The circadian clock that controls gene expression in Arabidopsis is tissue specific

Citation for published version:

Digital Object Identifier (DOI):
10.1104/pp.005405

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Plant physiology

Publisher Rights Statement:
RoMEO green

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
The Circadian Clock That Controls Gene Expression in Arabidopsis Is Tissue Specific

Simon C. Thain, Giovanni Murtas, James R. Lynn, Robert. B. McGrath, and Andrew J. Millar*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, United Kingdom (S.C.T., G.M., A.J.M.); Horticulture Research International, Wellesbourne, Warwick CV35 9EF, United Kingdom (J.R.L.); and Laboratory of Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021 (R.B.M.)

The expression of CHALCONE SYNTHASE (CHS) expression is an important control step in the biosynthesis of flavonoids, which are major photoprotectants in plants. CHS transcription is regulated by endogenous programs and in response to environmental signals. Luciferase reporter gene fusions showed that the CHS promoter is controlled by the circadian clock both in roots and in aerial organs of transgenic Arabidopsis plants. The period of rhythmic CHS expression differs from the previously described rhythm of chlorophyll a/b-binding protein (CAB) gene expression, indicating that CHS is controlled by a distinct circadian clock. The difference in period is maintained in the wild-type Arabidopsis accessions tested and in the de-etiolated 1 and timing of CAB expression 1 mutants. These clock-affecting mutations alter the rhythms of both CAB and CHS markers, indicating that a similar (if not identical) circadian clock mechanism controls these rhythms. The distinct tissue distribution of CAB and CHS expression suggests that the properties of the circadian clock differ among plant tissues. Several animal organs also exhibit heterogeneous circadian properties in culture but are believed to be synchronized in vivo. The fact that differing periods are manifest in intact plants supports our proposal that spatially separated copies of the plant circadian clock are at most weakly coupled, if not functionally independent. This autonomy has apparently permitted tissue-specific specialization of circadian timing.

Light is a key environmental signal for plants, regulating gene expression and development (Neff et al., 2000). Changes in fluence rate and light quality can occur unpredictably and rapidly during the day but have an underlying day-night cycle. Plants have evolved a circadian timing system that allows the anticipation of this predictable rhythm. When plants are deprived of environmental time cues and placed in constant (“free running”) environmental conditions, circadian rhythms persist with a period of around 24 h, often for many days (Millar, 1999; McClung, 2000; Murtas and Millar, 2000; Johnson, 2001). Within the circadian system of the whole organism, the term “circadian oscillator” has been used to denote the parts of the system responsible for rhythm generation. Light-dark signals entrain the oscillator via input phototransduction pathways, synchronizing its phase with the environmental light-dark cycle and also affecting its period. Rhythmic output from the oscillator controls a large number of physiological processes in plants (Lumsden and Millar, 1998). The abundance of 2% to 6% of RNA transcripts in Arabidopsis plants was scored as circadian-regulated in two recent microarray analyses, for example (Harmer et al., 2000; Schaffer et al., 2001).

The rhythmic expression of chlorophyll a/b-binding protein (CAB or Light-Harvesting Complex [LHCB]) genes has often been used as a marker for circadian regulation in plants (for review, see Fejes and Nagy, 1998), especially using firefly (Photinus pyralis) luciferase (LUC) reporter fusions (Millar et al., 1995a). CAB genes are strongly expressed in the mesophyll cells of photosynthetically active organs and in epidermal guard cells. However, physiological analysis shows that the plant circadian system comprises many copies of the circadian clock, with at least one clock in all the major plant organs and possibly one in most cells (Gorton et al., 1989; Kim et al., 1993; Mayer and Fischer, 1994). We use the term “circadian clock” to denote the smallest complete timing unit (comprising an oscillator with light input and output to overt rhythms). CAB rhythms, thus, reveal only a subset of clocks in the plant circadian system. The distributed circadian clocks are thought to function autonomously, because many circadian rhythms persist in isolated tissue explants (for example, Vaadia, 1960; Simon et al., 1976; Engelmann and Johnsson, 1978; Thain et al., 2000). Within a single

---

1 This work was supported by the Biotechnology and Biological Science Research Council (graduate studentship to S.C.T. and grant no. BI11209 to A.J.M.). The Warwick imaging facility is supported by the Gatsby Charitable Foundation, the Royal Society, and the Biotechnology and Biological Science Research Council (grants to A.J.M.).

2 Present address: Division of Environmental and Applied Biology, School of Life Sciences, University of Dundee, Dundee DD1 4HN, Scotland, UK.

3 Present address: Biogemma, Napoli, Italy.

* Corresponding author; e-mail Andrew.Millar@warwick.ac.uk; fax 44–24–7652–3701.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.005405.
Distinct Circadian Clocks Control Gene Expression in Arabidopsis

RESULTS

Expression of CHS:LUC in Roots and Leaves

Fusions of firefly LUC to the promoters of the white mustard (Sinapis alba) chalcone synthase (mCHS) and Arabidopsis chalcone synthase (CHS) genes were transformed into Arabidopsis plants of the Landsberg erecta (Ler) and C24 strains, respectively. Figure 1B shows that luminescence driven by the CHS promoter was evident in the leaves, hypocotyl, and roots of 12-d-old seedlings. Particularly high expression occurred in the shoot apical region and in young lateral roots. Younger, 5-d-old seedlings showed no CHS expression in the hypocotyl (data not shown), consistent with previous reports (Kaiser and Batschauer, 1995; Kaiser et al., 1995). The mCHS promoter had an identical pattern of expression (data not shown). In contrast, CAB:LUC activity was largely confined to green tissues, with a gradient down the hypocotyl and no detectable activity in the roots (Fig. ID).

The spatial pattern of CHS promoter activity was examined in greater detail in leaf sections from CHS:LUC plants and plants carrying a CHS-GUS fusion transgene. The CHS:LUC signal was highest in the upper epidermis, but both reporters showed patchy expression also in the palisade mesophyll layer (Fig.
The epidermal expression of CHS:Luc was about 2-fold higher than the mesophyll expression (in Fig. 1G, for example, average counts per pixel per minute were 0.36 for epidermis and 0.19 for upper mesophyll). A minor contribution from rhythmic CHS expression in the mesophyll would not be distinguished in our assays. Luminescence from the mesophyll is appreciably reduced by passage through the epidermis (Wood et al., 2001), indicating that the CHS:Luc rhythm preferentially reflects epidermal luminescence. CHS-GUS expression was also evident in all cell layers of root cross sections (data not shown).

The CHS Expression Rhythm Is Distinct from the CAB Expression Rhythm

The strong CHS:Luc activity in the root might make this a useful marker for circadian rhythms specifically in this organ, where root pressure and ion fluxes are the only previously reported, rhythmic markers (Vaadia, 1960; Parsons and Kramer, 1974; Gorr et al., 1995; Henzler et al., 1999). The circadian rhythm of CHS:Luc activity was tested under several fluence rates of constant light, because CHS mRNA levels increase in response to higher fluence rates (Feinbaum and Ausubel, 1988; Peter et al., 1991; Fu-
Transgenic seedlings were grown for 12 d on vertical agar plates under white light of 60, 150, or 250 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) fluence rate. Seedlings grown and assayed in white light of 60 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) showed low expression of \( CHS: \text{LUC} \) and \( mCHS: \text{LUC} \) (data not shown). Luminescence rhythms were measured after the plants were transferred to constant light of either the same fluence rates (Fig. 2, A and C), or of an increased fluence rate (from 150 to 250 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \); Fig. 2B). \( CHS: \text{LUC} \) activity showed circadian rhythmicity in aerial organs and roots under all conditions. The peak of activity on the 1st d in constant light occurred before predicted dawn under all conditions (at zeitgeber time [ZT] 20–22; ZT is defined as the number of hours since lights-on), 6 or 7 h before the peak of \( CAB \) expression. Transgenic plants carrying the \( mCHS: \text{LUC} \) fusion in the \( Ler \) background showed very similar luminescence rhythms (Fig. 2D).

The lighting conditions affected the amplitude of rhythmic \( CHS \) expression. The high-amplitude rhythm in the 1st d of constant light was followed by a reduction (damping) in the amplitude of the \( CHS: \text{LUC} \) circadian rhythm within two to three cycles. \( mCHS: \text{LUC} \) was less affected (Fig. 2D), possibly because of a difference between the \( C24 \) and \( Ler \) genetic backgrounds. \( CAB: \text{LUC} \) activity continued to cycle with high amplitude under constant light of all fluence rates. In plants transferred from lower to higher fluence rates of light (Fig. 2B), \( CAB: \text{LUC} \) luminescence levels were reduced compared with \( CHS: \text{LUC} \). When 12-d-old plants expressing \( CHS: \text{LUC} \) were transferred to continuous darkness, transcription from the \( CHS \) promoter was greatly attenuated (Fig. 2E). A single, high-amplitude peak at the expected phase was followed by rapid damping of \( CHS: \text{LUC} \) activity, with a complete loss of rhythmic amplitude by 72 h.

The \( CAB \) and \( CHS \) rhythms had different free-running periods under constant light. The lag (phase angle) between the rhythms, therefore, changed progressively during our experiments, most obviously when \( Ler \) plants were imaged on the 3rd to 7th d under constant light (Fig. 2D). The altered period was clear: The first peak of \( mCHS \) expression shown (at 68 h) occurred 10 h before the \( CAB \) peak (at 78 h), but by 147 h, the \( mCHS \) peak occurred with or slightly after that for \( CAB \). Period estimates were derived from luminescence rhythms measured in the aerial tissues, where both \( CHS: \text{LUC} \) and \( CAB: \text{LUC} \) are expressed (Table I). The period of the \( CHS \) rhythm (25.4 h) was significantly longer than the period of the \( CAB \) rhythm (23.7 h). A similar period difference was observed between \( CHS \) and \( CAB \) expression rhythms in the \( Ler \) background (as in Fig. 2D). In contrast, the period of rhythmic \( CHS \) expression in the root was not significantly different from that in the leaf, in either genetic background (Fig. 2; Table II; data not shown). These results suggested that the circadian system that controlled \( CHS \) in aerial organs was distinct from that controlling \( CAB \).

### Table I. Circadian period of gene expression in the aerial organs of det1

<table>
<thead>
<tr>
<th>LUC Marker</th>
<th>Genotype</th>
<th>Background</th>
<th>Period (h)</th>
<th>SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>( CAB ) +</td>
<td>C24</td>
<td>23.7\textsuperscript{a}</td>
<td>0.36</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>( CHS ) +</td>
<td>C24</td>
<td>25.0\textsuperscript{c}</td>
<td>0.41</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>( CHS ) det1</td>
<td>C24/Col</td>
<td>21.8\textsuperscript{d}</td>
<td>0.48</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>( CAB ) det1</td>
<td>C24/Col</td>
<td>20.7</td>
<td>0.39</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

The \( det1 \) mutant has a severe short-period phenotype for \( CAB \) expression (Millar et al., 1995b), along with other phenotypes principally related to light signaling (Chory and Petø, 1990; Pepper et al., 1994). \( toc1 \) is a short-period mutant that affects only clock-regulated processes (Millar et al., 1995a; Kreps and Simon, 1997; Somers et al., 1998b; Dowson-Day and Millar, 1999); \( TOC1 \) encodes one of the best candidates for a plant circadian oscillator component (Strayer et al., 2000; Alabadi et al., 2001). The \( CHS: \text{LUC} \) construct was crossed into these mutant backgrounds to test whether these genes are involved in the circadian systems that control both the \( CAB \) and \( CHS \) markers. \( det1 \) and \( toc1 \) mutant seedlings expressed \( CHS: \text{LUC} \) rhythmically in both leaves and roots under constant light (Fig. 3A; data not shown). Light-grown \( det1 \) seedlings express the \( CHS \) promoter in all leaf cell layers, and express \( CAB \) genes...
inappropriately in roots and in aerial tissue (Chory and Peto, 1990). CAB:LUC activity in det1 roots was barely detectable and was too low for us to measure circadian rhythms (data not shown). The det1 mutation prevents the damping of rhythmic CAB expression in darkness (Fig. 3B; Millar et al., 1995b). It had little effect on CHS expression, which damped out in the dark similarly in the mutant (Fig. 3B) and wild type (Fig. 2E). Consistent with previous data, the toc1 mutation did not affect the mean expression level or damping of CAB and CHS expression rhythms (data not shown; Millar et al., 1995a).

Quantitative comparisons showed that the period of CHS expression was longer than the period of CAB expression in both mutant backgrounds (Tables I and II), reinforcing the conclusion that separate clocks control these two promoters. However, both rhythmic markers were very similarly affected by the mutations (Tables I and II), with much shorter periods in mutant progeny than in wild-type controls. This result indicates that TOC1 and DET1 function similarly in the circadian clocks that regulate CAB and CHS.

DISCUSSION

Chalcone synthase is one of the key biosynthetic enzymes controlling anthocyanin formation. These flavonoid pigments function to protect plant cells from UV radiation and from pathogen attack, act as insect repellents, and are involved in plant-microbe and pollen-pistil signaling (Hahlbrock and Scheel, 1989). We used the noninvasive LUC reporter gene in fusions with the CHS promoter (CHS:LUC), to monitor the dynamic pattern of CHS expression (Fig. 1). Table II. Circadian period of LUC activity rhythms in toc1

Plants were maintained as described for Figure 2B and Table I. Period estimation and statistical comparisons were performed as described in “Materials and Methods.” All comparisons of toc1 versus wild type for a given marker and tissue, \( P < 0.0001 \); CHS in aerial tissues versus roots, \( P > 0.15 \) in both wild type and toc1. All lines are in the C24 background. The significance levels of \( t \) tests comparing the mean periods were as follows: \( ^{a} \text{CHS (+)} \) versus \( \text{CAB (+)}, P = 0.09 \); \( ^{b} \text{CAB (toc1)} \) versus \( \text{CHS (toc1)} \) in aerial organs, \( P < 0.005 \).

<table>
<thead>
<tr>
<th>LUC Marker</th>
<th>Genotype</th>
<th>Organs</th>
<th>Period</th>
<th>SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAB</td>
<td>+</td>
<td>Aerial</td>
<td>24.3(^{a})</td>
<td>0.08</td>
<td>4</td>
</tr>
<tr>
<td>CHS</td>
<td>+</td>
<td>Aerial</td>
<td>25.1</td>
<td>0.37</td>
<td>5</td>
</tr>
<tr>
<td>CAB</td>
<td>toc1-1</td>
<td>Aerial</td>
<td>19.2(^{b})</td>
<td>0.25</td>
<td>6</td>
</tr>
<tr>
<td>CHS</td>
<td>toc1-1</td>
<td>Aerial</td>
<td>20.6</td>
<td>0.23</td>
<td>5</td>
</tr>
<tr>
<td>CHS</td>
<td>+</td>
<td>Root</td>
<td>24.8</td>
<td>0.21</td>
<td>5</td>
</tr>
<tr>
<td>CHS</td>
<td>toc1-1</td>
<td>Root</td>
<td>21.2</td>
<td>0.26</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 3. Circadian rhythms of CHS:LUC activity in det1. Expression was assayed as in Figure 2. Seedlings were grown in LD (8, 16) at 20 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) to confirm the det1 phenotype. Seedlings were transferred to LD (12, 12) at 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), 2 d before the start of the experiment and to constant light of 250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 0 h (A) or to 250 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) at 0 h and constant darkness at 12 h (B). Luminescence data were analyzed from whole seedlings, without separate analysis of roots and aerial tissues. The data are representative of at least four independent experiments. White box on time axis, Light interval; black box, dark interval.

Regulation of CHS

The expression of CHS exhibited a very similar circadian rhythm in all tissues (Fig. 2). The phase of the circadian rhythm of CHS was earlier than CAB, with a peak occurring in the late subjective night (ZT 20–22). Chalcone synthase enzyme activity and mRNA abundance have previously been reported to exhibit diurnal and circadian regulation (Peter et al., 1991; Deikman and Hammer, 1995; Harmer et al., 1995).
Distinct Circadian Clocks Control Gene Expression in Arabidopsis

The reported phase of peak enzyme activity lags slightly behind the peaks of mRNA abundance and of transcriptional activity, suggesting that the circadian system principally regulates CHS expression at the transcriptional level. It may be advantageous for the plant to accumulate photoprotective pigments in advance of the daily photoperiod (Harmer et al., 2000); the timing of CHS transcription before dawn is consistent with this notion.

Increasing the fluence rate of white light from 150 to 250 μmol m$^{-2}$ s$^{-1}$ had little effect on the phase or period of CHS or CAB expression (Fig. 2; data not shown). However, mean expression levels were differentially affected by the lighting conditions. Plants entrained at 150 μmol m$^{-2}$ s$^{-1}$ but assayed at 250 μmol m$^{-2}$ s$^{-1}$ showed reduced levels of CAB:LUC activity compared with CHS:LUC activity (Fig. 2B), perhaps reflecting a requirement for increased photoprotection and reduced light-harvesting capacity. High-fluence rate light was required to maintain high expression levels of CHS transcription in wild-type plants: CHS:LUC activity was very low in plants assayed at 60 μmol m$^{-2}$ s$^{-1}$ (data not shown) or in darkness (Fig. 2E). The det1 mutation increases CAB expression levels in dark-adapted plants but had little effect on the level of CHS expression (Fig. 3B; Chory and Peto, 1990; Millar et al., 1995b). This presumably reflects a differential involvement of DET1 in the phototransduction pathways that regulate these promoters (Mustilli et al., 1999; Jenkins et al., 2001).

Differences in the Circadian Regulation of CHS and CAB

The rhythm of CHS expression was very similar in roots and in aerial organs under constant light, indicating that the clocks controlling CHS in these organs do not differ significantly. The period of CHS expression in aerial tissues was approximately 1.5 h longer than the period of CAB expression. This difference was maintained in two wild-type accessions and in the det1 and toc1 mutants (Tables I and II). Distinct circadian clocks, therefore, control the rhythms of CAB and CHS expression, although both are nuclear genes that could in principle respond to the same regulator. The phase of a single rhythm (free calcium concentration) has recently been shown to vary among tissues of transgenic tobacco (Nicotiana tabacum; Wood et al., 2001). The authors point out that a phase difference could result from tissue-specific clocks or from tissue-specific responses to a single, common clock. Where period differences are observed, the latter interpretation can be ruled out (Sai and Johnson, 1999).

It is not surprising that the period difference is small. Studies on cyanobacteria indicate that a clock with a period that matches the environmental light-dark cycle provides a competitive advantage (Ouyang et al., 1998). Balancing selection is, therefore, likely to maintain periods in a narrow range around 24 h. Consistent with this notion, we have previously shown that the similar periods of several Arabidopsis accessions are the result of balancing long- and short-period alleles at multiple loci (Swarup et al., 1999).

Period differences under constant conditions are relatively easy to measure. Under light-dark cycles, however, clocks with different periods will entrain to different phases (for example, Ouyang et al., 1998; Somers et al., 1998b). A clock with a longer period (such as that controlling CHS) will be set to a later phase, all else being equal. The longer period moves the peak of CHS expression away from midnight and toward dawn. If CAB was controlled by the same, longer-period clock, then the peak of CAB expression would also be later in the day, all else being equal. The delay between the peaks of CHS and CAB expression would thus be greater than we observe. Independent clocks allow the temporal sequence of metabolic processes to be fine-tuned: A smaller delay between the peaks of CHS and CAB expression might be one example of this. Such a flexible timing system has potential selective advantages (Roenneberg and Mittag, 1996), although these remain to be demonstrated experimentally.

Differential Regulation of Circadian Period

The circadian clocks that control CAB and CHS might differ fundamentally in their oscillator mechanism, or they might alternatively be separate copies of a common biochemical mechanism with only minor modifications leading to the period difference (Millar, 1998). det1 and toc1 mutations are thought to affect the circadian rhythm of CAB expression via the input pathway and the oscillator, respectively (Millar et al., 1995a, 1995b; Strayer et al., 2000; Alabadi et al., 2001). Each mutation shortens the circadian period of CHS in aerial organs in parallel with CAB (Tables I and II). This result shows that both circadian clocks share at least the TOC1 and DET1 functions, so the clocks are not radically different. A parsimonious explanation is that the CHS and CAB promoters are controlled by separate copies of the same clock mechanism (Fig. 4). The characteristics of circadian timing also vary among rodent organs, outside the brain (Yamazaki et al., 2000). Differences in input pathways might be particularly important in that case (Damiola et al., 2000; Stokkan et al., 2001). The same canonical clock genes seem to be involved in diverse anatomical locations (Ripperger et al., 2000).

CHS is expressed principally in the epidermal cell layer of wild-type aerial organs, whereas CAB is expressed in the mesophyll layers. It is most likely that their different circadian periods reflect tissue-specific modifications of the clock in epidermal and mesophyll cells, respectively. In support of this conclusion, we
have recently shown that the PHYTOCHROME B gene, which is expressed in the epidermis, also has a longer period than CAB (A. Hall, L. Kozma-Bognar, F. Nagy, and A.J. Millar, unpublished data). These data imply that epidermal circadian clocks are not tightly coupled to clocks in the mesophyll of the same leaf. We have previously demonstrated that several autonomous clocks exist within the mesophyll of a single leaf (Thain et al., 2000). Taken together, our results suggest that circadian control is local to one or a few cells, not widely coupled within or between tissues, at least for gene expression rhythms in the leaf.

The molecular cause of the observed period difference is unclear. Any circadian input pathway might contribute (Fig. 4). Photoreceptor genes are differentially expressed in the epidermal and mesophyll cell layers (Somers and Quail, 1995), and photoreceptors are known to alter circadian period in a dose-dependent manner (Somers et al., 1998b; Devlin and Kay, 2000). The light input pathways might thus control circadian period in a tissue-specific fashion. Observed distinctions between circadian rhythms suggest that further specialization of the circadian clock might be present in the cells that sense or respond to photoperiodic signals (for example, Salisbury and Denney, 1971; Fowler et al., 1999) or control rhythmic leaf movement (Hennessey and Field, 1992; Fowler et al., 1999; Park et al., 1999). Rhythmic reporters that peak at various phases in restricted spatial patterns are required to determine how much the circadian clock is modulated for specialized timing functions. Recent microarray experiments suggest many candidate promoters (Harmer et al., 2000; Schaffer et al., 2001), which can now be tested in detail using LUC fusions.

**MATERIALS AND METHODS**

**Plant Material, Media, and Growth Conditions**

Experiments were performed with transgenic Arabidopsis seedlings carrying the native firefly (Photinus pyralis) LUC gene under the control of one of two chalcone synthase promoters. Arabidopsis plants of the C24 accession carrying the native Arabidopsis CHS promoter fused to the LUC gene have been described (Michelet and Chua, 1996). The Columbia transgenic line carrying a fusion of the same Arabidopsis CHS promoter fragment to GUS was also described previously (Hartmann et al., 1998). Transgenic plants of the Ler accession carried the promoter of white mustard (Sinapis alba) CHS1 from -907 to +26 bp (Batschauer et al., 1991), fused to the LUC gene in the pMON 721 binary vector backbone. Four independently transformed lines were tested with very similar results (data not shown). The C24:LUC transgene introgressed from C24 into the Ler background has been described (Somers et al., 1998b). CHS:LUC from the C24 accession was crossed with the det1-1 mutant in the Columbia background (from Dr. Joanne Chory, Salk Institute). det1-1 mutant and wild-type plants were selected from the F2 population on the basis of their morphology; mutant scoring was confirmed in the F3 generation. The line carrying CAB:LUC in the det1-1 background has been described (Millar et al., 1995b). CHS:LUC in C24 was crossed to the toc1-1 mutant that carries CAB:LUC in the C24 background (Millar et al., 1995a). Homozygous mutant F2 progeny were selected by scoring short-period CAB:LUC luminescence in leaves; among these, seedlings carrying CHS:LUC were selected by scoring luminescence in roots. The CAB:LUC reporter was removed by segregation in the F3, resulting in greatly reduced luminescence in leaves. The presence of CHS:LUC and absence of CAB:LUC was confirmed by PCR using transgenic-specific primers designed from published sequences (data not shown).

Seedlings were grown at 22°C in 12:12-h light-dark cycles (unless otherwise indicated) on agar medium containing 3% (w/v) Suc under cool-white fluorescent lights of the fluence rates indicated for each figure. Our standard conditions (approximately 60 µmol m⁻² s⁻¹) gave low CHS expression (data not shown); higher fluence rates of 150 to 250 µmol m⁻² s⁻¹ increased expression from these constructs, so all subsequent experiments were conducted within this range.

**Localization of CHS Expression**

Histochemical localization of GUS (Fig. 1E) was performed as described (Jefferson et al., 1987). Ruthenium red counterstaining was carried out by standard techniques. Tissue sections were prepared for light microscopy using a Technovit 7100 embedding kit (Kulzer, Wehrheim, Germany). High-resolution imaging of LUC activity was performed essentially as described (Hall et al., 2001). The image in Figure 1, G and H, was captured through a Fluor 20x objective (Zeiss, Jena, Germany) by a 30-min exposure on a cooled back-thinned CCD in a liquid-nitrogen-cooled camera (LN/CCD-512-TKB with ST138 controller, Roper Scientific Ltd., Marlou, UK). Camera background has been removed from Figure 1G. The specific signal in this image ranges from 2 (dark blue) to 24 (white) counts per pixel. Dark current was undetectable and readout noise was 1.8 counts (σ). The lowest luminescence levels have been clipped in Figure 1H to reveal the tissue outline.

**Imaging of LUC Biomolecules Rhythms and Statistical Analysis**

After luciferin pretreatment (Millar et al., 1992), seedlings were sprayed before each image with 10 mM β-luciferin (Promega, Madison, WI) in 0.01% (w/v) Triton X-100 and left in darkness for 5 min to allow chlorophyll chemiluminescence to decay. Biomolecules were detected by ultra-low-light cameras (VIM C2400-47, Hamamatsu, Bridgewater, NJ) and Roper Scientific LN/CCD-512-TKB with ST138 controller), as described (Millar et al., 1992; Michelet and Chua, 1996; Hall et al., 2001). The data from both cameras were similar, although absolute luminescence counts are not correctly comparable because of the different image acquisition methods (for example, in Figs. 2 and 3). Data are expressed as detected photons per plant per 25-min image, derived from data from groups of 10 to 20 plants. Data in Figures 2 and 3 have been processed with a three-point, boxcar filter. Period estimates from the raw data were produced by the last Fourier transform nonlinear least squares method (FFT-NLLS, Plautz et al., 1997). The first 24 h
of each time course was excluded from the period estimation to exclude any transient effects of the transfer to continuous light.

To derive mean period estimates from the results of seven experiments, the data of Table I were analyzed using residual maximum likelihood (REML) (Patterson and Thompson, 1971) in the statistical package Genstat 5 (Payne et al., 1993). REML can be thought of as a generalization of analysis of variance to unbalanced designs. Data were weighted for analysis by the reciprocal of the estimated variance of the circadian period for the trace, which was derived from FFT-NLLS (Millar et al., 1995a). The data were analyzed with each line taken as a fixed effect and experiment and trace within experiment as random effects. The significance of differences between pairs of treatments were assessed using t tests, based on se of the differences derived from REML, rather than the se of individual means presented in Table I (Patterson and Thompson, 1971). The mean periods and se of Table II are derived from the same variance-weighting procedure on period estimates from FFT-NLLS for replicated samples in a separate experiment.

Waveforms in the data that are unusually close to a cosine wave can give very low estimated ses from FFT-NLLS and, thus, gain disproportionate weight in the variance-weighted means. The analysis was repeated with revised weights that were derived by adding 0.1 to the original, estimated se to reduce the effects of such rare estimates. The conclusions of the analysis were not altered by this procedure, and the data presented are weighted using the original estimates.

ACKNOWLEDGMENTS

We thank Profs. Gareth Jenkins and Nam-Hai Chua for helpful discussions and materials and members of the chronobiology group at Warwick for discussions and assistance with imaging experiments.

Received March 12, 2002; accepted April 22, 2002.

LITERATURE CITED


Distinct Circadian Clocks Control Gene Expression in Arabidopsis
Thain et al.


