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The *Atger*3 promoter confers circadian clock-regulated transcription with peak expression at the beginning of the night

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Abstract

In *Arabidopsis thaliana*, steady-state abundance of the *Atger*3 transcript encoding a germin-like cell wall protein follows a circadian rhythm, reaching its highest level at the beginning of the night. As a first step towards dissecting the molecular mechanisms underlying these transcript oscillations, the *Atger*3 genomic locus was characterised. Transcriptional fusions of 1.8 kb and 967 bp *Atger*3 promoter fragments to the β-glucuronidase (GUS) reporter gene mediate high-amplitude circadian oscillations of the GUS transcript in transgenic *Arabidopsis*. 5′ deletion to −490 greatly reduces overall transcript abundance while retaining a basal oscillation. Further deletion to −299 abolishes preferential GUS expression in the evening. Taken together, these data indicate that clock-response elements contributing to high-amplitude *Atger*3 oscillations largely reside between −299 and −967. Histochemical staining for GUS activity indicates that the *Atger*3 promoter is active in cotyledons, young leaves, petioles, the inflorescence axis, pedicels, sepals, ovary, style and siliques but not in roots, petals and anthers.

Introduction

In most organisms, many cellular functions follow an endogenous daily rhythm. This adaption to the alternation of day and night allows anticipation rather than mere reaction to reiterative changes in ambient light and temperature. These approximately 24 h, 'circadian', rhythms continue under constant external conditions. Under the natural cycle of light and darkness they are synchronised to follow these external changes to ensure cellular functions to occur at an appropriate time of the day (Bünning, 1977; Sweeney, 1987). In this way, interfering reactions such as photosynthesis and nitrogen fixation in cyanobacteria can be directed to different phases of the light/dark cycle (Mitsui *et al.*, 1986). Rhythmic processes in higher plants include leaf movement (Engelman *et al.*, 1992), stem growth (Lecharny and Wagner, 1984), stomatal opening (Gorton *et al.*, 1989), photosynthetic activity (Hennessey and Field, 1991), photoperiodic flower induction (Bünning, 1936) as well as rhythmic expression of specific genes (for a review, see Beator and Kloppstech, 1996).

Circadian regulation of mRNAs has first been demonstrated for those encoding the light-harvesting chlorophyll *a*/*b*-binding proteins (*Lhc*) (Kloppstech, 1985; Piechulla and Gruissem, 1987). *Lhc* transcript levels increase before dawn, peak in the morning and are almost undetectable throughout most of the night. These oscillations persist in the absence of external timing cues, when plants are transferred to continuous light, indicating an endogenous control. Run-on transcription experiments in isolated nuclei demonstrated that the *Lhc* transcription rate is under circadian control in tomato and maize (Giuliano *et al.*, 1988; Taylor, 1989). Reporter gene fusions have led to the identification of *cis*-acting promoter elements mediating circadian regulation of *Lhc* expression in several plant species (Fejes *et al.*, 1990; Millar and Kay, 1991; Carré and Kay, 1995; Ono *et al.*, 1996; Piechulla *et al.*, 1998).

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AJ132237.

For another morning-specific gene in *Arabidopsis* encoding ribulose-bisphosphate carboxylase activase, a promoter fragment extending from −313 to +13 relative to the transcription start site has been shown to mediate a low-amplitude circadian oscillation (Liu *et al.*, 1996).

Although recently a few clock-controlled genes have been identified that reach their highest expression in the evening (Redinbaugh *et al.*, 1990; Carpenter *et al.*, 1994; Heintzen *et al.*, 1994a,b; Zhong and McClung, 1996), the molecular basis of their regulation has not been investigated. Characterisation of such genes, with mRNA cycling roughly antiphase to the *Lhc* phase, would permit the establishment of additional rhythmic molecular markers in *Arabidopsis* and thus would help to address questions of how temporal information from the endogenous clock is conveyed on differently phased output rhythms (for reviews, see McClung, 1998; Staiger and Heintzen, 1999).

In a systematic search for clock-regulated genes in *Sinapis alba* we previously identified a transcript with peak abundance about 12 h after onset of illumination (Heintzen *et al.*, 1994a). This transcript codes for a cell-wall protein that shows homology to germin. Germin has first been found to be expressed during germination in wheat (Lane *et al.*, 1992). It turned out to be almost identical to barley oxalate oxidase and to have the same enzymatic activity. Therefore it has been suggested to contribute to cell wall remodelling, by virtue of the enzymatic breakdown of oxalate with concomitant release of hydrogen peroxide (Lane *et al.*, 1993). Subsequently, several germin-type proteins have been described that are expressed at various developmental stages, upon diverse stress treatments and in response to fungal infection in cereals (Hurkman *et al.*, 1994; Dumas *et al.*, 1995; Hurkman and Tanaka, 1996; Zhou *et al.*, 1998; Berna and Bernier, 1999). As H_2O_2 serves as second messenger in the control of defence gene expression and contributes to oxidative cross-linking of cell wall proteins, germin-type proteins have been implicated as part of plant defence mechanisms.

Germin-like proteins (GLPs) have also been identified in dicotyledonous plants (Michalowski and Bohnert, 1992; Heintzen *et al.*, 1994a). In *Arabidopsis*, expressed sequence tags for at least twelve GLP genes have been found (Membre *et al.*, 1997; Carter *et al.*, 1998). So far no oxalate oxidase activity has been demonstrated for these proteins and their exact function has remained elusive.

Circadian oscillations have been found for the transcript encoding the germin-like cell wall protein *Sa*GLP in *Sinapis alba* (Heintzen *et al.*, 1994a) and its *Arabidopsis* counterpart *At*GER3 (Membre *et al.*, 1997; D. Staiger, unpublished) as well as for a transcript in *Pharbitis nil* (Ono *et al.*, 1996). Diurnal regulation of mRNA abundance was also shown for a germin-like protein in barley (Vallelian-Bindschedler *et al.*, 1998).

In order to begin to understand the clock regulation of the *Atger*3 gene, we have isolated the genomic sequence from *Arabidopsis* and tested the 5′-upstream region for rhythmicity and organ-specific expression using β-glucuronidase reporter gene fusions in transgenic *Arabidopsis*.

Materials and methods

Isolation of a genomic Atger*3 clone*

The *Atger*3 clone was isolated from a λ EMBL4 library (kindly provided by Dr Christiane Nawrath and Dr Csaba Koncz) by standard plaque screening procedures. Hybridisation with an *Atger*3 cDNA fragment, labelled with the Prime it II kit (Stratagene), was performed according to Yang *et al.* (1993). Final washes were done with 0.2× SSC at 62 °C.

An *Eco*RI fragment comprising the *Atger*3 locus was isolated from plaque-purified phage and subcloned into pBSK− (Stratagene). The sequence of both strands was determined by dideoxy sequencing (Sanger *et al.*, 1977).

Primer extension

The transcription start site was determined by primer extension. An antisense oligonucleotide spanning positions 70 to 99 was end-labelled with γ-^{32}P ATP and polynucleotide kinase and hybridised to 2 μg polyadenylated *Arabidopsis* RNA from plants harvested at zt12 (*zeitgeber* time 12; 12 h after lights on). Reverse transcription and analysis on 6% polyacrylamide urea gels was performed essentially according to Ausubel *et al.* (1987).

Chimeric gene constructs

Constructs are schematically depicted in Figure 3. To generate the translational *Atger*3 fusion with β-glucuronidase, an *Nco*I site was engineered at the ATG start codon by PCR. For construct TL, the 1.9 kb 5′-upstream region was inserted in front of the GUS gene in a vector based on pRT104 (Töpfer *et al.*, 1987).

For the transcriptional fusions, a *Bgl*II site was engineered at the transcription start site by PCR. The tobacco mosaic virus omega element (Gallie *et al.*, 1987) was used as a heterologous 5′-untranslated leader. The 1.8 kb promoter region (construct TR) and the truncated fragments Δ1 and Δ2 (cf. Figure 3) were cloned between the *Bgl*II and *Sma*I site in pRT104 containing a *Sma*I-*Bgl*II linker upstream of the omega element/GUS gene fusion.

Cauliflower mosaic virus (CaMV) 35S RNA enhancer fusions were constructed by inserting the *Atger*3 promoter subfragments spanning −299 to +3 and −134 to +3 via blunt-ended restriction sites at their 5′ end and the newly introduced *Bgl*II site at the transcription start site between the *Eco*RV site (−90) of the CaMV enhancer and the omega element in front of GUS in a vector based on pRT104 to yield the constructs CaMV-Δ3 and CaMV-Δ4, respectively.

All constructs were verified by sequencing. The promoter-GUS fusions were subcloned into pBin19 (Bevan, 1984) and transformed into *Agrobacterium tumefaciens*.

Transformation of Arabidopsis *plants*

The translational fusion was introduced into *A. thaliana* ecotype C24 by *Agrobacterium*-mediated root transformation (Valvekens *et al.*, 1988). The transcriptional fusions were transformed into *A. thaliana* ecotype Columbia by vacuum infiltration (Bechtold *et al.*, 1993). Kanamycin-resistant seedlings were selected and grown to flowering in soil. Southern blot analysis verified that the transgenic plants contained intact *Atger*3-GUS fusion constructs.

Northern blot kinetics

T2 seeds were germinated on 0.5× MS plates (Murashige and Skoog, 1962) containing 1% sucrose and 50 μg/ml kanamycin. Resistant plantlets were transferred to 0.5× MS plates. Three to four weeks after sowing, plants were harvested at 4 h intervals throughout a light/dark cycle (LD), as indicated in the figure legends, and on the second day after transfer to continuous light (LL).

RNA isolation and northern blotting were performed as described (Heintzen *et al.*, 1994a, b, 1997). After determination of OD_{260} the individual RNA samples were adjusted to the same concentration (1 μg/μl) to increase accuracy of the photometer reading and double-checked by a second OD measurement. 15 μg of total RNA were separated on 1.2% agarose formaldehyde gels. Blots were consecutively hybridized with the β-glucuronidase-coding region, the *Atger*3 cDNA and a 26S rDNA probe to confirm equal loading.

Histochemical staining

Samples collected at the time of the circadian maximum (zt12) were infiltrated with 0.1% 5-bromo-4-chloro-3-indolyl glucuronide in 50 mM sodium phosphate buffer pH 7 containing 0.1% Triton X-100, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 10 mM EDTA and stained at 37 °C overnight (Jefferson *et al.*, 1996). Subsequently, samples were washed with 50 mM sodium phosphate and cleared of chlorophyll with 70% ethanol.

To generate tissue sections, specimens were fixed with 0.25% glutaraldehyde/3% paraformaldehyde in 0.1 M sodium phosphate buffer and stained with Ruthenium Red. Samples were dehydrated in a graded ethanol series and embedded in Technovit 7100 methacrylate resin according to the supplier's instructions (Kulzer Heraeus, Friedrichsdorf, Germany). Sections were inspected with an Axiophot microscope.

Results

Isolation of the A. thaliana *germin-like protein 3 gene*

200 000 pfu of an *A. thaliana* genomic library in EMBL4 were screened with an *Atger*3 cDNA fragment which we previously isolated from an evening-specific λgt10 library (Membre *et al.*, 1997; D. Staiger, unpublished). Nine positive clones were detected, which fell into two classes. Restriction mapping and Southern hybridisation indicated that these clones largely overlapped, with one class extending about 1 kb further at the 5′ end.

A 6.6 kb *Eco*RI fragment of the larger clone was subcloned into pBSK− (Stratagene) and mapped in detail. A 2.9 kb *Eco*RI-*Kpn*I fragment was identified containing the entire coding region as well as 1.9 kb upstream of the ATG start codon. Collinearity of the clone with the *A. thaliana* genomic sequence was proven by Southern blotting: digests of *A. thaliana* DNA with *Eco*RI/*Xho*I and *Xmn*I/*Sna*BI were probed with a radioactively labelled fragment covering the 5′-untranslated and putative promoter region. Identical bands were recognised by this probe in the genomic DNA as well as in the clone (not shown).

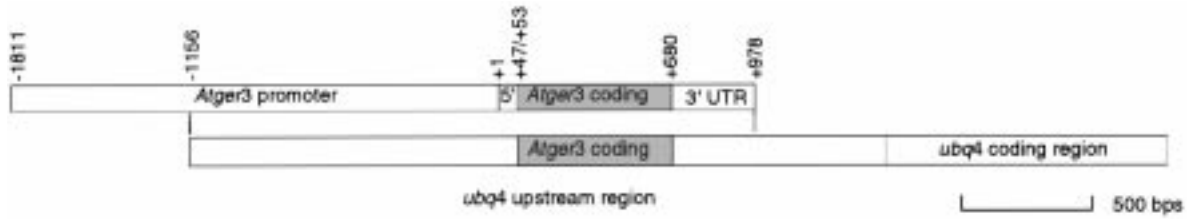

Figure 1. Scheme showing the relative location of the *Atger*3 genomic region (accession number AJ132237) and the ubiquitin4 genomic region (accession number U33014; Burke *et al.*, 1988; Callis *et al.*, 1995). Numbering is relative to the *Atger*3 transcription start site (+1), as determined by primer extension (cf. Figure 2). The overlap between the two genomic regions is delineated by the dotted line.

Sequence analysis

The sequence of both strands was determined after generating suitable subclones. A scheme of the *Atger*3 genomic fragment comprising 1.9 kb of upstream region, the entire coding region and 0.3 kb downstream of the stop codon is presented in Figure 1. Comparison with the *Atger*3 cDNA (accession number Y12673; Membre *et al.*, 1997) revealed the absence of introns. Two presumptive ATG translation initiation codons are found in frame at positions 47 and 53 relative to the start site of transcription. The deduced amino acid sequence contains an extracellular targeting peptide of 18 to 20 amino acids.

The *Atger*3 deduced amino acid sequence shares 67% sequence identity with the coding region of an auxin-binding protein, ABP20, from *Prunus persica* (Ohmiya *et al.*, 1988), indicating that among the *Arabidopsis* germin-like proteins *At*GER3 is related to ABP20.

Database searches indicated that the closest neighbour located downstream of *Atger*3 is the *ubiquitin*4 gene (accession number U33014; Burke *et al.*, 1988; Callis *et al.*, 1995; Carter *et al.*, 1998; F. Bernier, personal communication). The *Atger*3 promoter starting from position −1156 as well as the coding sequence and the 3′-untranslated region are entirely contained within the described polyubiquitin genomic region (cf. Figure 1). The 3′ end of our genomic clone is located 555 bp upstream of the *ubiquitin*4 ATG start codon.

Determination of the transcription start site

Poly(A) containing RNA from plants harvested at the circadian maximum (zt12) was used to delineate the transcription start site by primer extension. The RNA was hybridised to an end labelled antisense oligonucleotide spanning positions 70 to 99 within the coding region and was reverse-transcribed. Two strong and one weaker extension products clustered around bases 1757–1760 were detected (Figure 2), suggesting heterogeneity in the mRNA 5′ ends or premature stops of the reverse transcriptase. The C corresponding to the longest extension product was taken as the transcription start site and numbered +1. The identified mRNA 5′ end conforms well to a consensus transcription start site CTC**A**TCA deduced from 79 plant genes (Joshi, 1987).

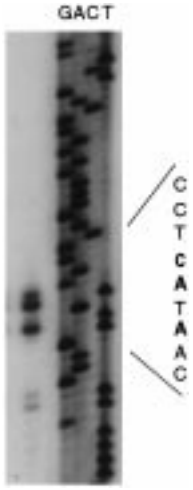

Figure 2. Determination of the *Atger*3 transcription start site. The gel shows primer extension products using an antisense oligonucleotide spanning positions 70 to 99 adjacent to dideoxy sequencing ladders (lanes G, A, C, T). Part of the sequence (inverse of the sequencing reaction) is indicated with the C and A residues corresponding to the extension products in bold.

Clock-response elements are located in the 5′-flanking region

To determine whether clock-responsiveness of the *Atger*3 gene resides within the 5′-flanking region, the 1.9 kb fragment upstream of the ATG start codon was fused to the β-glucuronidase open reading frame (Figure 3). Transgenic T2 plants harbouring this translational fusion construct TL were grown in 8 h light/16 h

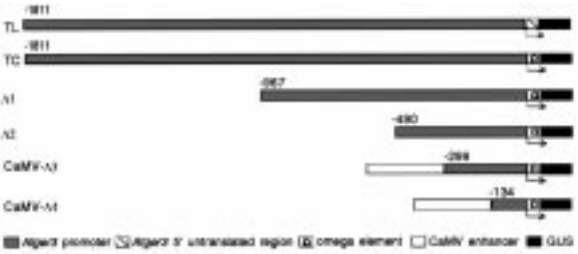

Figure 3. Scheme of the chimeric promoter-GUS constructs. Numbering is relative to the *Atger*3 transcription start (+1), as determined by primer extension (cf. Figure 2). All constructs contain the CaMV 35S RNA terminator. TL, translational fusion; TC, transcriptional fusion; Δ, deletion.

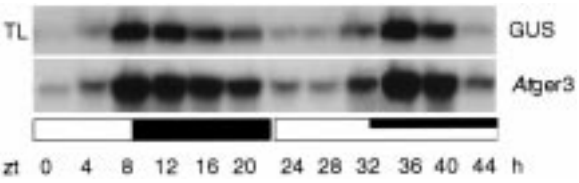

Figure 4. The *Atger*3 upstream region mediates oscillations of a linked β-glucuronidase reporter transcript. Plants harbouring the translational fusion TL of *Atger*3 (−1811 to +47) linked to the GUS coding region were grown in short days (8 h light) and subsequently transferred to continuous light (LL). Plants were harvested at 4 h intervals in light/dark cycles and on the second day in LL. The northern blot was hybridised with the GUS-coding region (upper row) and the endogenous *Atger*3 cDNA (lower row). Light and dark periods are represented by open and solid bars, respectively. The inserted bar in LL indicates the time interval corresponding to darkness during light/dark cycles (subjective night). Zt, zeitgeber time; h, hours after transfer to LL.

dark cycles and subsequently transferred to continuous light. Steady-state abundances of the β-glucuronidase reporter transcript as well as of the endogenous transcript were determined by northern analysis. Figure 4 shows that the β-glucuronidase transcript under control of the *Atger*3 upstream region oscillates in light/dark cycles, reaching its highest level around zt8 to zt12 (upper row), similar to the behaviour of the endogenous *Atger*3 transcript (lower row). These oscillations persist in constant light, indicating that the sequence upstream of the ATG start codon contains elements involved in circadian regulation of the *Atger*3 gene. Almost identical GUS mRNA kinetics were observed for four independent transgenic lines (not shown).

Transcriptional control of Atger*3 oscillations*

To determine whether the transcription of the *Atger*3 gene is regulated by the clock, 1.8 kb of the promoter were fused to the omega element as a heterologous 5′-untranslated region (construct TC; Figure 3). T2 plants of ten independent kanamycin-resistant transformants were tested for GUS mRNA levels. High GUS transcript levels are found in the evening (zt12) whereas almost no GUS transcript is detectable in the morning (zt0–zt4). A detailed RNA kinetic for a representative line harvested at four-hour intervals in 16 h light/8 h dark cycles and on the second day after transfer to continuous light is shown in Figure 5. The temporal pattern of GUS mRNA levels under control of the *Atger*3 promoter (upper row) corresponds to the oscillations of the endogenous *Atger*3 transcript (lower row), demonstrating that the transcription of the *Atger*3 gene is under control of the endogenous clock. Maximal *Atger*3 expression occurs around zt12 to zt16 in long-day conditions (16 h light) as compared to zt8 to zt12 in short-day conditions (8 h light) (cf. Figure 4; Heintzen *et al.*, 1997).

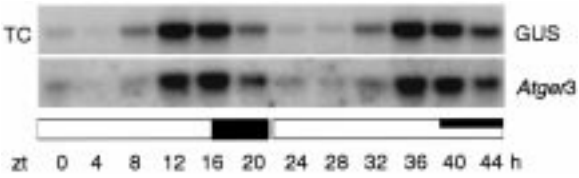

*Figure 5. Atger*3 transcript oscillations are generated at the transcriptional level. Plants harbouring 1.8 kb of the *Atger*3 promoter fused to the tobacco mosaic virus omega element as a heterologous 5′-untranslated region in front of GUS (construct TC) were grown in long days (16 h light) and subsequently transferred to continuous light (LL). Plants were harvested at 4 h intervals in light/dark cycles and on the second day in LL. The northern blot was hybridised with the GUS-coding region (upper row) and the endogenous *Atger*3 cDNA (lower row). Light and dark periods are represented by open and solid bars, respectively. The inserted bar in LL indicates the time interval corresponding to darkness during light/dark cycles (subjective night). Zt, zeitgeber time; h, hours after transfer to LL.

The peak-to-trough difference in expression was retained when the promoter was shortened to −967: GUS expression of independent transgenic lines harbouring construct Δ1 is significantly higher in plants harvested at the circadian maximum (zt12) than in plants harvested at the circadian minimum (zt4), shown for five representative lines in Figure 6A. Further truncation to −490 resulted in a large drop in expression level. GUS mRNA levels still are slightly higher in the evening (zt12) than in the morning (zt4), indicating that a basal circadian regulation is retained in construct Δ2 (Figure 6 B).

To be able to discern whether shorter promoter deletion would drive circadian oscillations, the 35S CaMV enhancer was positioned in front of these short fragments to increase overall transcript abundance

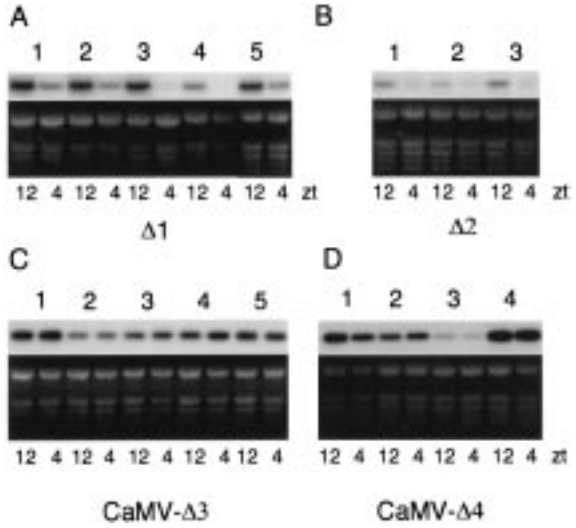

Figure 6. GUS expression driven by truncated *Atger*3 promoter fragments. Independent lines of transgenic plants harbouring deletion constructs Δ1 (A), Δ2 (B), CaMV-Δ3 (C) and CaMV-Δ4 (D) (cf. Figure 3) were grown under long-day conditions (16 h light/8 h dark). RNA was isolated from plants harvested at the circadian maximum (zt12) and the circadian minimum (zt4). The northern blot was hybridised with the GUS-coding region. Note that the blot with construct Δ2 shown in B was exposed about five times as long as the blot with Δ1 shown in A. The ethidium bromide-stained RNA gels are shown below each autoradiagram as loading control.

(Figure 3). This approach was also applied to determine nodule-specific *cis* elements within a soybean leghaemoglobin promoter (Stougaard *et al.*, 1987). Transgenic plants harbouring the constructs CaMV-Δ3 with a deletion to position −299 and CaMV-Δ4 ending at −134 were analysed for GUS mRNA levels at the circadian maximum (zt12) and the circadian minimum (zt4). GUS transcript levels were not significantly different in plants harvested at zt12 as compared to plants harvested at zt4, as shown for representative lines of each construct in Figures 6C and 6D, although the individual lines displayed some variation in overall expression levels. Taken together, these data indicate the presence of at least two *cis*-acting elements contributing to high-amplitude *Atger*3 oscillations within the promoter, residing between positions −299 and −490 and between −491 and −967, respectively.

Histochemical localisation of GUS expression

Histochemical staining for GUS activity in transgenic *Arabidopsis* harbouring the transcriptional fusion TC revealed GUS expression in cotyledons (Figure 7A, B), young leaves, petioles (B), flowers and pedicels (C, D) as well as siliques (E). No GUS expression was found in roots (A, B). In the inflorescence axis, highest expression for the translational fusion TL is found in the subepidermal cortical cells (F, G). In flowers, GUS expression is found in the sepals except the vascular tissue (I). Expression is also found in the subepidermal cell layers of the ovary and style (K, L) but is absent from petals and anthers (I, M). In the siliques, staining is found in the parenchymal cells (H).

Discussion

Previous work has identified a transcript encoding the germin-like cell wall protein *Sa*GLP in *Sinapis alba* that oscillates with peak abundance in the evening (Heintzen *et al.*, 1994a). Here we characterise the 5′-upstream region of its *Arabidopsis* counterpart *Atger*3. In particular, we wanted to determine whether the promoter mediates circadian clock regulation at the transcriptional level.

We could show that 1.9 kb upstream of the translation start codon (construct TL) mediate oscillations of a linked GUS reporter transcript with peak abundance at the end of the daily light period. The oscillations persist under continuous light. No attempts were made to monitor GUS expression in continuous darkness in the transgenic plants: *Atger*3 expression rapidly damps to levels below the detection limit in constant dark, similar to the *Sa*glp transcript (Heintzen *et al.*, 1994a; D. Staiger, unpublished observation).

Clock regulation occurs predominantly at the level of transcription, as the *Atger*3 promoter fragment from −1811 to +3 relative to the transcription start site linked to a heterologous 5′-untranslated leader (construct TC) directs rhythmic GUS mRNA expression. Clock regulation of gene expression in higher plants appears to occur mostly at the level of transcription. Rhythmic transcription has also been found for catalase3 in maize (Redinbaugh *et al.*, 1990) and the *Lhc* genes in wheat, maize, tomato and *Arabidopsis* (Giuliano *et al.*, 1988; Nagy *et al.*, 1988; Taylor, 1989; Millar and Kay, 1991). In the case of the *Arabidopsis Lhc*1*3 (*cab*1) gene a post-transcriptional control mechanism seems to operate in addition to rhythmic transcription, leading to a relatively constant steady-state *Lhc*1*3 transcript level (Millar and Kay, 1991). For nitrate reductase2 in *Arabidopsis*, nuclear run-on experiments revealed predominantly post-transcriptional control of the mRNA oscillations (Pilgrim *et al.*, 1993).

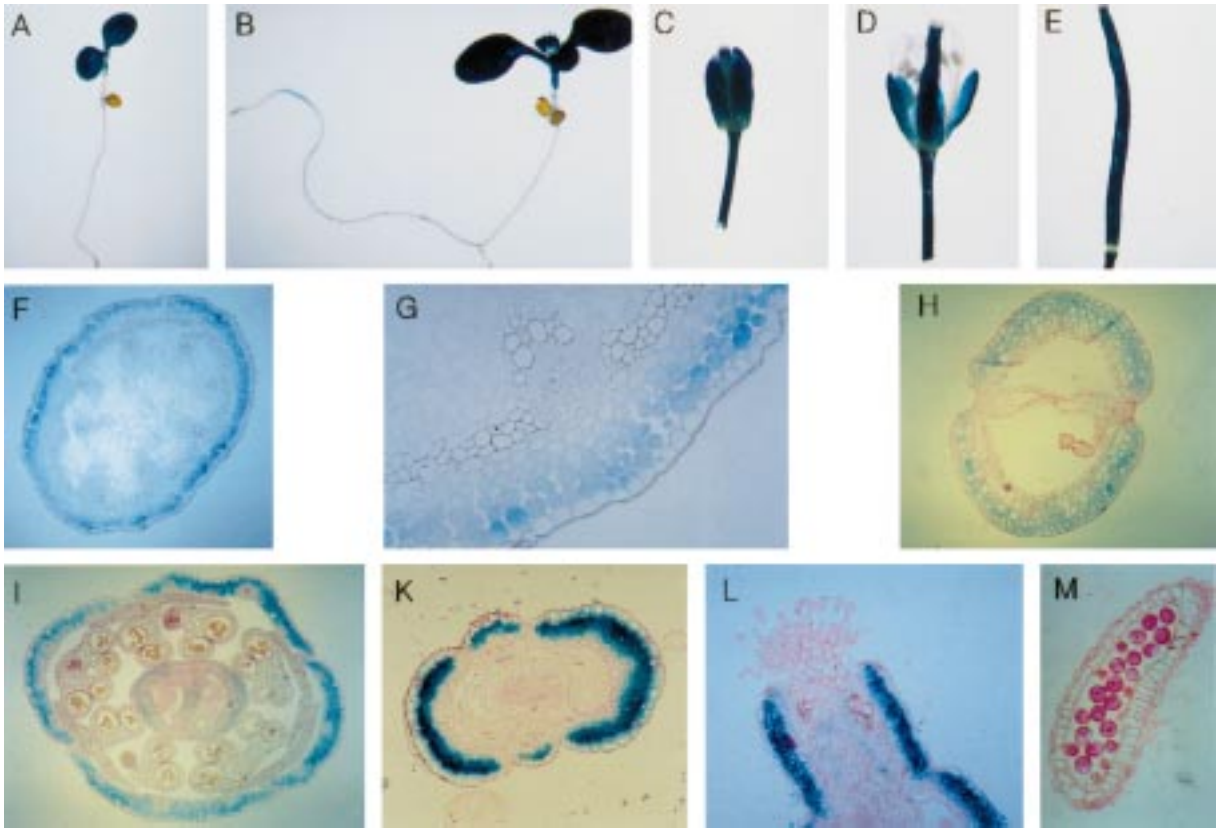

Figure 7. Histochemical localisation of GUS activity in transgenic *Arabidopsis* plants harbouring the transcriptional *Atger*3-GUS fusion TC (A–E) or the translational *Atger*3-GUS fusion TL (F–M), respectively, (cf. Figure 3). A, 6-day old seedling; B, 11-day old plant; C, flower bud; D, flower; E, silique; F, cross-section through inflorescence axis; G, detail of F; H, cross-section through silique; I, cross-section through flower; K, cross section through ovary; L, longitudinal section through style at anthesis; M, longitudinal section through anther.

Circadian-regulated expression with a high peak-to-trough difference is also detected when the *Atger*3 promoter is shortened to −967 (construct Δ1). Construct Δ2 retaining 490 bp upstream of the transcription start site is still expressed at a slightly higher level at the circadian maximum as compared to the circadian minimum although overall transcript abundance is greatly reduced. Constructs CaMV-Δ3 and CaMV-Δ4 with 5′-end points at −299 and −134, respectively, show about equal expression at the circadian maximum and the circadian minimum. Taken together, these data indicate that sequence elements contributing to preferential expression at the beginning of the night largely reside between −967 and −299. A clock response element mediating a basal oscillation seems to be present between −299 and −490. A second element contributing to high-amplitude cycling is located between −491 and −967. The weak expression of construct Δ2 precluded a definite decision whether motifs located within the −491/−967 region would act as general activating sequences or selectively boost expression at the time of the circadian maximum. This distinction may be possible with the use of more sensitive assay techniques such as *in vivo* measurements of promoter-luciferase fusions (Carré and Kay, 1995). Formally we cannot entirely exclude that an additional element mediating a low-level amplitude oscillation may have gone unnoticed in the CaMV constructs because such a clock response element might not have acted in concert with the CaMV enhancer.

Recently, the MYB-type protein CCA1 originally identified as a *trans*-acting factor binding to a phytochrome-responsive promoter element of an *Arabidopsis Lhc* gene has been shown to be part of a negative feedback loop involved in rhythm genesis (Wang *et al.*, 1997; Wang and Tobin, 1998). CCA1 overexpression negatively affects the oscillations of transcripts with different phases such as *Lhc* and the endogenous *cca*1 transcript that peak in the morning and the *Atgrp*7/*ccr*2 transcript peaking later in the day.

Whether CCA1 or related factors also regulate the *Atger*3 transcript with maximal expression at zt12 is not known. Interestingly, one motif identical to the CCA1 binding site AA$^{A}/_{C}$AATCT, as determined previously (Wang *et al.*, 1997; Wang and Tobin, 1998), is located at −197 and four additional sequence elements displaying a 7 out of 8 bp identity are present at −315, −478, −780 and −1011 of the *Atger3* promoter.

An almost perfect direct repeat of an 18 bp sequence is found between −1091 and −1046. Its significance for *Atger*3 expression in not known at present.

The *Atger*3 promoter also mediates organ-specific expression of a linked GUS reporter gene. Expression was found in cotyledons and young leaves, but not in roots. In a northern blot analysis *Atger*3 mRNA previously was detected in leaves, flower buds and open flowers, but not in siliques (Membre *et al.*, 1997). This difference might be due to a different developmental state. In the inflorescence axis, expression is found in the subepidermal cortical cell layers. The expression pattern closely resembles the spatial expression of the mustard counterpart *Sa*GLP, as determined by *in situ* hybridisation (Heintzen *et al.*, 1994a). In flowers, expression was found in sepals, ovary and style but not in petals and anthers. This organ-specific expression pattern could be due either to transcriptional activation or negative regulation in distinct organs. Further dissection of the promoter is required to distinguish between these possibilities.

*At*GER3 previously has been mapped to chromosome 5 between the nga106 and g4560 markers (Membre *et al.*, 1997). Database searches with the genomic clone now revealed a tight head-to-tail linkage of the *Atger*3 gene to the polyubiquitin genomic region (Burke *et al.*, 1988; Callis *et al.*, 1995; Carter *et al.*, 1998). The 3′ end of the *Atger*3 clone is located 555 bp upstream of the *ubiquitin*4 ATG start codon, thus leaving ca. 850 bp intergenic region between the *Atger*3 stop codon and the *ubiquitin*4 start codon. Notably, the *Atger*3 coding region is contained within a 2 kb DNA fragment that previously has been used as a 5′-specific hybridisation probe for the *ubiquitin*4 gene (Burke *et al.*, 1988). This 5′ probe detected a 1.35 kb transcript whereas the ubiquitin coding regions additionally detected mRNAs of 1.9, 1.7 and 0.85 kb. Differences in organ-specific expression levels were revealed for the 1.35 kb transcript depending on the choice of the hybridisation probe. Notably, in heat-stressed *Arabidopsis* plants a small decrease of the 1.35 kb band was detected with the coding region as probe, whereas a large decrease relative to untreated plants was observed upon hybridisation with the 5′ probe. From this finding the authors concluded that an additional transcript must be present in the 1.35 kb size class whose regulation is distinct from that of *ubiquitin*4. It may be interesting to determine whether part of this differential behaviour might be due to the oscillating *Atger*3 transcript.

In conclusion, we have shown that the *Atger*3 promoter mediates cycling of a linked reporter gene with preferential expression at the beginning of the night. Sequence motifs contributing to high-amplitude cycling reside largely between positions −299 and −967. The *Atger*3 promoter should prove useful to generate markers for clock output differentially phased to the well known oscillations of *Lhc* promoter activity with peaks in the morning.

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