2G34400
AT5G56310
AT5G13230
AT4G32430
AT2G27610
AT1G16480
AT1G26900
AT3G57430
AT4G18750
AT2G44880
AT4G30700
AT3G22690
AT2G42920
AT3G46790
AT4G37170
AT3G05340
AT5G50390
AT3G29290
AT1G30610
AT1G74850
AT3G09650
AT5G10690
AT5G46100
AT1G52620
AT5G59900
AT2G37230
AT4G18520
AT1G71460
AT5G25630
001-114-A12 (rice)
PnC401 (Pharbitis)
AtC401
AT1G11900
AT5G28340
AT3G60960
AT4G21170
AT2G01860
AT2G17670
AT5G04810
AT1G19720
AT3G23020
AT3G59040
AT1G01970
AT2G35130
AT2G01360
AT1G07590
AT1G02370
AT3G13150
AT3G62540
AT1G11630
AT1G55890
AT4G26800
AT1G62910
AT1G63080
AT1G63150
AT1G62590
AT1G09680
AT4G20740
AT1G06270
AT1G07740
AT1G09900
AT2G36240
AT1G06710
AT5G38730
AT2G15820
AT1G79540
AT1G71210
0.5

Figure 3. Schematic representation of the genomic and cDNA structures of *AtC401*, *PnC401*, AT5G25630 and rice 001-114-A12. Exons are indicated by open boxes. The conserved C401 domain and point of intron insertion were indicated by gray boxes and black arrowheads, respectively.

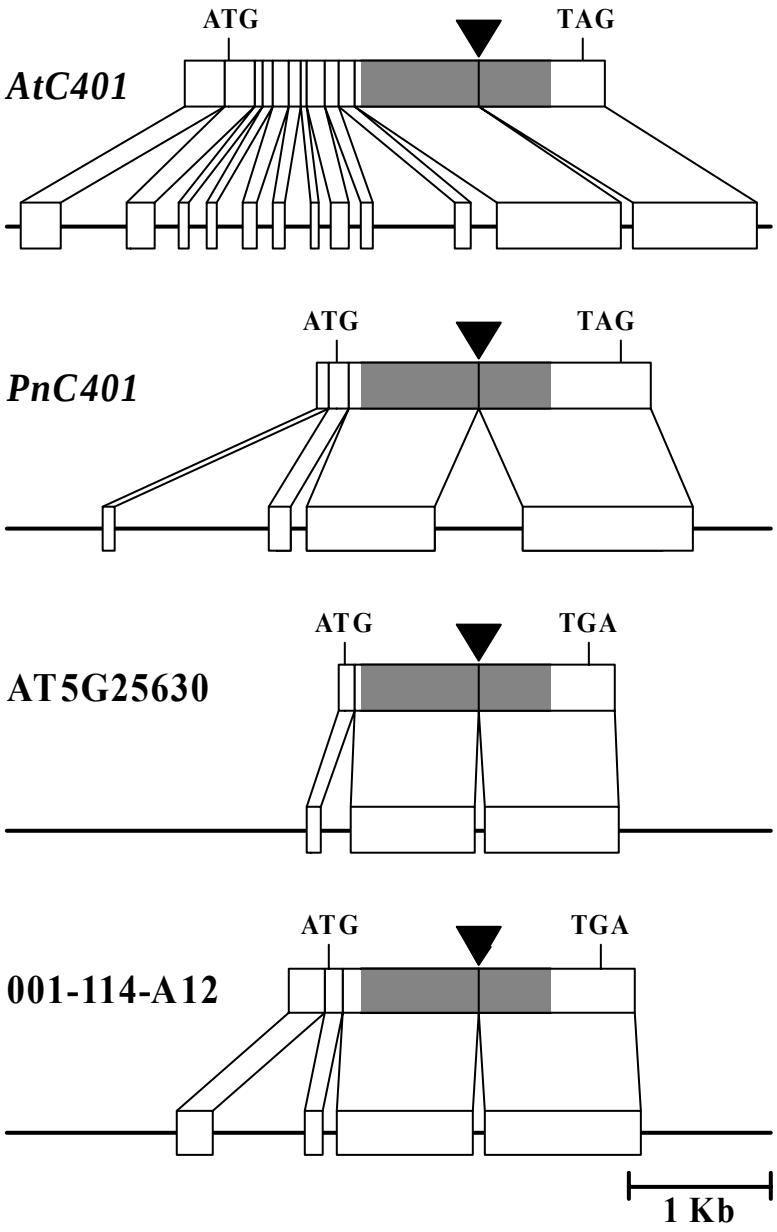

ATG
TAG
AtC401
ATG
TAG
PnC401
ATG
TGA
AT5G25630
ATG
TGA
001-114-A12
1 Kb

Figure 4. Mapping of the transcriptional initiation site of *AtC401* gene. Primer extension analysis was performed using total RNA from the whole plants harvested at CT0 with a primer described at Fig. 5. Four lanes at the left side show size marker produced by sequence analysis reaction using the same primer and the genomic clone of *AtC401* gene. Primer extension lane (indicated PE) shows a major product by arrowhead.

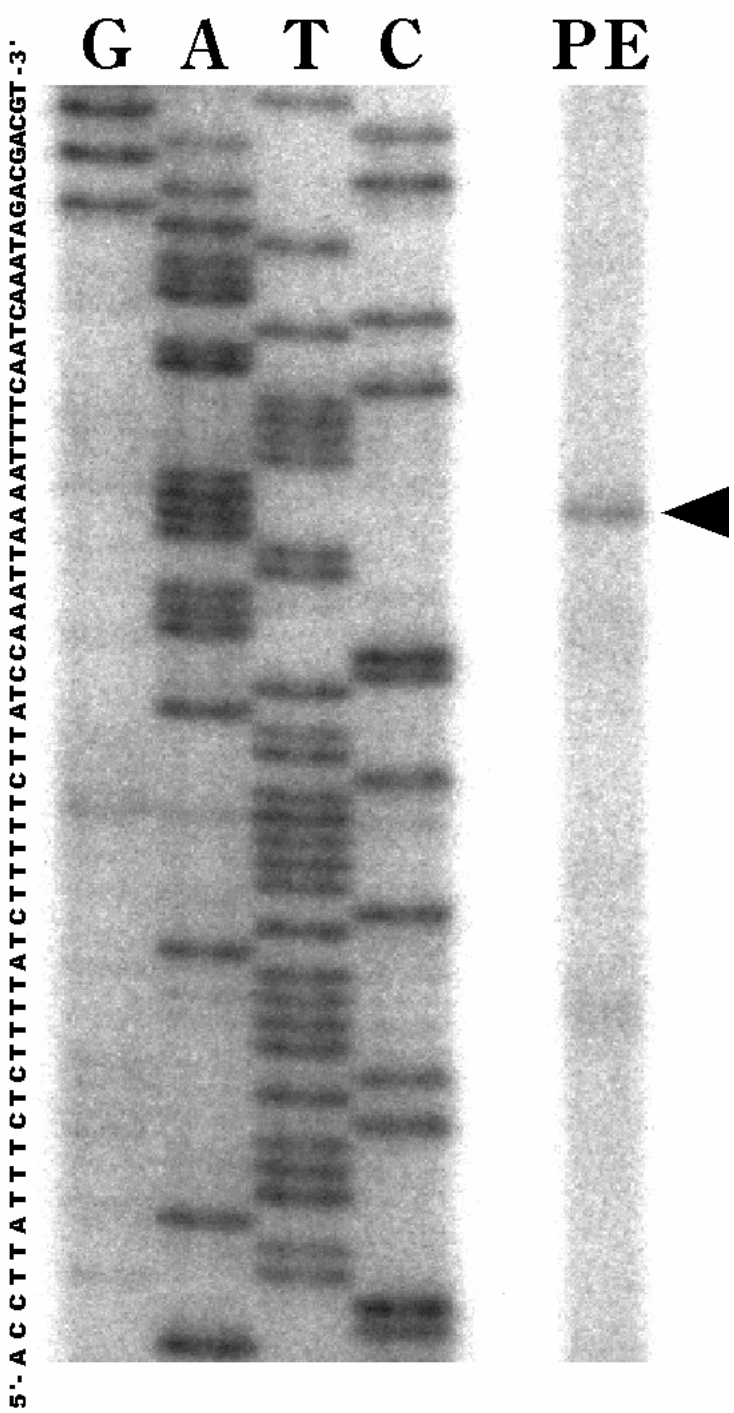

G A T C
PE
5'- A C C T T A T T T C T C T T T T T A T C C A A A T T A A A A T T T T C A A T C A A A T A G A C G A C G T -3'

Figure 5. Nucleotide sequence and schematic representation of the 5' flanking region of the *AtC401* gene. The sequence is numbered with the first nucleotide of the transcription initiation site as +1. The transcriptional initiation site is shown with black arrowhead and TA-rich sequence is in about 35 bp upstream (indicated by dots). The open arrow and arrowhead show the primer for the primer extension and 5'-end of the 5' RACE product, respectively. A motif similar to CCA-binding (CCA1b-like), a CIRCADIANLELHC motif and an initiator consensus are boxed. Arrows indicate tandem repeats of GATA and GATA-related motifs at the transcriptional region. Exons of *AtC401* gene are indicated by underline and the coding region is italicized. Exons of AT5G21170 gene are indicated by dashed underline and the stop codon for translation is indicated by asterisk.


CIRCADIANLELHC
-897 CAAGGTGATTGTGGACGGTGAATCCAAATACATCCCAGATTTACCCTTTGTAGCAGACGAAGTTGGCAATGTCTGTAACA
-817 TTCTCGATGTTCATGTAAGTTACTTTCCCACCTCTTTTTTTCTCATGTTAAATCTATCAATCAGCTTAACAGTATCTGCA
-737 ATCATTATGCATGCAGAACTTTGTGCCAGAAAACCCAGAAAGCATAGTGGAGTTTGAGGCGCCACCATCACCTGATCATA
-657 GCTACGGTCAAACCCTACCAGCCGCAGAAGATTACGCGAAAGAGCCACTGGCGGTCCACCTCAGCTTCATCTAACACTTC
-577 TTGGCACTACTGAAGAGACAGCCATAGCCACAAAGCCTCAACATGTGGTGCTTAACCATGTGTTCATAGAGCAAGGATGG
-497 ACTCCTCAATCCATAGTAGCTTTGGGTTTAACCCACAGGTTCGAGTCTAAGTACATAACTGTTGTCCTCTACAAACCGCT
-417 CACACGGTAACCCTAAAAATTCAGTGCCACATTGTTCTCTCTCTTTCTTTCTTTTTCATCTCTCAATTATGTGAAAACAT
-337 GTTCTTTGTTCATAGTGACTAGTGAGTGCCCAACATTTGAGTTTATTAACCTCTTTCTTACCTGGTTTGTGGTGCTTGTG
-257 TACAAAGCTTCTCTCATTGTAACGATTGTTGTTTTATTTTCTGCAAAAACTGTAAAATACATTCGCTAATAATGGCTATA
CIRCADIANLELHC
-177 ACAAGCTTATCTGCTAGCAACCAAACACATAAATCCTGTTTTTTTTCTTCTTCTGACAAAACGTTTGCGAAAGTGACAAA
CCA1b-like
-97 TTTTTGAAAATAATCTTTCTTTTTGGTATTGGAAAAGGCTAACCAGTTTGAAAGTGTACTCATAATAAATCCACGTCGTC
+1
-17 TATTTGATTGAAAATTTTAATTTGGATAAGAAAAAGATAAAAGAGAAATAAGGTGAGAAAAAGTGAGCCACACGATGGAG
64 TATGGCGGAAGAAGCATCGACCGAGTAATTGAGACGCTCTGTTTCCAATTACTACTCTCTCGAGCTCCTCCGCTCTGCAT
144 TGTCGCCGAGAGTTCCTTTCTTGTCCGACAAACTACTTCTTCTCCTTCGATAAGCCAATTCTGCGCCATAGATTTTTGAA
224 ATCTACTGCCGGAAAGGCTGCATGCGATTTCAACTCCCTTTTCCCGGGACAATTCGCAGGTGTGTATTCCTGAAGCTCCG
304 TTGTACTATACTACTATTCCATGTTTCACTTCGAACGAAGAATCAATCATTCGGTAAATTGGATAATTTGGTCACTTGTT
384 CATGACCTGTACATAATCAAATGTGTAGGAGGACGATGATGAATTTGTAGCATGAACTATCGACGTCATGATTCTAGAAA
464 TTTATGTTGGAATTAAAGGGCCCAATGAGAAACATACCCAACAATTTATGCTGTTGCTGAAAAGCTTTGCCATCGCATTG
544 TGGATTTCAGCTGTTTTTGCCTTGTCTTTATCACATTTGACGCCATAGTACTATTTGTGTGTATTCTCGTATTTTACAGT
624 TTCTTTGTCCCTTTTGCATTAGTTTCTGTGAATTGAGAAAAGAAACACTCTGTTGCCTGCACTGTTGGGTTCTTTGGAGA
704 TAGAATTCTAACATTGACATTACATTTTCATGAAACAGCTTGTGAAGAAGATCAAGAGGTGAACTCTGAGTGATGAGTGA
784 GCCAAAGGTCAGGCGTTGGGTTGGCAAATATGAGGTTGGAAGATTGATCGGTGAATGTAATTTTGGGAAACTGCGGAGT

Figure 6. Free-running rhythm of bioluminescence of *Arabidopsis* seedlings transformed with the reporter construct. A: Scheme of the reporter constructs used for the transformation. The box and circle indicate CIRCADIANLELHC motif and CCA1-binding like motif, respectively. The arrowheads indicate the transcriptional initiation site of *AtC401* gene. The numbers indicate the position referred to the transcriptional initiation site of *AtC401* gene. The *Arabidopsis* genomic DNA fragments were transcriptionally fused to the firefly *luciferase* (*luc*+) (Promega) reporter gene. Each construct was transformed into the Col-0 ecotype of *Arabidopsis* by floral-dip method, and T2 seedlings were used for the experiment. Transgenic seedlings were entrained for 5 days in 12 h light/12 h dark cycles before being released and imaging in continuous light. B, C and D show the observed bioluminescence of the transgenic seedling containing the d1-luc+, d2-luc+ and d3-luc+, respectively. The different colored plots show the bioluminescence of genetically independent transgenic lines. Plots represent the average data from 24 individual seedlings.

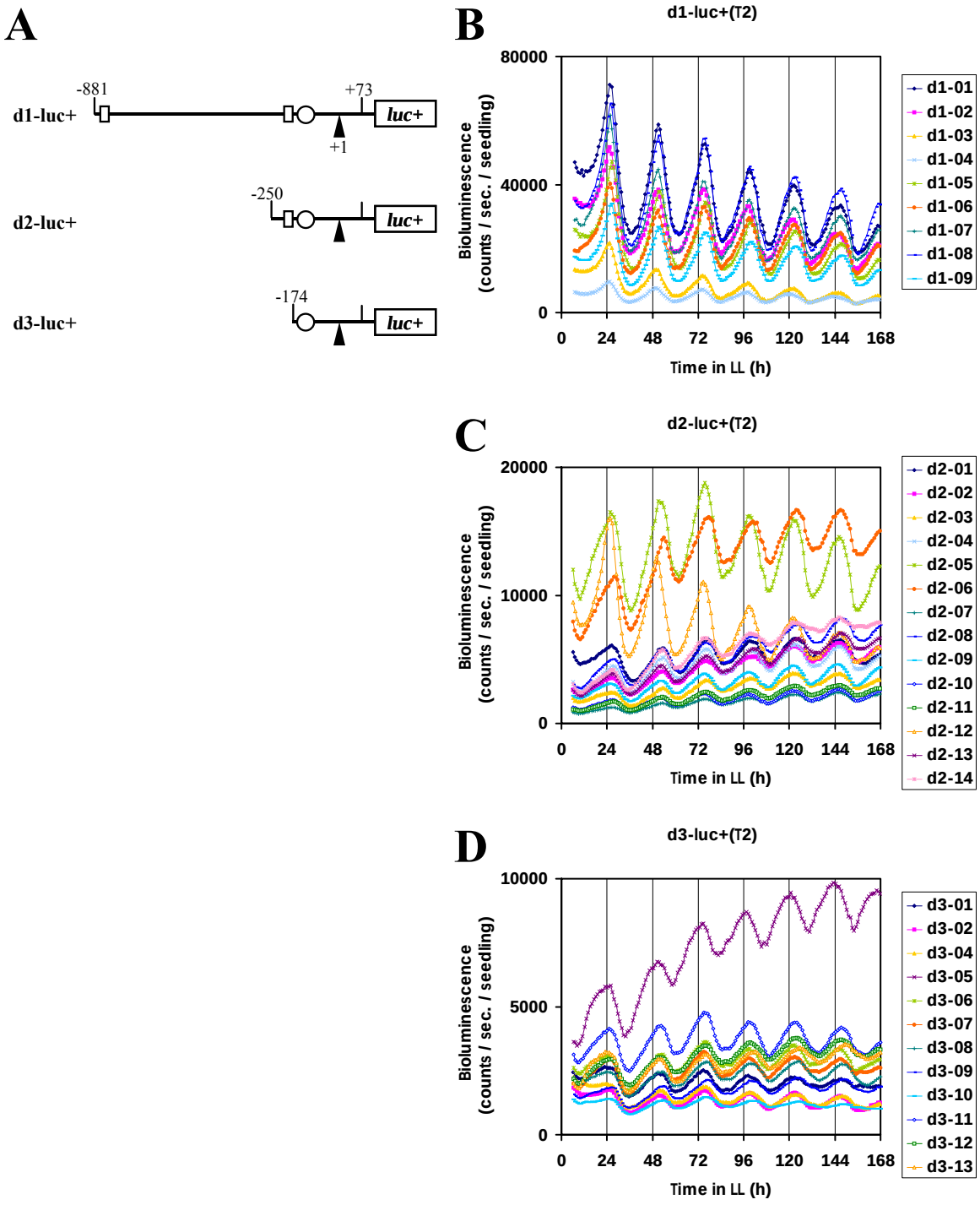

A
-881
+73
d1-luc+
luc+
+1
-250
d2-luc+
luc+
-174
d3-luc+
luc+
B
d1-luc+(T2)
Bioluminescence (counts / sec. / seedling)
80000
40000
0
0 24 48 72 96 120 144 168
Time in LL (h)
d1-01
d1-02
d1-03
d1-04
d1-05
d1-06
d1-07
d1-08
d1-09
C
d2-luc+(T2)
Bioluminescence (counts / sec. / seedling)
20000
10000
0
0 24 48 72 96 120 144 168
Time in LL (h)
d2-01
d2-02
d2-03
d2-04
d2-05
d2-06
d2-07
d2-08
d2-09
d2-10
d2-11
d2-12
d2-13
d2-14
D
d3-luc+(T2)
Bioluminescence (counts / sec. / seedling)
10000
5000
0
0 24 48 72 96 120 144 168
Time in LL (h)
d3-01
d3-02
d3-04
d3-05
d3-06
d3-07
d3-08
d3-09
d3-10
d3-11
d3-12
d3-13

Figure 7. The mRNA levels of the reporter *luciferase* gene. Seedlings were grown under 12 h light / 12 h dark cycles at 22° C, and subjected to the photoperiodic treatment: A: 12 h light/ 12 h dark cycle; B: continuous light condition. The aerial part of ten seedlings were harvested every 4 h during photoperiodic treatments and used for extraction of RNA. Total RNA (10 μg per lane) was fractionated by gel electrophoresis and allowed to hybridization to partial fragment of *luciferase* (*luc*+) or *AtC401* cDNA. The lower columns of each panel shows UV image of rRNA stained by SYBR® Gold (Molecular Probes).

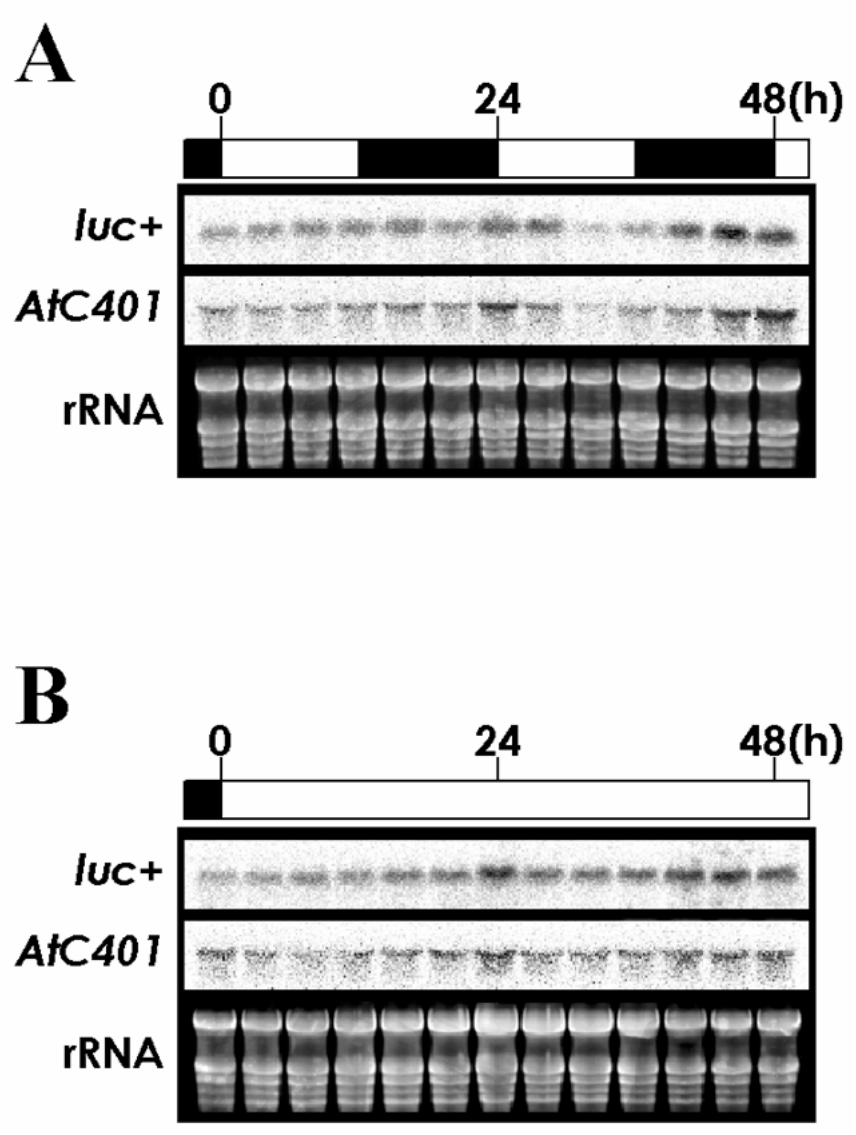

A
0
24
48(h)
luc+
AtC401
rRNA
B
0
24
48(h)
luc+
AtC401
rRNA

Figure 8. Luminescence image of d3-luc+ transgenic plants. Transgenic plants were cultured in 12 h/12 h light/dark cycles for 5 days (A, B), 2 weeks (C, D) or 6 weeks (E, F). Photon counting was performed at 12 h after transferred to continuous light condition. The luminescence images represent bioluminescence for 20 min. with pseudocolor, indicated below (A, C and E). B, D and F were bright images corresponding with A, C and E, respectively. Arrowheads in F indicate the youngest rosette leaves. Bars indicates 1 cm.

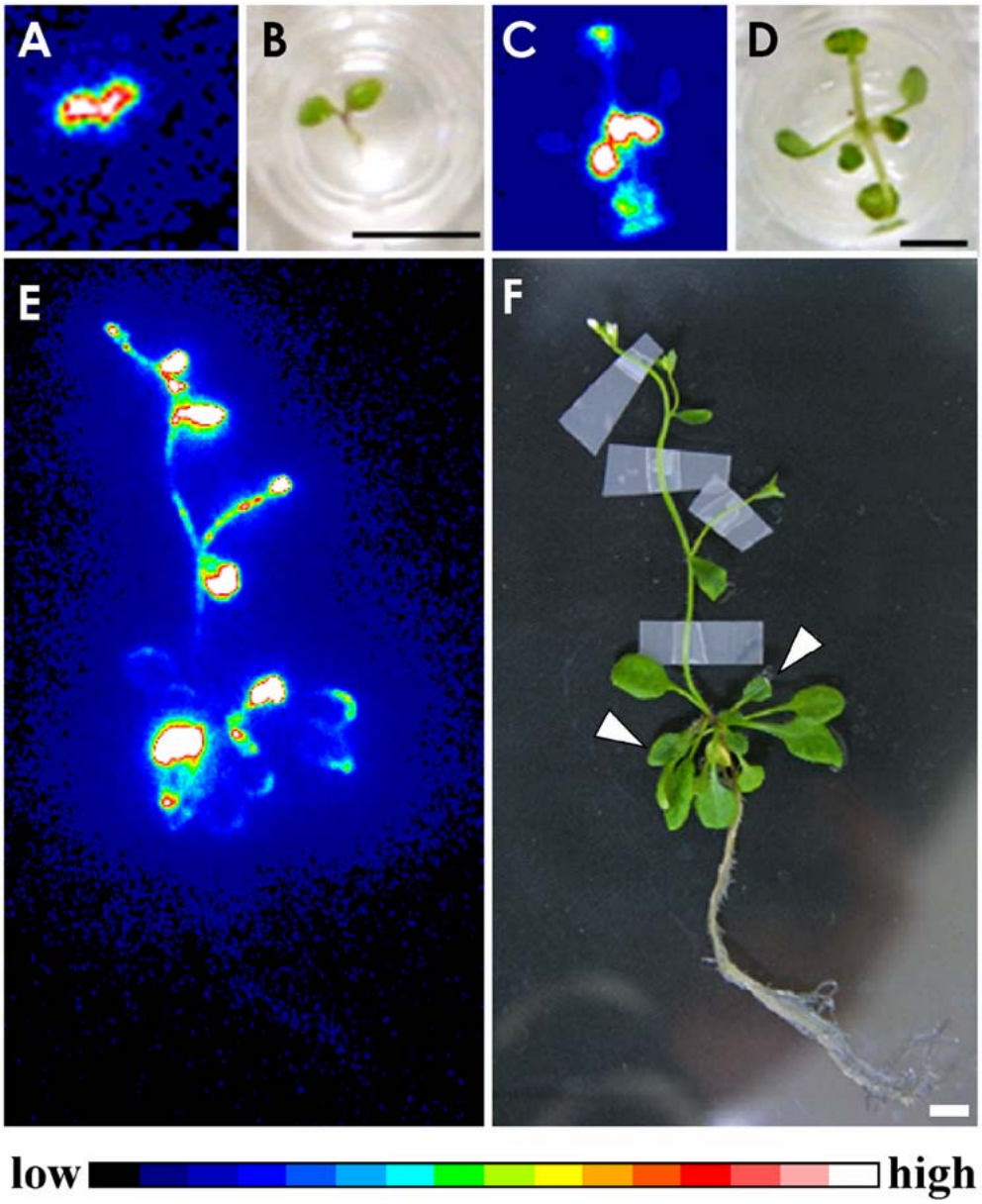
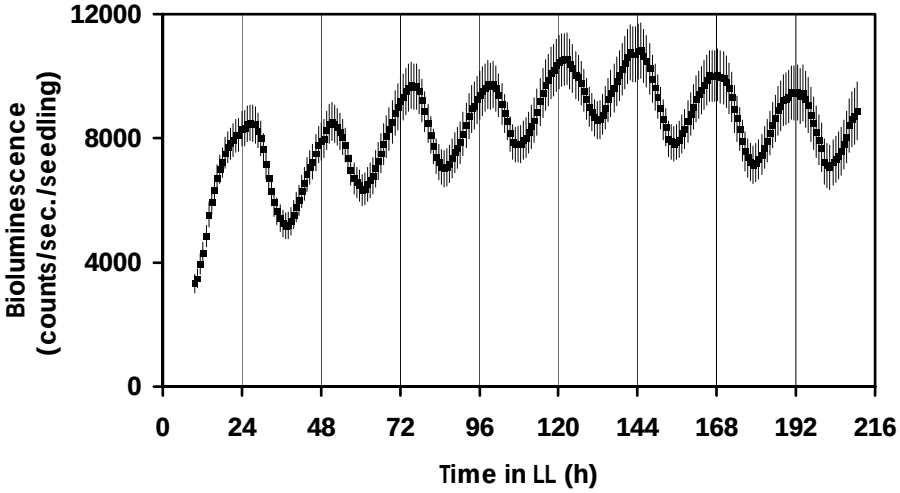

A
B
C
D
E
F
low
high

Figure 9. Free-running bioluminescence oscillation of homozygotic d3-luc+ transgenic seedlings. The homozygotic transgenic seedlings containing the d3-luc+ were used for the experiment in A. Transgenic seedlings were entrained for 5 days in 12 h light/12 h dark cycles before being released and imaging in continuous light. Plots represent the average data from 96 individual seedlings. The bioluminescence of the CAB2-luc+ transgenic seedlings is shown in B. Error bars indicate the standard errors.

A


d3-luc+ (d3-5-1)
12000
8000
4000
0
Bioluminescence (counts/sec./seedling)
0
24
48
72
96
120
144
168
192
216
Time in LL (h)

B

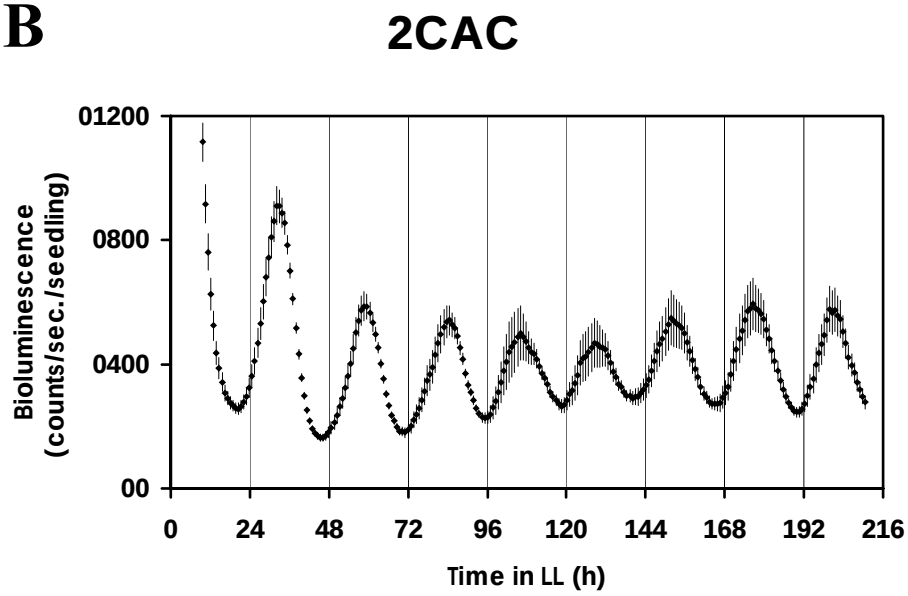

2CAC
01200
0800
0400
00
Bioluminescence (counts/sec./seedling)
0
24
48
72
96
120
144
168
192
216
Time in LL (h)

Figure 10. Effects of sucrose on free-running rhythm of *AtC401* expression in continuous dark (DD). Transgenic plants containing d3-luc+ (A) or CAB2-luc+ (B) were sown on an agar plate containing half-strength Murashige-Skoog's medium containing 0.8% (w/v) agar, with 3%, 1%, or no sucrose (indicated by filled circle, open circle, and cross, respectively). The CAB2-luc+ transgenic line (C24 background) was kindly provided by Dr. S.A. Kay (The Scripps Research Institute, La Jolla, CA, USA). After five days of entrainment to 12 h light/ 12 h dark cycles at 22° C, seedlings were transferred to DD. Bioluminescence was counted at equal intervals using a TopCount NXT (Perkin-Elmer Biosystems, Foster City, CA, USA). Each plot is the mean value of the bioluminescence of 45-48 seedlings.

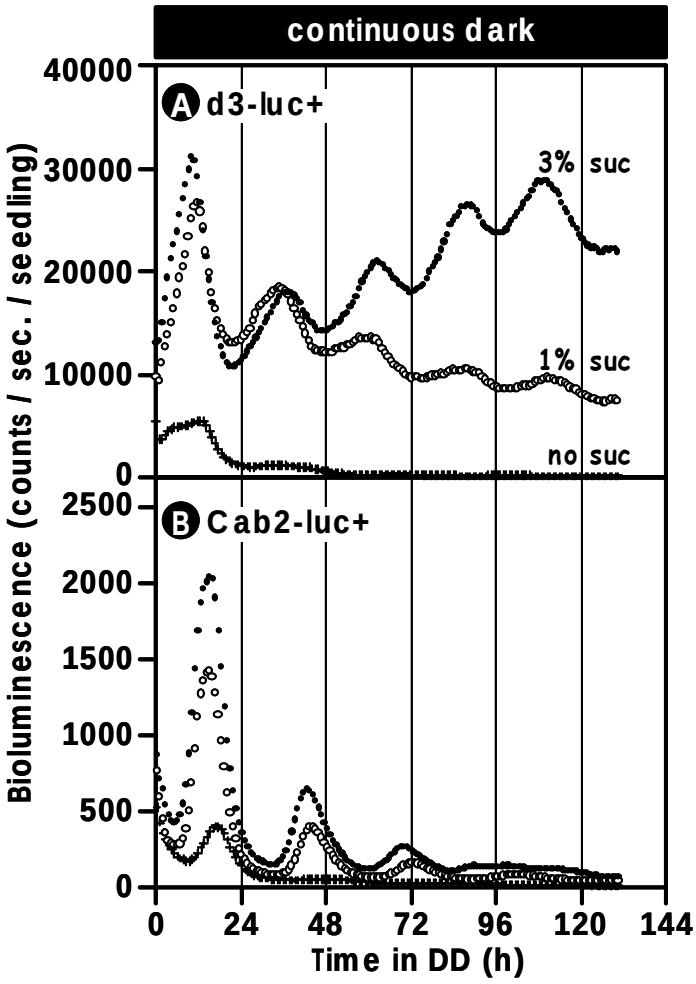

continuous dark
A d3-luc+
3% suc
1% suc
no suc
B Cab2-luc+
Bioluminescence (counts / sec. / seedling)
Time in DD (h)
40000
30000
20000
10000
0
2500
2000
1500
1000
500
0
0
24
48
72
96
120
144

Figure 11. Effects of sucrose on free-running rhythm of *AtC401* expression in continuous light (LL). Transgenic plants containing d3-luc+ (A) or CAB2-luc+ (B) were sown on medium with 3%, 1% or no sucrose (indicated by filled circle, open circle, and cross, respectively). After five days of entrainment to 12 h light/ 12 h dark cycles at 22° C, seedlings were transferred to LL. Bioluminescence was counted at equal intervals using a TopCount NXT. Each plot is the mean value of the bioluminescence of 45-48 seedlings.

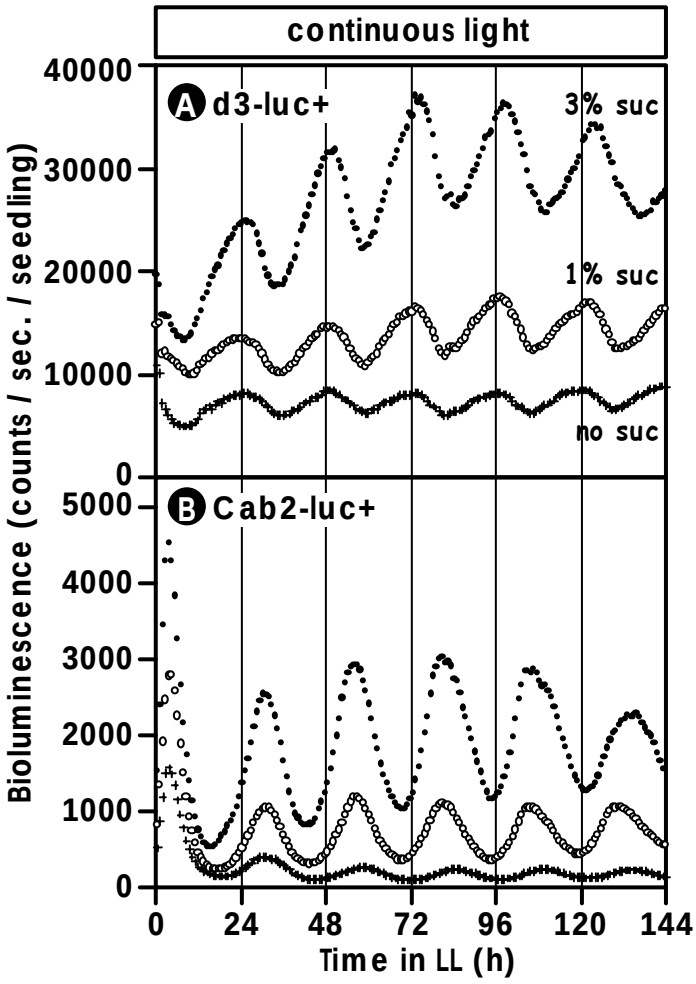

continuous light
A d3-luc+
3% suc
1% suc
no suc
B Cab2-luc+
Bioluminescence (counts / sec. / seedling)
Time in LL (h)
0
10000
20000
30000
40000
1000
2000
3000
4000
5000
24
48
72
96
120
144

Figure 12. Effects of sucrose on period of *AtC401* expression. Periods of d3-luc+ (A and C) and CAB2-luc+ (B and D) free-running expression in LL are plotted against the Rel-Amp values. The medium contained no sucrose (A and B) or 3% sucrose (C-E). Periods of the d3-luc+ free-running expression in DD are shown in (E). Each plot indicates the period of an individual seedling. Periods were estimated using the fast Fourier transform-nonlinear least square method (FFT-NLLS) (Plautz *et al.*, 1997).

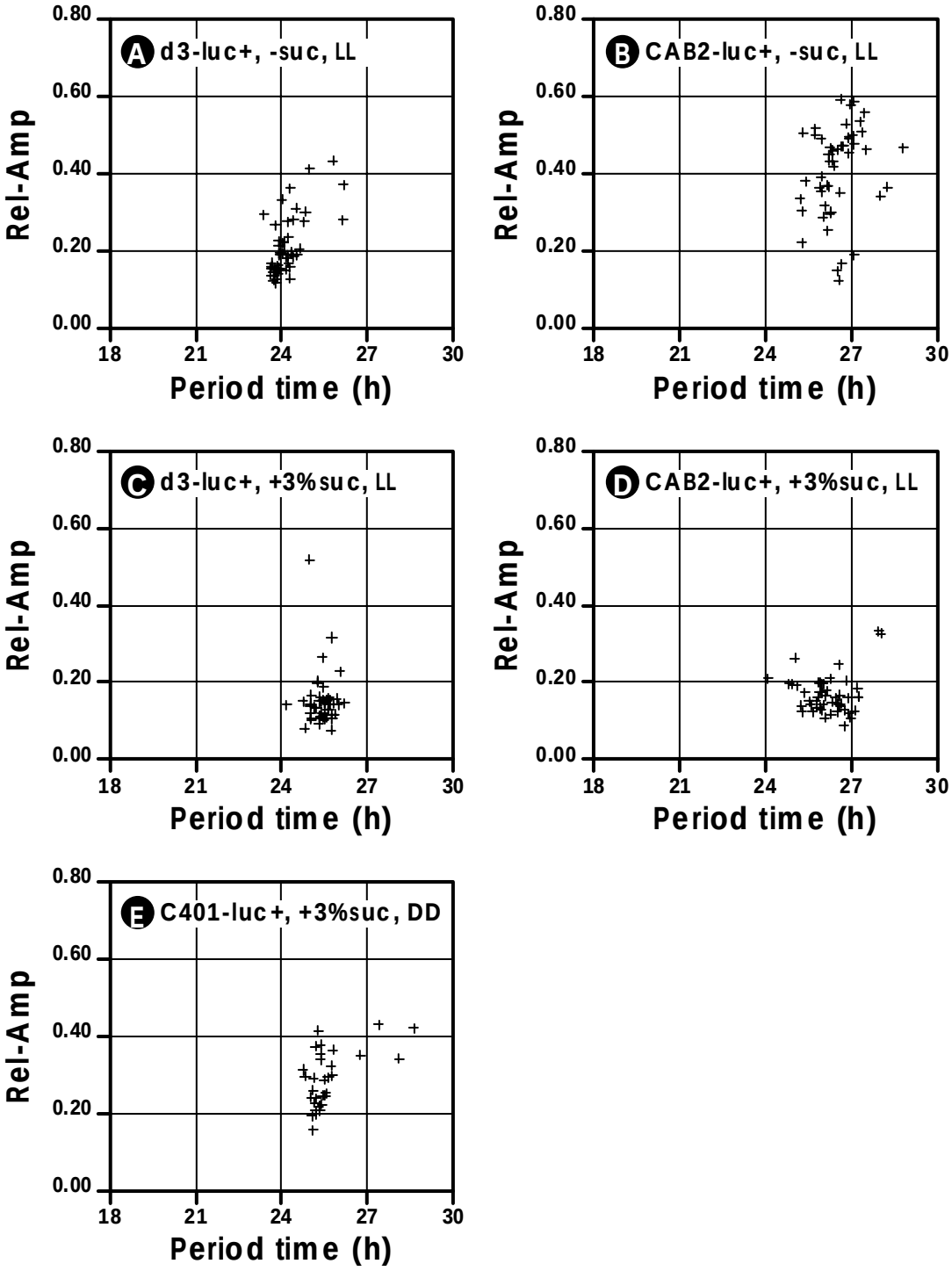

A d3-luc+, -suc, LL
B CAB2-luc+, -suc, LL
C d3-luc+, +3%suc, LL
D CAB2-luc+, +3%suc, LL
E C401-luc+, +3%suc, DD
Rel-Amp
Period time (h)
0.00
0.20
0.40
0.60
0.80
18
21
24
27
30

Figure 13. Effects of sucrose on *AtC401* mRNA expression. *Arabidopsis* plants were sown on the agar plate containing half-strength Murashige-Skoog's medium containing 0.8% (w/v) agar, with 3% (B, D and F) or no sucrose (A, C and D). Seedlings were grown under 12 h light / 12 h dark cycles at 22° C, and subjected to various photoperiodic treatment: (A and B) 12 h light/ 12 h dark cycle; (C and D) continuous light condition; (E and F) continuous dark condition. The aerial part of ten seedlings were harvested every 4 h during photoperiodic treatments and used for extraction of RNA. Total RNA (10 μg per lane) was fractionated by gel electrophoresis and allowed to hybridization to partial fragment of *AtC401* cDNA. The lower columns of each panel were shown UV image of rRNA stained by SYBR® Gold (Molecular Probes).

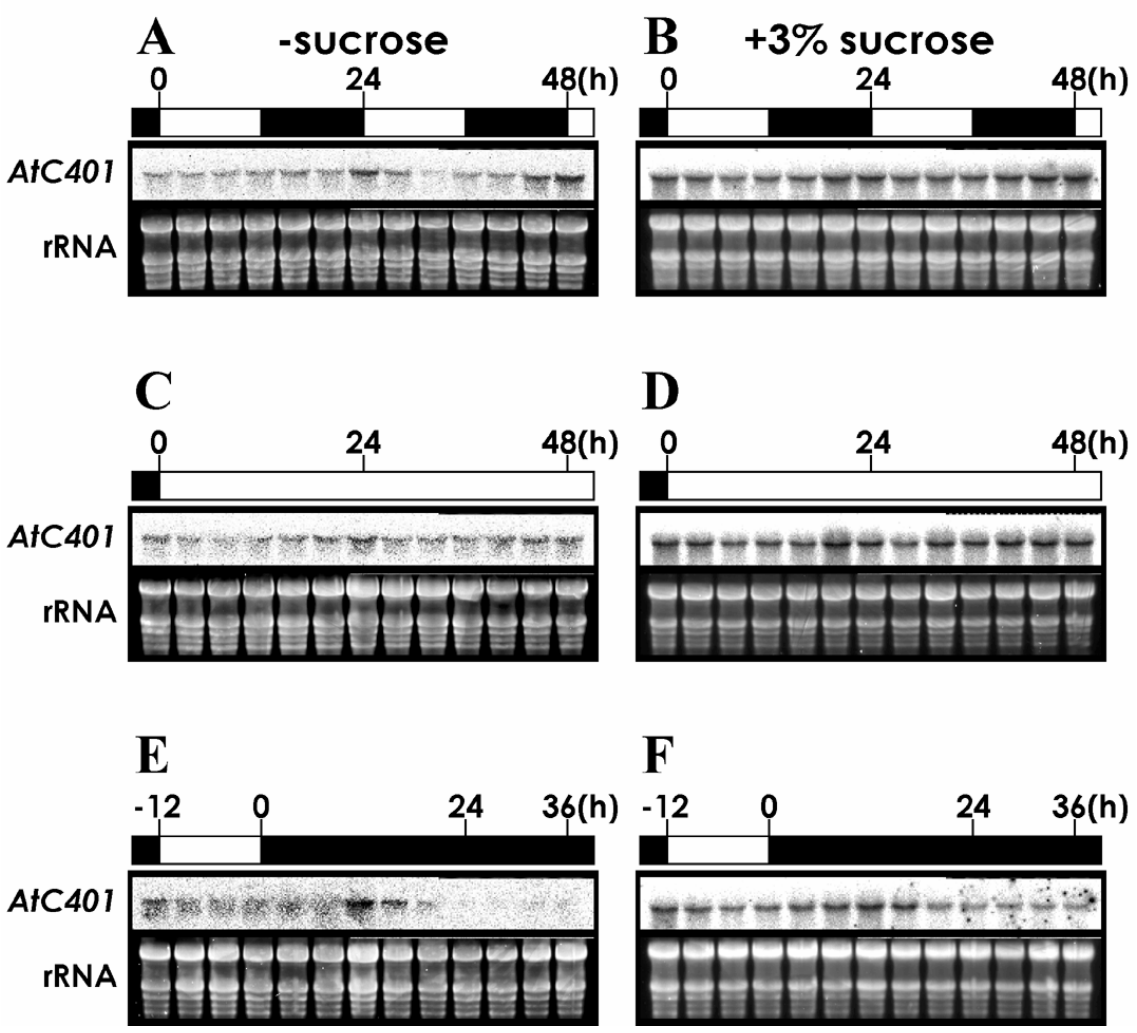

A
-sucrose
0
24
48(h)
AtC401
rRNA
B
+3% sucrose
0
24
48(h)
C
0
24
48(h)
AtC401
rRNA
D
0
24
48(h)
E
-12
0
24
36(h)
AtC401
rRNA
F
-12
0
24
36(h)

Figure 14. Free-running rhythms of the reporter expression in DD under different temperature conditions. After five days of entrainment to 12 h light/ 12 h dark cycles at 22° C, seedlings were transferred to a temperature-controlled dark box at the indicated temperature. Bioluminescence of 24-48 seedlings was counted for 20 min. every 2 h using the AQUACOSMOS/VIM system. Each plot is the mean value of the bioluminescence of 36 seedlings at 12° C (A), 16° C (B), 22° C (C), 26° C (D), or 30° C (E). The Rel-Amp values were estimated using the FFT-NLLS. The estimated fit-curves are drawn in the panels.

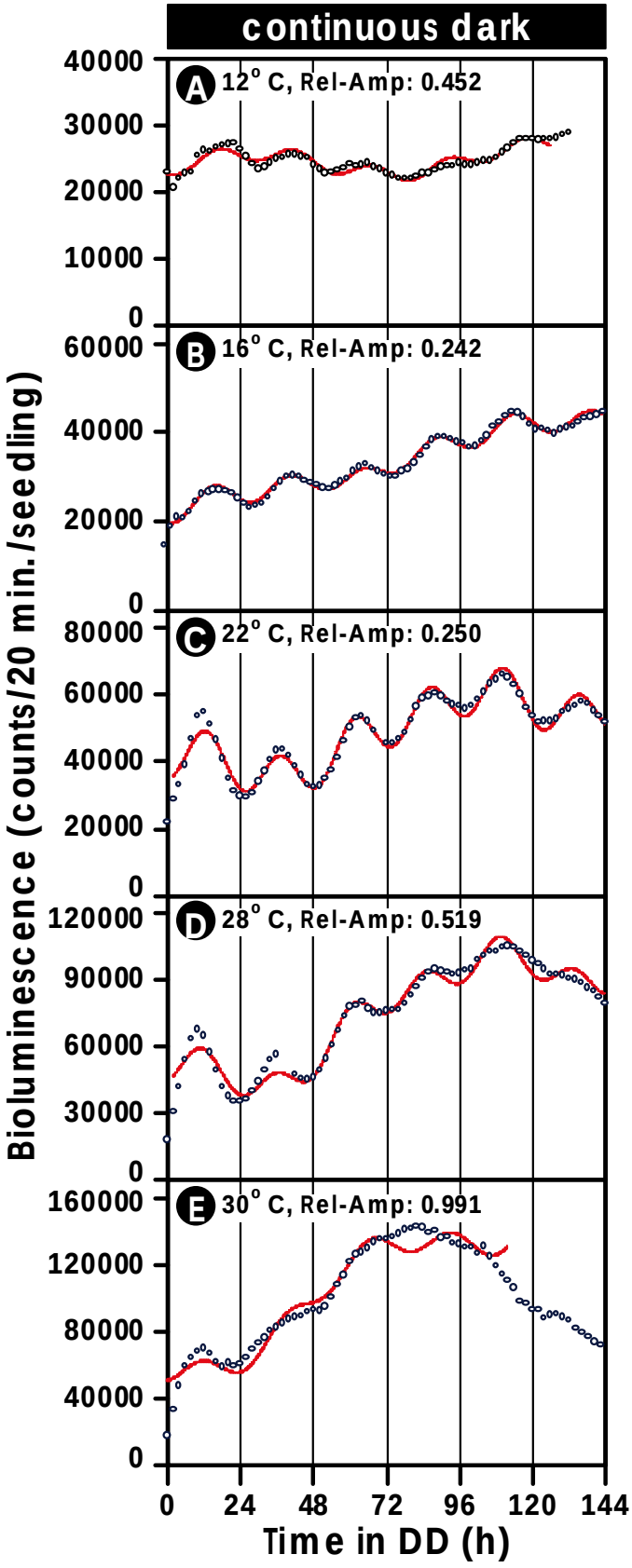

continuous dark
A 12° C, Rel-Amp: 0.452
B 16° C, Rel-Amp: 0.242
C 22° C, Rel-Amp: 0.250
D 28° C, Rel-Amp: 0.519
E 30° C, Rel-Amp: 0.991
Bioluminescence (counts/20 min./seedling)
Time in DD (h)
0
24
48
72
96
120
144

Figure 15. Estimated period of free-running rhythms of the reporter expression in DD under different temperature conditions. Period times of the free-running rhythms of the reporter expression shown in Fig. 14 were estimated using FFT-NLLS. Errors indicate 95% confidence intervals of the period estimated.

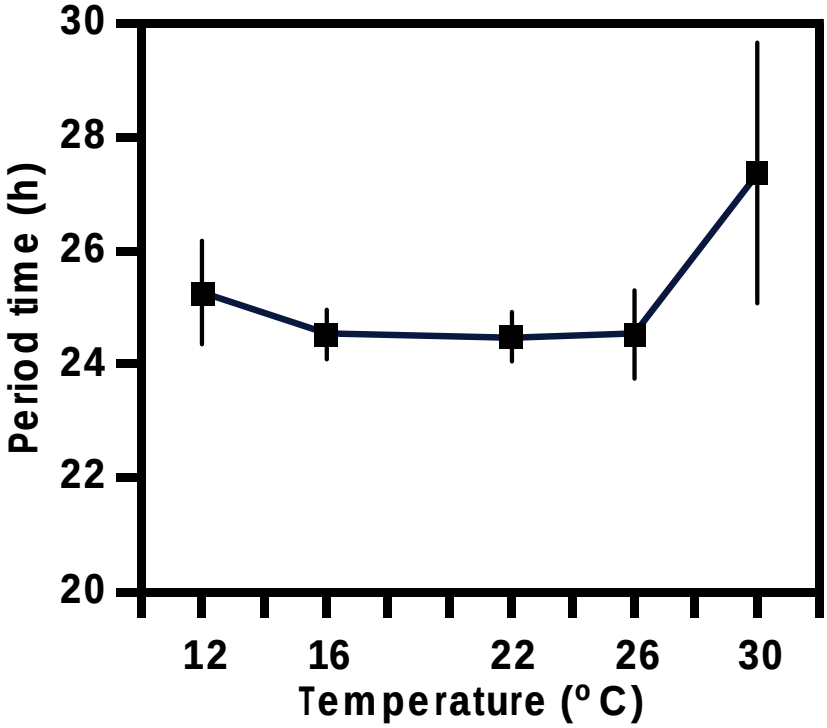

30
28
26
24
22
20
Period time (h)
12
16
22
26
30
Temperature (ºC)

Figure 16. Effect of non-circadian photoperiod on the entrainment of the reporter expression. (A-C) Before transfer to LL, transgenic plants were cultured for five days under a circadian photoperiod, 12 h light/12 h dark (A), an ultradian photoperiod, 8 h light/8 h dark (B), or an infradian photoperiod, 16 h light/16 h dark (C). The bioluminescence of d3-luc+ (filled circle) and CAB2-luc+ (open circle) are indicated. Bioluminescence was counted at equal intervals using a TopCount NXT. Each plot is the mean value of the bioluminescence of 36 seedlings. The media used were sucrose freee. (D) Estimated periods of the free-running rhythms. Periods were estimated from individual seedlings using the FFT-NLLS. The mean values of estimated periods are shown with standard deviations.

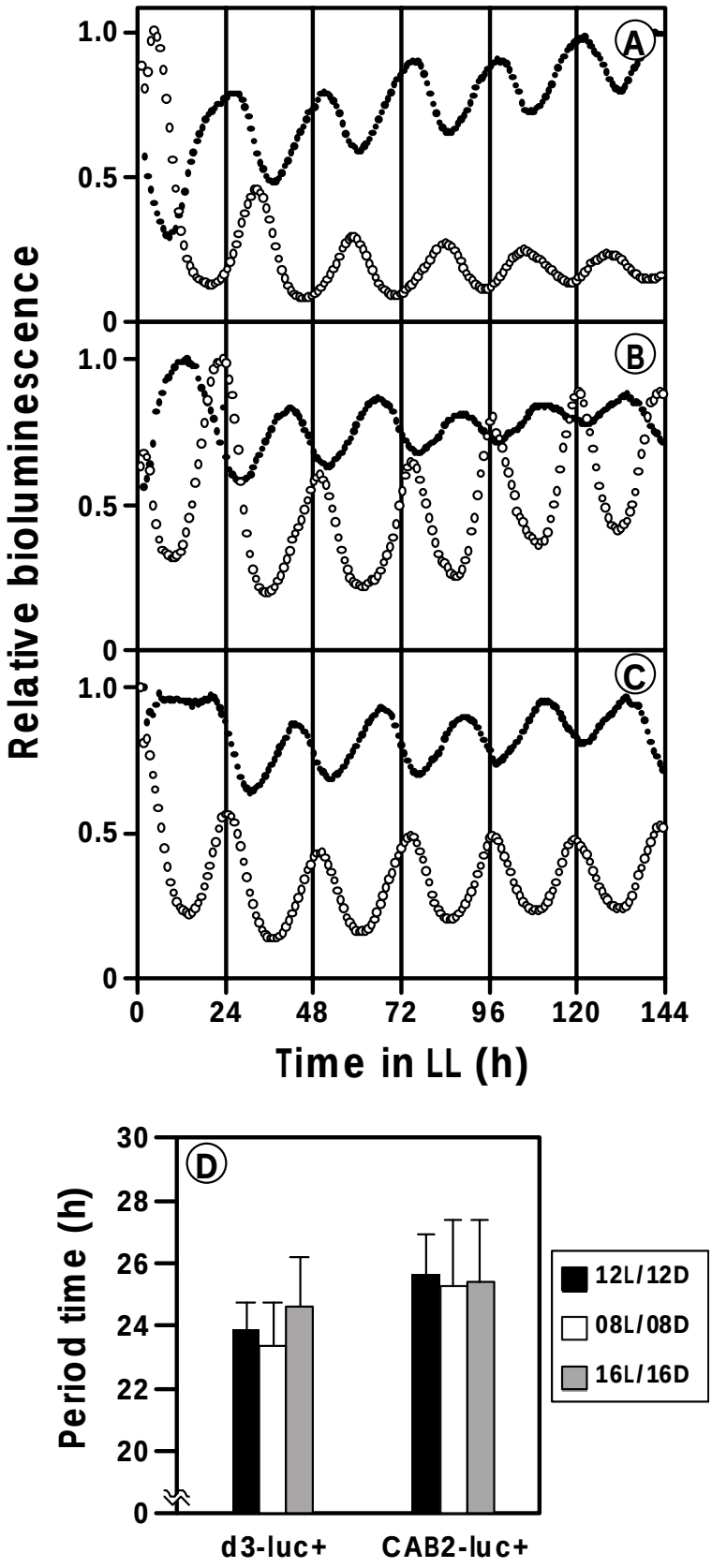

Relative bioluminescence
1.0
0.5
0
A
B
C
0
24
48
72
96
120
144
Time in LL (h)
D
Period time (h)
30
28
26
24
22
20
0
d3-luc+
CAB2-luc+
12L/12D
08L/08D
16L/16D

Figure 17. Phase response of d3-luc+ expression to red light pulse. Seedlings were transferred to DD after five days of entrainment to 12 h light/ 12 h dark cycles at 22° C. Beginning after one full day in DD, individual plates of seedlings were exposed to 10-min red light pulses at 4-h intervals for 28 h. Phase shifts were calculated as described by Covington *et al.* (2001). Phase response curve of phase shifts of d3-luc+ expression (± pooled SE) elicited by light pulses plotted against the circadian times (CT) at which the light pulses were administered. Phase advances are plotted as positive values, and delays are plotted as negative values.

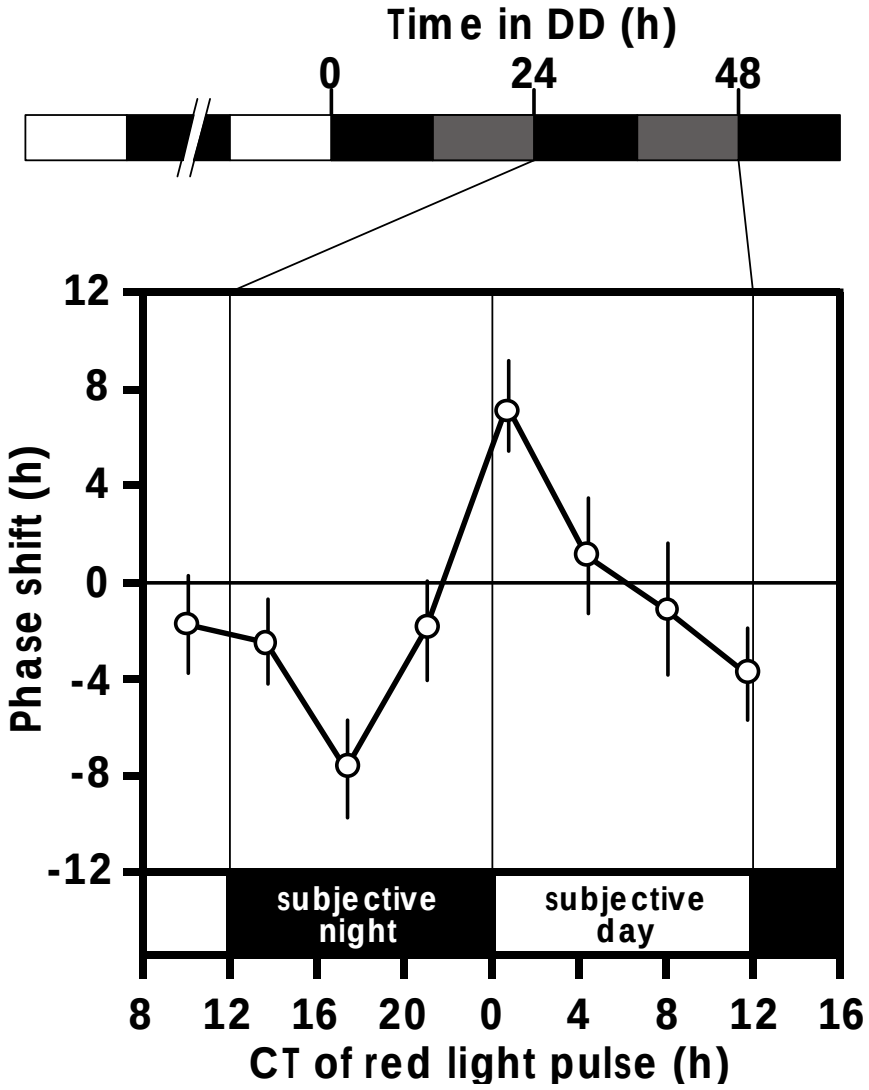

Time in DD (h)
0
24
48
12
8
4
0
-4
-8
-12
Phase shift (h)
subjective night
subjective day
8
12
16
20
0
4
8
12
16
CT of red light pulse (h)

Figure 18. Effect of CCA1-binding like motif on circadian transcription of *AtC401*. (A) Scheme of the reporter constructs used for transformation. The circle in d3-luc+ indicates the CCA1-binding like motif. *Arabidopsis* plants were transformed with the d3-luc+ or d4-luc+ reporter construct. The arrowheads indicate the transcriptional initiation site of *AtC401* gene. The numbers indicate the position referred to the transcriptional initiation site of *AtC401* gene. (B) A homozygotic line with the d4-luc+ construct (T3) was selected and used for the experiment. Transgenic seedlings were entrained for 5 days in 12 h/12 h light/dark cycles before being released and imaging in continuous light. Plots represent the average data from 96 individual seedlings. (C) The bioluminescence of homozygotic d3-luc+ transgenic seedlings. Error bars indicate the standard error.

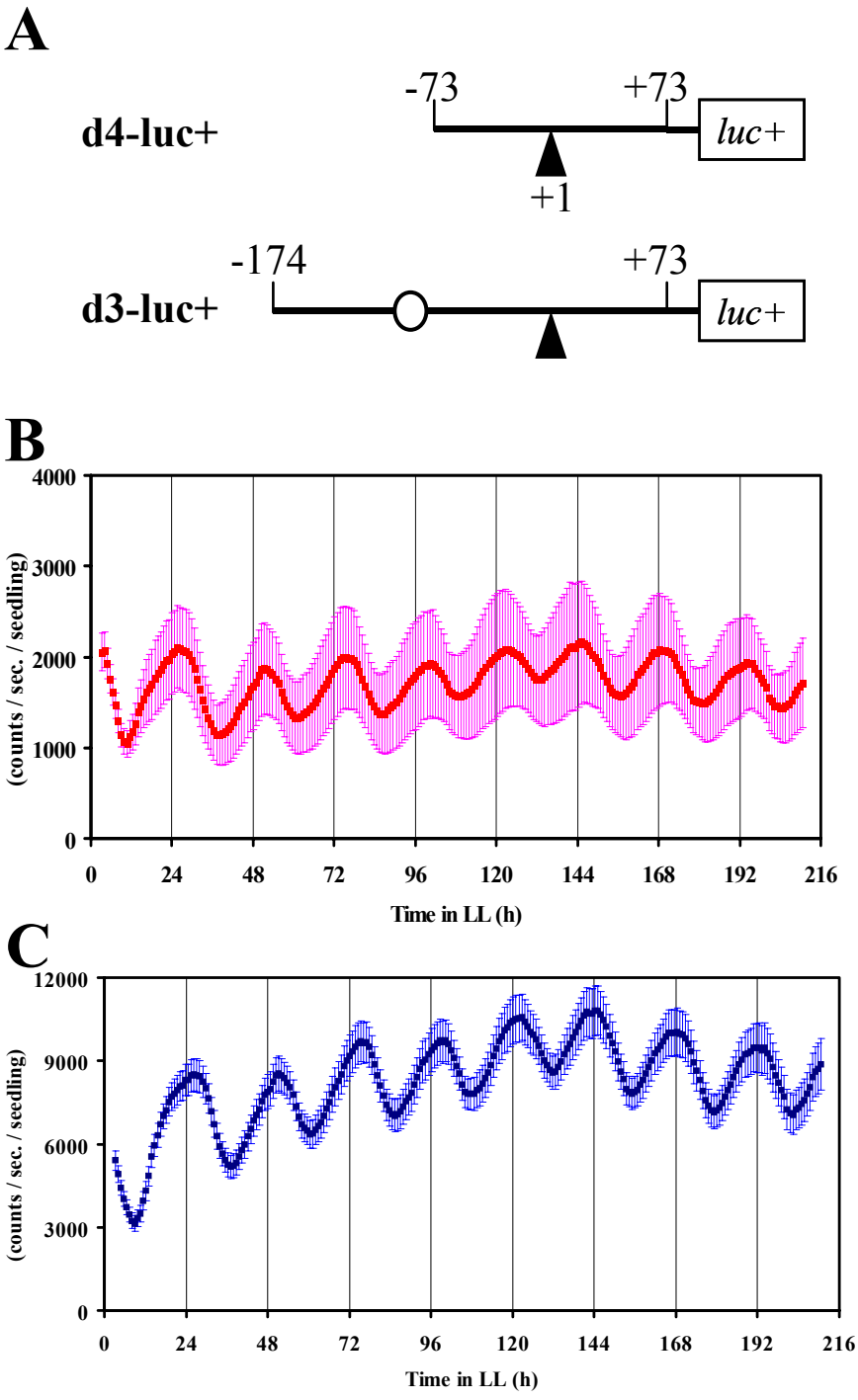
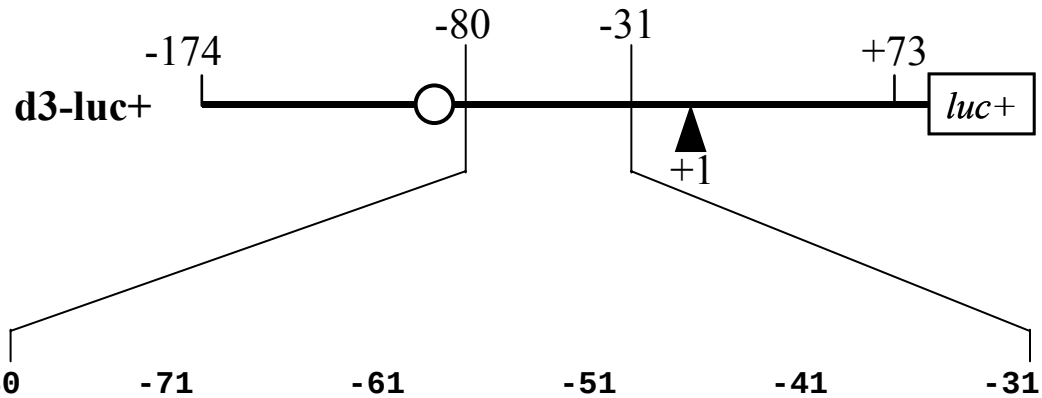

A
d4-luc+
-73
+73
luc+
+1
d3-luc+
-174
+73
luc+
B
Bioluminescence (counts / sec. / seedling)
4000
3000
2000
1000
0
0
24
48
72
96
120
144
168
192
216
Time in LL (h)
C
Bioluminescence (counts / sec. / seedling)
12000
9000
6000
3000
0
0
24
48
72
96
120
144
168
192
216
Time in LL (h)

Figure 19. Linker-scanning analysis of 5' upstream region of *AtC401*. (A) The scheme of the linker scan constructs. The circle indicates CCA1-binding like motif and the arrowhead indicates the transcriptional initiation site of *AtC401* gene. The numbers indicate the position referred to the transcriptional initiation site of *AtC401* gene. Every reporter constructs were transformed into *Arabidopsis* plants and T2 seedlings were used for experiment. Transgenic seedlings were entrained for 5 days in 12 h/12 h light/dark cycles before being released and imaging in continuous light. (B-G) The observed bioluminescence of the transgenic seedlings containing the linker-scanning construct, m1 to m6, respectiveely. The bioluminescence was plotted by relative value. The different colored plots show the bioluminescence of genetically independent transgenic lines. Plots represent the average data from 24 individual seedlings. (H) The observed bioluminescence of the homozygotic d3-luc+ transgenic seedlings.

A




```
  -80      -71        -61        -51        -41        -31
   CTTTTTGGTA TTGGAAAAGG CTAACCAGTT TGAAAGTGTA CTCATAATAA
m1 ggaattcc-- ---------- ---------- ---------- ----------
m2 --------gg aattcc---- ---------- ---------- ----------
m3 ---------- ------ggaa ttcc------ ---------- ----------
m4 ---------- ---------- ----ggaatt cc-------- ----------
m5 ---------- ---------- ---------- --ggaattcc ----------
m6 ---------- ---------- ---------- ---------- ggaattcc--
```

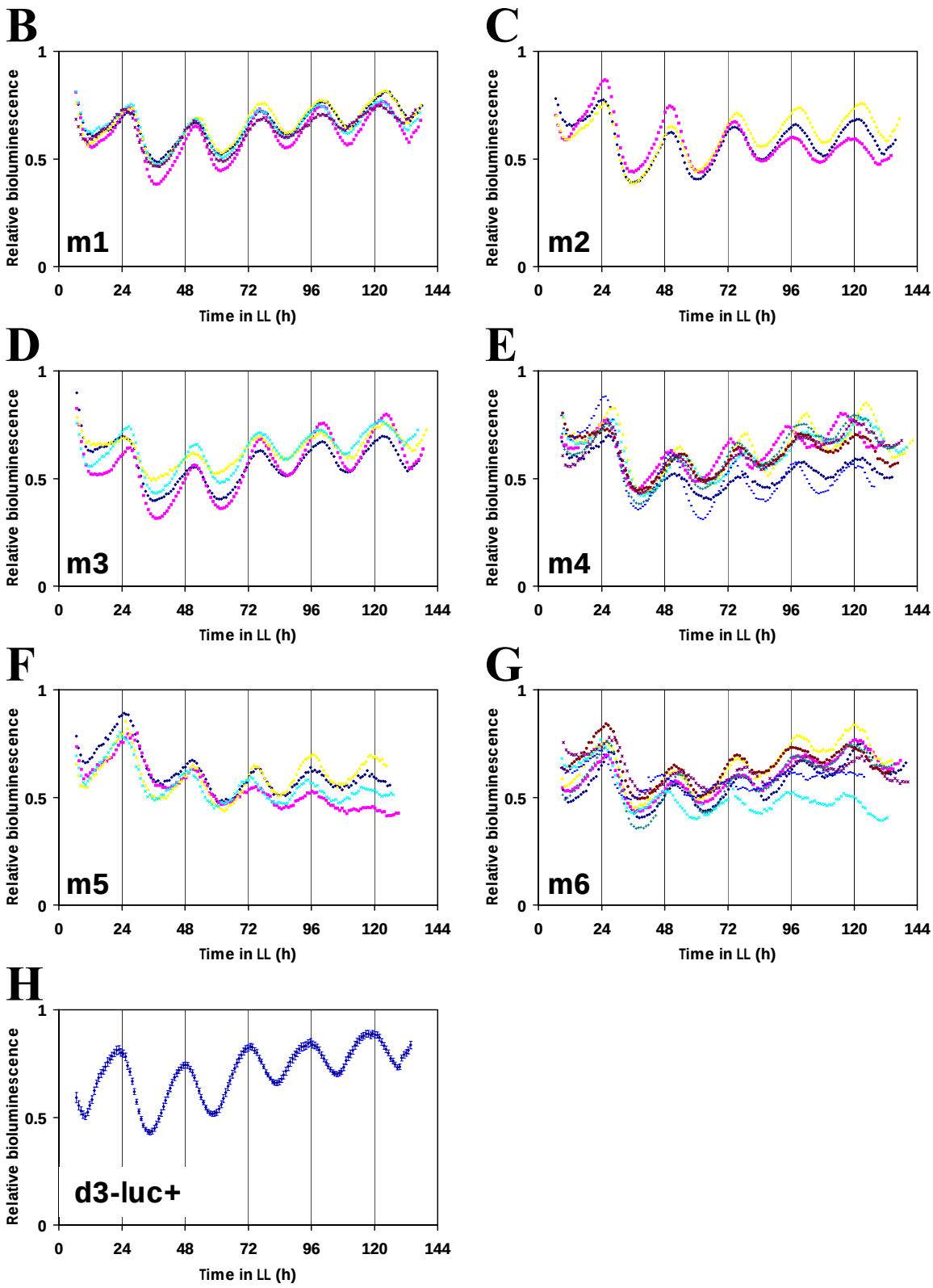
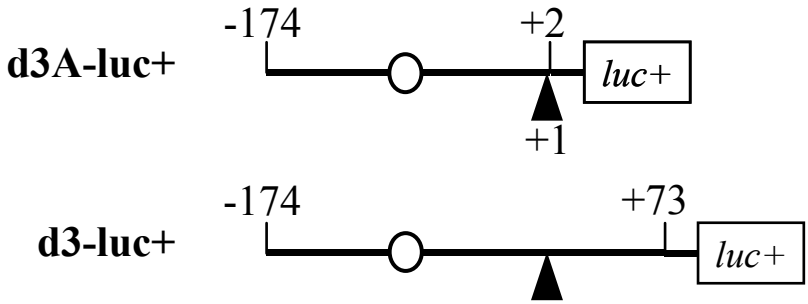
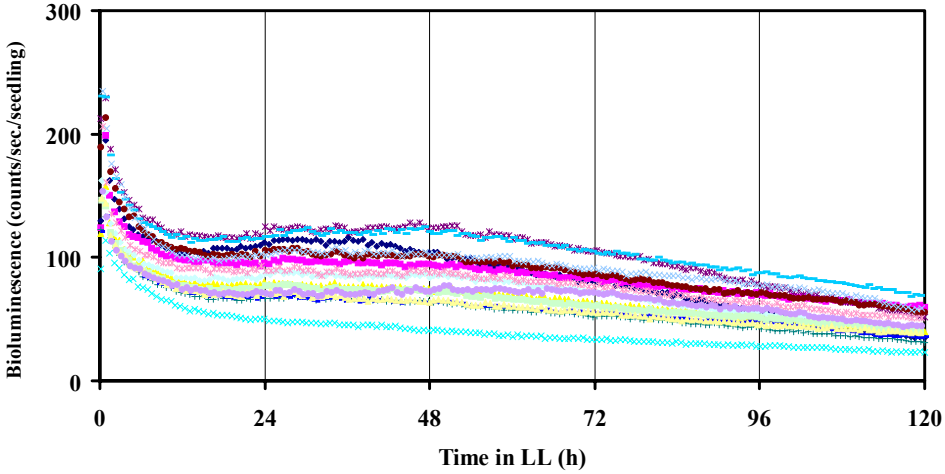

B
C
D
E
F
G
H
m1
m2
m3
m4
m5
m6
d3-luc+
Relative bioluminescence
Time in LL (h)
0
0.5
1
24
48
72
96
120
144

Figure 20. Effect of the deletion of 5' UTR on circadian transcription of *AtC401*. Scheme of the d3A-luc+ reporter construct used for transformation is shown in (A). The circle indicates CCA1-binding like motif and the arrowhead indicates the transcriptional initiation site of *AtC401* gene. The numbers indicate the position referred to the transcriptional initiation site of *AtC401* gene. d3A-luc+ construct was transformed into *Arabidopsis* plants, and T2 seedlings were used for experiment. Transgenic seedlings were entrained for 5 days in 12 h/12 h light/dark cycles before being released and imaging in continuous light. The observed bioluminescence of the d3A-luc+ transgenic plants is shown in (B). The different colored plots show the bioluminescence of genetically independent transgenic lines. Plots represent the average data from 24 individual seedlings. The bioluminescence of homozygotic d3-luc+ transgenic seedlings is indicated in (C). Error bars indicate the standard error.

A


d3A-luc+
-174
+2
luc+
+1
d3-luc+
-174
+73
luc+

B


Bioluminescence (counts/sec./seedling)
300
200
100
0
0
24
48
72
96
120
Time in LL (h)

C

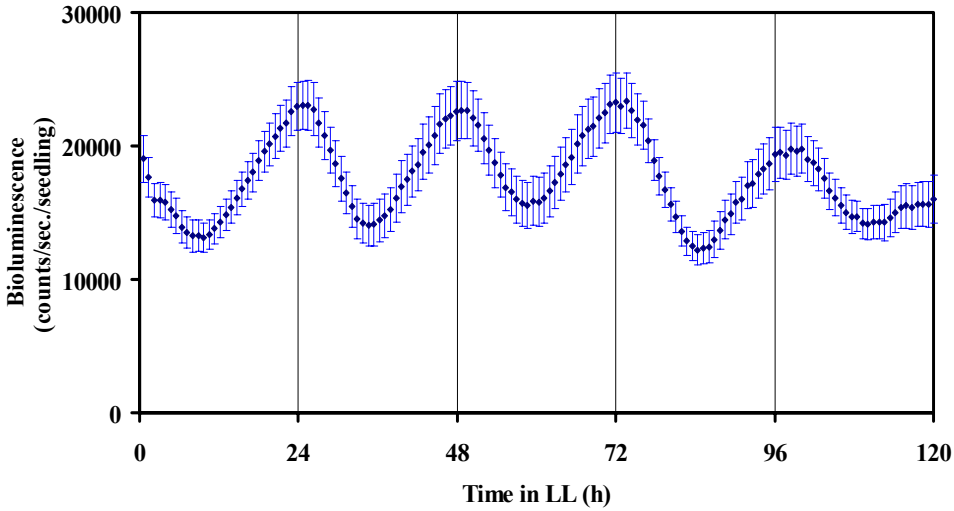
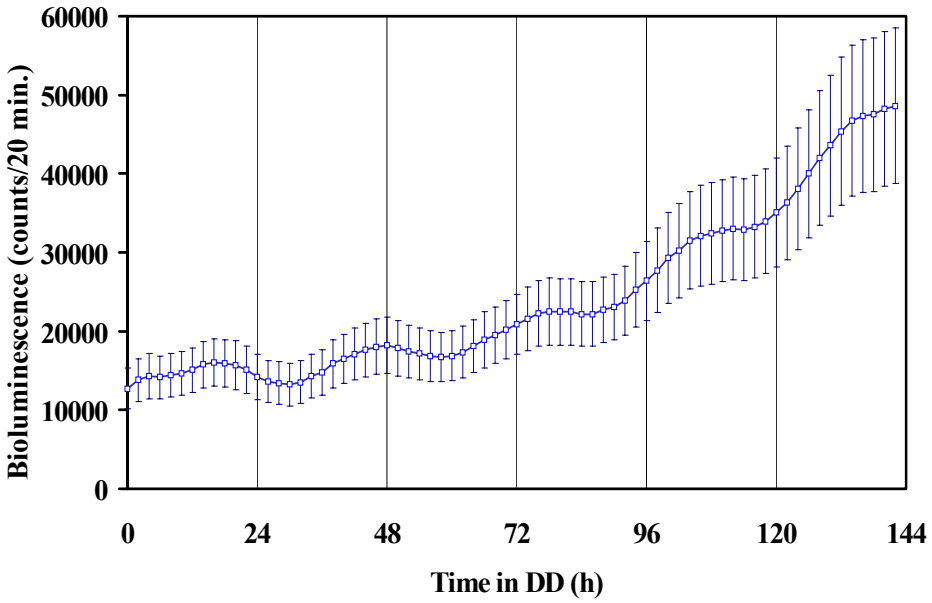

Bioluminescence (counts/sec./seedling)
30000
20000
10000
0
0
24
48
72
96
120
Time in LL (h)

Figure 21. Free-running bioluminescence rhythm of T87 cells transformed with the d3-luc+ construct in DD. T87 cells were transformed with the d3-luc+ construct using the *Agrobacterium*-mediated method. For luciferase assay, transgenic T87 cells were transferred onto the agar medium containing 0.1 mM luciferin and entrained for 3-5 days to 12 h light/ 12 h dark cycle. After entrainment, cells were transferred into the dark box and monitored the bioluminescence every 2 h. Photon counting was performed using the AQUA COSMOS system (Hamamatsu Photonics) and the luminescence was accumulated for 20 min.. (A) The observed bioluminescence of the transgenic T87 cells. (B) Average rate of bioluminescence changes in every 4 h. Plots represent the average data from 24 genetically independent transgenic cell lines. Error bars indicate the standard error.

A


60000
50000
40000
30000
20000
10000
0
Bioluminescence (counts/20 min.)
0
24
48
72
96
120
144
Time in DD (h)

B

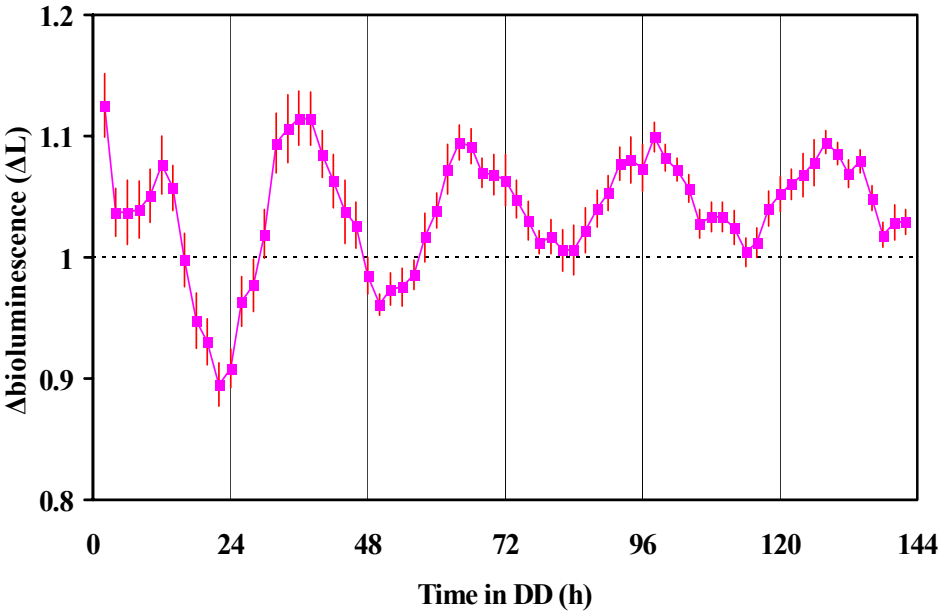

1.2
1.1
1
0.9
0.8
Δbioluminescence (ΔL)
0
24
48
72
96
120
144
Time in DD (h)

Figure 22. Scheme of the reporter constructs transformed into T87 cells. The circle indicates CCA1-binding like motif and the arrowhead indicates the transcriptional initiation site of *AtC401* gene. The numbers indicate the position referred to the transcriptional initiation site of *AtC401* gene.

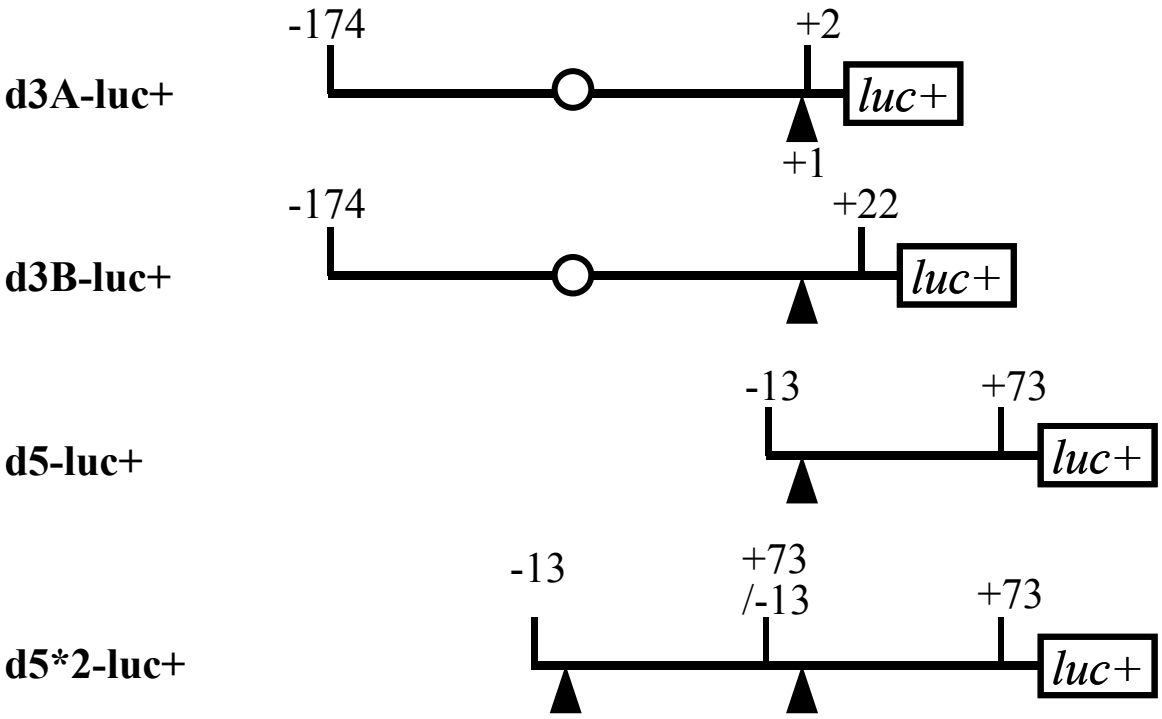
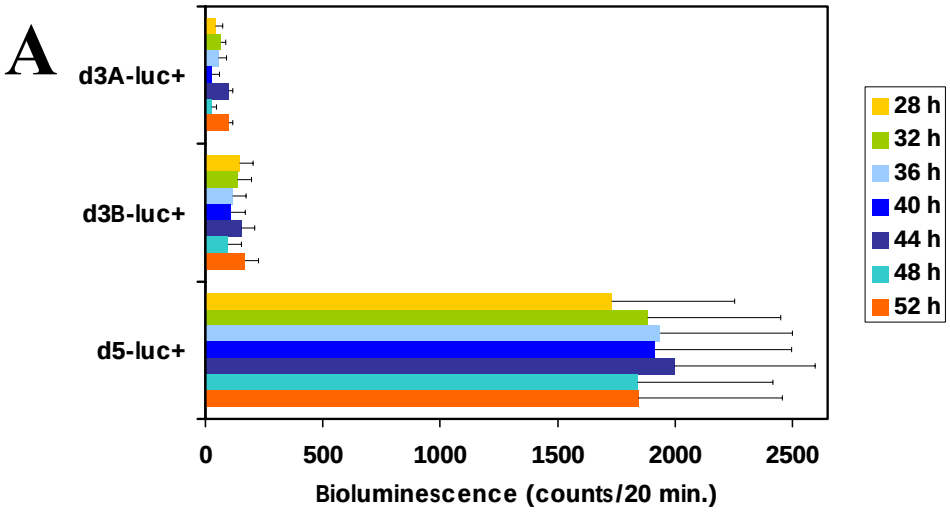
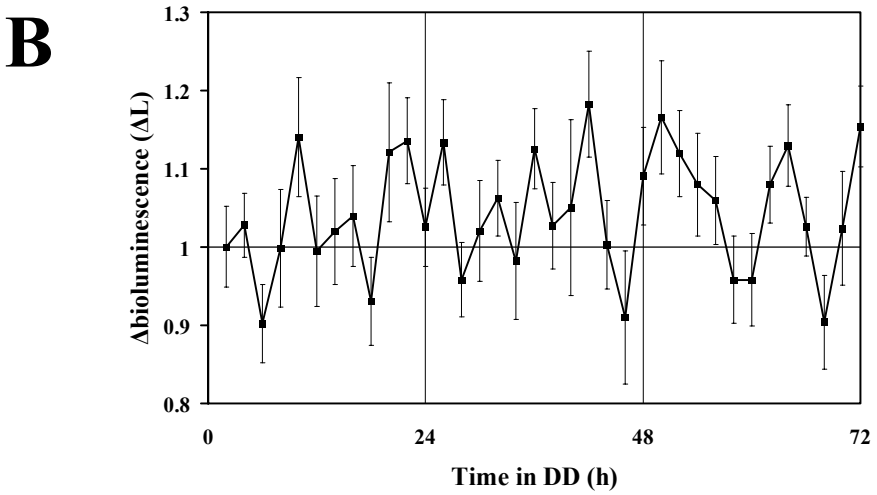

d3A-luc+
-174
+2
luc+
+1
d3B-luc+
-174
+22
luc+
d5-luc+
-13
+73
luc+
d5*2-luc+
-13
+73
/-13
+73
luc+

Figure 23. Effects of 5' UTR fragment on the transcription level and circadian regulation of *AtC401*. The genetically independent 18-26 transgenic cell lines for each construct were used for the experiment. (A) The bioluminescence levels of the transgenic T87 cells. The average data of the observed bioluminescence (counts per 20 min.) at 28, 32, 36, 40, 44, 48, 52 h after transfer to DD are represented. (B and C) The fluctuation of the bioluminescence levels of the transgenic cells containing d3B-luc+ (B) and d5-luc+ (C) reporter construct. The rate of change of the bioluminescence level is represented against the time in DD. The FFT-NLLS analysis evidenced the periodic oscillation in the observed data of d5-luc+ (Rel-Amp=0.478), but not found in d3B-luc+. Error bars indicate the standard error.


A
d3A-luc+
d3B-luc+
d5-luc+
28 h
32 h
36 h
40 h
44 h
48 h
52 h
0
500
1000
1500
2000
2500
Bioluminescence (counts/20 min.)


B
1.3
1.2
1.1
1
0.9
0.8
ΔBioluminescence (ΔL)
0
24
48
72
Time in DD (h)

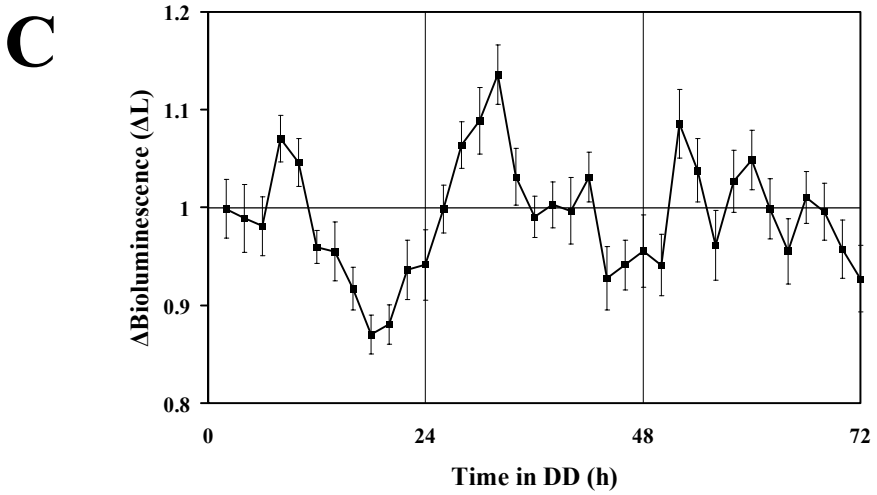
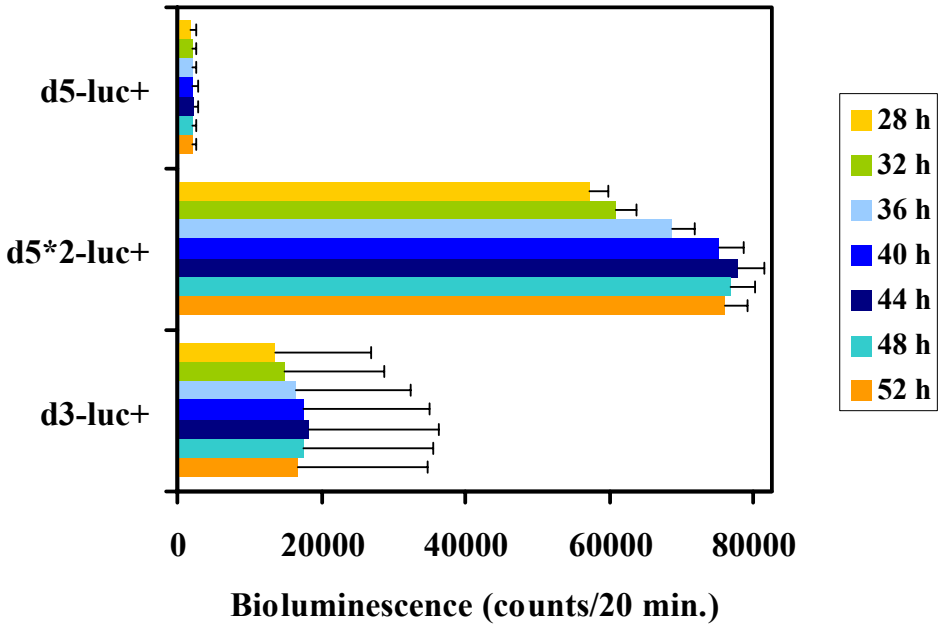

C
1.2
1.1
1
0.9
0.8
ΔBioluminescence (ΔL)
0
24
48
72
Time in DD (h)

Figure 24. Regulation of the circadian expression of *AtC401* by the d5 fragment.

The genetically independent 24 transgenic cell lines for each construct were used for the experiment. (A) The bioluminescence levels of the transgenic T87 cells. The average data of the observed bioluminescence (counts per 20 min.) at 28, 32, 36, 40, 44, 48, 52 h after transfer to DD are represented. (B) The fluctuation of the bioluminescence levels of the transgenic cells containing d5*2-luc+ reporter construct. The rate of change of the bioluminescence level is represented against the time in DD. The FFT-NLLS analysis evidenced the periodic oscillation in the observed data (Rel-Amp=0.214). Error bars indicate the standard error.

A


d5-luc+
d5*2-luc+
d3-luc+
28 h
32 h
36 h
40 h
44 h
48 h
52 h
0
20000
40000
60000
80000
Bioluminescence (counts/20 min.)

B

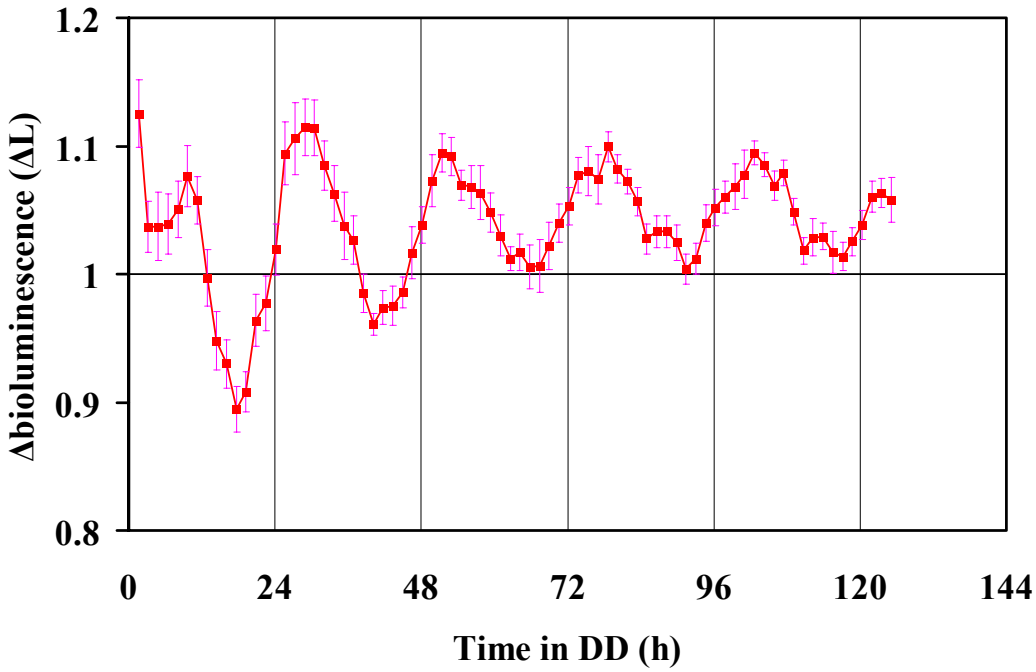

1.2
1.1
1
0.9
0.8
Δbioluminescence (ΔL)
0
24
48
72
96
120
144
Time in DD (h)

Figure 25. A model of the cis-acting transcriptional regulation factors in the *AtC401* promoter. The sequence of the d5 fragment is shown. The d5 fragment contains an initiator motif (indicated by red circle) and the tandem repeat of the GAT/AAA motif (indicated by green corns). The region indicated asterisk is a 20 bp fragment that recovered the bioluminescence level of d3A-luc+. Based on the results obtained in this study, a model of the cis-acting transcriptional regulation factors in the *AtC401* promoter was made: (1) the initiator motif should be required for the binding of the general transcription machinery; (2) the tandemly repeated GAT/AAA motif should be related to circadian regulation. The complex composed of the general transcription machinery and the trans-acting factor(s) bound on these motifs should regulate the circadian transcription of *AtC401*.

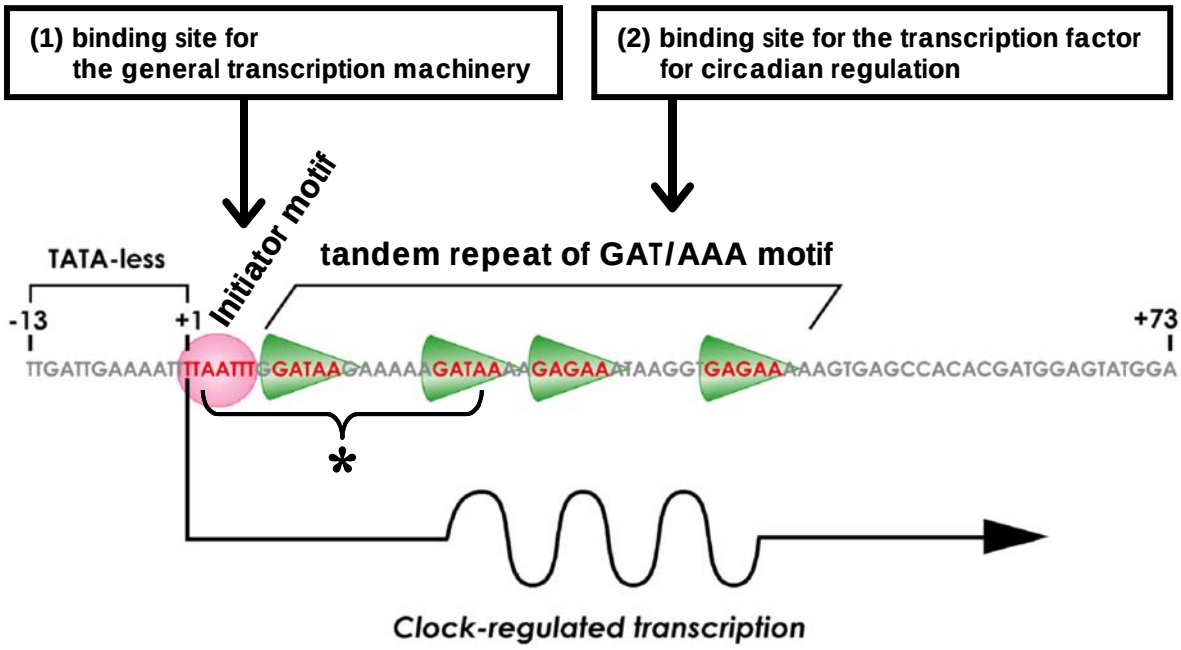
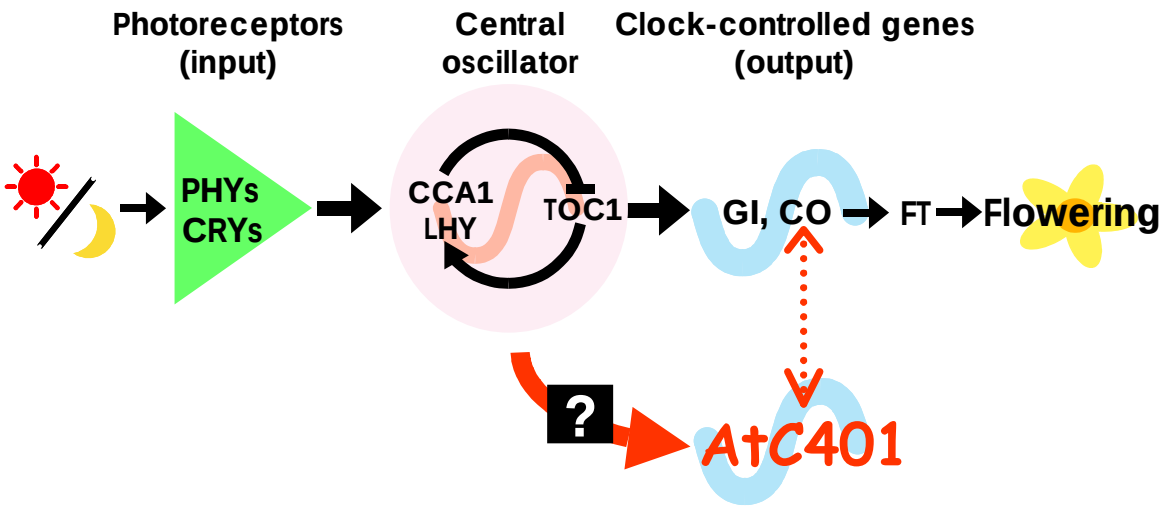

(1) binding site for
the general transcription machinery
(2) binding site for the transcription factor
for circadian regulation
Initiator motif
TATA-less
tandem repeat of GAT/AAA motif
-13
+1
+73
TTGATTGAAAATTTTAATTTCGATAAGAAAAAGATAAAAGAGAAATAAGGTGAGAAAAAGTGAGCCACACGATGGAGTATGGA
*
Clock-regulated transcription

Figure 26. A schematic drawing of the location of AtC401 in the process of photoperiodic induction of flowering and circadian rhythm. *AtC401* is characterized as a novel out-put factor that is regulated by the central circadian oscillator through the different pathway from GI and CO. AtC401 might regulate flowering through the interaction with CO.


Photoreceptors
(input)
Central
oscillator
Clock-controlled genes
(output)
PHYs
CRYs
CCA1
LHY
TOC1
GI, CO
FT
Flowering
?
AtC401

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