A Clock- and Light-Regulated Gene That Links the Circadian Oscillator to LHCB Gene Expression

Yao Xu and Carl Hirschie Johnson

Department of Biological Sciences, Box 1812-B, Vanderbilt University, Nashville, Tennessee 37235

We have identified and characterized a novel tobacco gene, called ZGT (from the Chinese phrase zhong guang tiaokong, or clock and light controlled), that is regulated by the circadian clock and light. ZGT transcripts have alternate forms that are differentially expressed in different tissues. ZGT is expressed rhythmically in light/dark cycles and in constant light. Constitutive expression of ZGT sustains the expression of the clock-controlled LHCB1*1 gene in constant darkness, when it would normally dampen, but does not affect LHCB1*1 expression in constant light. ZGT expression is induced rapidly by light, and overexpression of ZGT increases the sensitivity of the circadian oscillator to brief light pulses. The ZGT promoter includes a G-box motif that is found in many light-regulated promoters in plants and is the same as the E box described for rhythmically regulated promoters of animal circadian clock genes. The ZGT promoter also includes “evening element” motifs that are correlated with circadian control of plant genes. We postulate that light- and clock-regulated expression of ZGT acts as a coupling agent between the central circadian oscillator and rhythmic LHCB1*1 expression and that it may function as a component in plant phototransduction pathways.

INTRODUCTION

Circadian (daily) rhythms are a crucial adaptation of organisms to consistent changes in their environment, in particular to the daily changes of illumination, temperature, humidity, and so on. Circadian rhythms are governed by an internal, autonomous oscillator that can be entrained by environmental cues. The daily light/dark (LD) cycle is the dominant time cue that provides temporal information to circadian oscillators. In most animals, circadian rhythms are expressed in both constant darkness (DD) and constant light (LL; at least in dim constant light). In contrast, many observable rhythms in plants dampen rapidly in DD. Therefore, for plant rhythms, light is both a temporal cue that indicates the time of day and a conditional requirement for the expression of the output rhythms (Johnson et al., 1998; Johnson, 2001).

Among the plant rhythms that dampen rapidly in DD are rhythms of gene expression. For example, circadian rhythms of mRNA abundance that are expressed robustly in LL but dampen rapidly in DD are known for many genes, including those encoding the light-harvesting protein–pigment complex (LHC) gene family, catalase, the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, nitrate reductase, and many others (Deng et al., 1990; Pilgrim and McClung, 1993; Pilgrim et al., 1993; Watillon et al., 1993; Zhong et al., 1997; Fejes and Nagy, 1998). The case of catalase mRNA rhythms is particularly interesting. There are at least six different catalase isoenzymes in Arabidopsis, of which at least two, CAT2 and CAT3, are controlled by a circadian oscillator. In LD, both CAT2 and CAT3 mRNA abundances are robustly rhythmic, but upon transfer to DD, both mRNA rhythms dampen quickly. The intriguing observation is that CAT2 mRNA dampens rapidly to a low steady state abundance (as is true for most known circadian clock–regulated plant genes) but CAT3 mRNA dampens to a high steady state abundance (Zhong et al., 1997). Mutations in both the PHYA and CRY1 genes affect the damping characteristics of catalase mRNA rhythms (Zhong et al., 1997).

The plant gene family for which mRNA abundance rhythms were first reported and that remains the best characterized is the one encoding the proteins of the LHC. Daily variations in the mRNA level of LHC transcripts with a peak in the early daytime were first reported by Kloppstech (1985), and subsequent studies demonstrated that this rhythm persists in LL (Otto et al., 1988). It is now known that the expression of many members of the LHC family exhibits circadian rhythmicity that rapidly dampens in DD (Kellmann et al., 1993; Fejes and Nagy, 1998). Light stimulation of phytochrome and possibly other photoreceptors is necessary to maintain the robust expression of these LHC genes (Fejes and Nagy, 1998). Most of the members of the LHC family are regulated by transcriptional control, and the promoter regions of a few members of the family have been analyzed.
found a robust daily rhythm of ral expression pattern was analyzed by RNA gel blotting, we (Figure 1). In the LL time course, the peak levels of the plants grown in LD cycles and in LL. Figure 1 shows that in rhythmic coupling factor between a central circadian oscillator and gene, which we call sion—dampen in DD. We have discovered a new plant gene, which we call ZGT, from tobacco, whose overexpression maintains the expression of the LHCB1*1 promoter in DD and that affects the sensitivity of the circadian oscillator to phase-resetting light signals. The expression of ZGT is induced rapidly by light pulses and is controlled by the circadian clock. We postulate that ZGT acts as a light-dependent coupling factor between a central circadian oscillator and rhythmic LHCB1*1 expression and that it also may act as an amplifier of photoreceptive input to circadian oscillators.

RESULTS

Tobacco ZGT Is a New Gene That Is Expressed Rhythmically

We identified a new gene from a tobacco genomic library that we have named ZGT, from the Chinese phrase zhong guang tiaokong (clock and light controlled). When its temporal expression pattern was analyzed by RNA gel blotting, we found a robust daily rhythm of ZGT mRNA abundance in plants grown in LD cycles and in LL. Figure 1 shows that in LD 12:12 (lights on from LD time 0 [LDT 0] to LDT 12, and lights off from LDT 12 to LDT 24/0), the peak levels of the ZGT transcript occurred at approximately LDT 8 (8 hr after dawn) and the mRNA abundance was at its lowest level at approximately LDT 0 (dawn) in a time-course experiment in which RNA was collected at 4-hr intervals. To determine if the expression of this transcript is circadian, we performed time-course collection of RNA from tobacco plants exposed to LL (after entrainment by an LD cycle). The RNA gel blot hybridized with a ZGT probe revealed a robust circadian rhythm of ZGT mRNA abundance for at least 2.5 days in LL (Figure 1). In the LL time course, the peak ZGT mRNA abundance was in the middle of subjective day (RNA was sampled every 6 hr in the LL experiment). The same blots probed for rRNA (as a loading control) showed a constant level. In a similar time-course experiment performed in DD, the level of ZGT mRNA expression was very low and appeared to be constitutive (data not shown). These data indicate that ZGT expression is controlled by a circadian biological clock and is light dependent.

Because ZGT is expressed robustly with a rhythmic pattern, we were interested in further characterizing this gene and its possible role in plant circadian systems. On the basis of the original genomic clone, we first determined the nucleotide sequence of the ZGT DNA fragment and its 5' and 3' flanking regions. The full-length cDNA sequence of ZGT was determined by rapid amplification of cDNA ends (RACE). Overlapping 5' and 3' RACE cDNA sequences indicated that ZGT has a single open reading frame encoding a putative ZGT protein with 291 amino acids, whose structural features are depicted in Figure 2A. Database searches demonstrated that the deduced ZGT protein does not share significant sequence similarity to any known proteins, except a putative homolog in Arabidopsis that was released recently to the sequence database (GenBank accession number CAB87751; see Figure 2B). We will refer to this Arabidopsis sequence as pZGT (for putative ZGT); it shows 61% identity and 74% similarity in amino acid sequence to that of tobacco ZGT based on Gapped BLAST and PSI-BLAST analyses (Altschul et al., 1997). Near the C terminus of tobacco ZGT, we found an aspartate- and glutamate-rich (DE-rich or DE-repeat) region. These DE-rich sequences also are found in a number of other proteins, such as nucleolar transcription factors (GenBank accession numbers P25976, P25977, P25979, P25980, and P17480), nucleolins (GenBank accession numbers P13383, P15771, P19338, JH0148, and BAA86200), GTPase activating proteins (GenBank accession numbers P46061 and NP_010454), and some kinases (GenBank accession numbers T28145, CAA65449, O15066, and Q61771).

The ZGT Promoter Contains Conserved Light- and Clock-Regulated Motifs

Between positions −147 and −142 of the ZGT 5’ flanking region, we found a palindromic hexanucleotide DNA sequence, CACGTG, that is one version of the light-regulated motif known as a G-box core (Chattopadhyay et al., 1998; Martínez-García et al., 2000). Within the 680-bp 5’ flanking region of the Arabidopsis pZGT gene, we also found four putative G-box motifs located at −84 to −79, −263 to −258, −334 to −321, and −675 to −670 relative to the first codon (numbered as +1) of its putative translational start site. The presence of this core target element for light signaling in the ZGT promoter is consistent with its playing a role in the light regulation of ZGT, which we demonstrate below (see Figure 5). Intriguingly, the same consensus CACGTG sequence functions as a circadian enhancer motif in Drosophila and mammalian circadian clock gene promoters—in which case it has been called an E box. In Figure 2C, we compare the flanking sequences of the G box or E box (G/E box) in the ZGT promoter with those from various clock- and/or light-regulated genes. In the case of the Arabidopsis pZGT gene, we chose the motif closest to its putative "evening element" motif (see below and Figure 2D) for comparison. The tobacco ZGT promoter has flanking sequences downstream of the G/E box that are highly conserved relative to those downstream of the G/E box in the
Circadian Clock Coupling Factor 1413


In addition, putative evening elements AAAATATCT (EE1, /H11002178 to /H11002186) and AATATCT (EE2, /H11002231 to /H11002237) were found in an antisense orientation in the ZGT promoter (Figures 2A and 2D). This evening element, a conserved nine- or seven-nucleotide (AA)AATATCT motif in promoters for circadian control of plant gene expression, was recently identified by computational analysis of a number of cycling genes in Arabidopsis that have peak expression near dusk (Harmer et al., 2000). There are also four other putative evening elements in the ZGT promoter in a sense orientation (within /H11002870 bp of the transcriptional start site) that differ by only one nucleotide from the core seven-nucleotide consensus sequence (Figure 2D). Arabidopsis pZGT has only the one putative evening element in its promoter, as shown in Figure 2D.

These alternate terminations give rise to two isoforms of ZGT transcripts, ZGT-s and ZGT-l, that were detected in a long-running RNA gel blot assay (Figure 3C). ZGT-l is 147 bp longer than ZGT-s in the 3’ untranslated region. These alternate forms of the ZGT mRNA exhibited a tissue-specific pattern. At LDT 8, ZGT-l transcripts were expressed approximately equally in all tissues examined, whereas ZGT-s was transcribed differentially in different tissues (Figure 3C). In leaf tissues, levels of the ZGT-s transcript were much higher than those of the ZGT-l transcript. Both transcripts showed similar expression levels in stem tissues, whereas levels of the ZGT-s transcript were very low in root tissues. These results suggest that the 3’ untranslated region of the ZGT gene may play a regulatory role in development.

ZGT Promoter Activity Is Rhythmic

Because the abundance of ZGT mRNA clearly cycles under both light/dark and free-running conditions (Figure 1), we were interested to know if its promoter was regulated by the circadian clock. To continuously monitor the circadian activity of the ZGT promoter, we created transgenic tobacco plants expressing the fusion gene P_{ZGT}::luc, in which the expression of a cDNA encoding the firefly luciferase structural gene (luc) is driven by a ZGT 5’ flanking region (from

Figure 1. Daily and Circadian Rhythms of ZGT Transcript Abundance.

The daily rhythms are shown at left and the circadian rhythms at right.
(A) Tobacco plants were first grown in a greenhouse under natural LD conditions for 6 weeks and then entrained to LD 12:12 for three cycles before harvesting RNA in LD or released to LL at 50 μE m⁻² sec⁻¹ for harvesting RNA in LL. Total RNA was collected from leaves at the indicated LD or LL time points. RNA gel blots (20 μg/lane) were first probed with a ZGT fragment and then stripped and rehybridized with an rDNA probe to normalize the loading. The open and closed bars beneath the blots represent the light and dark periods, respectively, and the hatched bars indicate subjective nights in LL.
(B) Quantification of the data shown in (A). The maximum transcript level was taken as 1.0.

ZGT mRNA Has Alternate Termination Sites and Tissue-Specific Expression Patterns

Our 3’ RACE analysis revealed two different termination sites for ZGT transcripts, each having a conserved AGTCTT motif just before the poly(A) tail (Figures 2A, 3A, and 3B).
Figure 2. Structure of the Tobacco ZGT Gene.

(A) Scheme of the ZGT gene is shown at the top. The coding region of the ZGT gene is denoted by an open box, and its translational initiation and termination codons are marked as ATG (starting at +74) and TAA (starting at +946), respectively. The transcriptional start site is numbered as +1 and indicated by an arrow. The hatched box represents the aspartate- and glutamate-rich region (DE Rich) near the C terminus. The location of the putative G/E box in the 5' flanking region is depicted by a closed square, and the two closed circles denote the positions of two putative evening elements (EE1 and EE2). The two HindIII restriction sites are labeled. The lengths of the two transcripts, ZGT-1 and ZGT-s, are shown immediately below. AGTTT indicates the conserved nucleotide motif in the 3' end of these two cDNAs, which occurs just before the poly(A) sequence.

(B) Sequence alignment of the entire coding region of the tobacco ZGT gene (GenBank accession number AF368237) and a putative homolog.
Expression of the ZGT Gene Is Light Regulated and Light Dependent

The rhythmic expression of many plant genes is dependent on light, and our RNA gel blot analyses showed that ZGT mRNA expression was light dependent, because ZGT expression in DD was very low compared with that in LD or LL. Therefore, we set out to determine if the activity of the ZGT promoter is affected by light. The rhythmicity of ZGT promoter activity as reported by a P<sub>ZGT</sub>::luc construct in transgenic seedlings damped in constant darkness (Figure 5A). After LL was restored, however, the rhythm of P<sub>ZGT</sub>::luc luminescence recovered immediately, indicating that a crucial light signal is involved in the expression of the ZGT gene. Because the luminescence of the P<sub>ZGT</sub>::luc reporter responded so rapidly to the DD-to-LL transition (Figure 5A), we examined the response and sensitivity of ZGT gene expression to light pulses. As illustrated in Figure 5B, a single brief light pulse was able to rapidly activate the ZGT promoter activity of transgenic seedlings that had been in darkness for an extended period (80 hr). Even a 5-min light pulse elicited a significant increase in ZGT promoter activity that continued for at least 8 hr, and a 1-hr light pulse provoked a greater than twofold increase of luminescence that required many hours in darkness to dissipate (Figure 5B). Light-induced accumulation of ZGT transcripts was confirmed by RNA gel blot analysis (Figure 5C). These results show that the expression of the ZGT gene is light inducible and further suggest that the regulation of its photosresponsiveness occurs at least partially via changes in transcriptional rates. These results are consistent with the hypothesis that the G/E box in the ZGT promoter is recognized by light-regulated transcription factors such as PIF3 (Martínez-García et al., 2000) and HY5 (Chattopadhyay et al., 1998).

Expression of ZGT Sustains Circadian Oscillations of LHCB Expression in DD but Has Little Effect in LL

To determine if the ZGT gene plays a role in the plant circadian system, the ZGT structural gene was expressed under the control of the cauliflower mosaic virus 35S promoter found in the Arabidopsis database (GenBank accession number CAB87751) by using the FASTA algorithm (Pearson and Lipman, 1998) in the GCG package (Genetics Computer Group). Identical amino acids are indicated by vertical lines, and similar amino acids are indicated by dots. Spacers and gaps are indicated by dashes.

(A) Alignment of the flanking sequences of putative G boxes (or E boxes) in the promoters of tobacco ZGT (from −151 to −138; this study, GenBank accession number AF368237), a putative Arabidopsis ZGT homolog (pZGT; GenBank accession number CAB87751), Arabidopsis CCA1 (U28422), Arabidopsis LHY (AJ006404), Drosophila vri (Y11837), Drosophila per (D00009), Drosophila tim (U37018), and the mouse vasopressin gene (M88354). The central CACGTG cores are outlined in black. To clarify the alignments, the sequences labeled with asterisks are inverted relative to their promoters. Identical nucleotides in at least four of the eight flanking sequences are shaded in gray.

(B) Comparison of putative evening elements (EE) in the tobacco ZGT and Arabidopsis pZGT promoter regions. The consensus sequence for the nine-nucleotide AAAATATCT EE that was found to be a conserved motif in the promoters of various cycling genes in Arabidopsis (Harmer et al., 2000) is shown at the bottom. Single asterisks denote the sequences that are on the antisense strand relative to their promoters. Nucleotide locations relative to the transcription start site are shown to the right (the coordinate with double asterisks indicates the distance from the transcription start site, because the transcriptional start site for Arabidopsis pZGT is not yet determined). The EE cores are outlined in black. Identical nucleotides in the flanking regions of at least three of the seven sequences are shaded in gray.
fused to a tobacco mosaic virus translational enhancer (Ω) sequence (Figures 6A and 6B). Expression of the 35S promoter is constitutive in plants during the circadian cycle (Millar et al., 1992). The ZGT gene was expressed in transgenic tobacco harboring the P_{LHCB}:luc luminescence reporter (Anderson and Kay, 1995) to determine whether the expression of ZGT would affect LHCB expression. We compared the rhythmic luminescence expression of the P_{LHCB}:luc reporter in seedlings between single transformants with P_{LHCB}:luc (wild type) and double transformants with P_{LHCB}:luc and P_{35S}:ZGT (OX/ZGT). P_{LHCB}:luc reporter luminescence was monitored in either LL or DD after entrainment by LD 16:8 cycles. Under DD conditions, the circadian luminescence rhythms damped rapidly in wild type, whereas circadian oscillation was sustained stably in the ZGT gene–expressing seedlings (Figure 6D, OX/ZGT). However, constitutive expression of the ZGT gene did not significantly affect the circadian rhythms of LHCB promoter activity in LL conditions (Figure 6C).

The fact that constitutive expression of ZGT sustains the rhythmic expression of the clock-controlled and light-responsive LHCB gene in DD encouraged us to determine whether the ZGT protein may be involved in the circadian system via either an input pathway and/or an output pathway. To determine if the expression of ZGT can affect the sensitivity of the circadian oscillator to light signals via the input pathway, we compared the magnitude of light-induced phase resetting in the wild-type versus the OX/ZGT background. First, we measured a phase response curve to 30-min white light pulses and found that both wild type and OX/ZGT exhibited very similar phase response curves (data not shown). However, when we chose a specific phase (circadian time 19[CT 19]) that

Figure 3. Mapping and Expression of Two Transcripts of the ZGT Gene.

(A) and (B) The 3' ends of cDNA sequences of the two ZGT transcript isoforms, ZGT-s and ZGT-l, respectively, determined by 3' RACE from total RNA in leaf tissues collected at LDT 8. Lanes G, A, T, and C indicate the reactions for sequencing nucleotides. The conserved 3' end motif of these two transcripts is underlined.

(C) Tissue-specific expression patterns of ZGT-s and ZGT-l were revealed by RNA gel blot analysis, using a ZGT hybridization probe. Total RNA was isolated at LDT 8 from root, leaf, or stem tissues. Equal amounts of total RNA (20 μg/lane) were loaded in each lane. The two ZGT transcripts are indicated by arrows.
gave reproducible phase shifts for both lines, we found that they showed different responses to light pulses whose duration ranged from 3 to 30 min. As shown in Figure 7, the magnitude of the light-induced phase shift increased with pulse duration. The phase shifts of OX/ZGT were statistically greater than those of wild type at the $P = 0.0001$ level, as analyzed by analysis of variance (randomized block design). Therefore, OX/ZGT seedlings are significantly more sensitive to phase resetting by brief light pulses than are wild-type seedlings.

**Figure 4.** Circadian Rhythmicity of ZGT Promoter Activity.

(A) ZGT promoter activity was monitored with the luminescence reporter construct $P_{ZGT}::luc$. The diagram shows the T-region in the construct $p_{ZGT}/Luc$ for $P_{ZGT}::luc$ expression. The black box represents the 5’ flanking sequence of the ZGT gene from −950 to +73 ($P_{ZGT}$). The open box denotes the coding region for firefly luciferase (LUC). The gray box indicates the nopaline synthase polyadenylation region. LB and RB represent T-DNA left and right repeats, respectively. The stippled box to the left of the $P_{ZGT}::luc$ cassette is an expression cassette of the neomycin phosphotransferase gene. Arrows indicate the direction of transcription.

(B) Images of transgenic tobacco seedlings (3 weeks old) expressing the fusion gene $P_{ZGT}::luc$ captured every 4 hr in LL (labeled in circadian time [CT]). Seedlings had been entrained to LD 16:8 and then transferred to LL, and the images shown are for 24 to 44 hr in LL. Intensity is encoded in pseudocolor: blue is dim luminescence, and green/yellow is bright luminescence.

(C) Rhythmic luminescence of transgenic tobacco seedlings harboring $P_{ZGT}::luc$ or $P_{LHCb}::luc$ reporter constructs in LL (seedlings had been entrained to LD 16:8 before release into white LL). At least six channels (one seedling per channel) were monitored continuously for luminescence for each transformant, and representative data are shown. Time 0 is the beginning of LL (subjective dawn; i.e., the end of the last dark interval in LD 16:8).
Table 1. Comparison of the Luminescence Period, Amplitude, and Phase of Reporter Expression between P_{ZGT}:luc and P_{LHC}:luc Transgenic Seedlings in LL

<table>
<thead>
<tr>
<th>Period (hr)</th>
<th>Phase Difference (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>Peak</td>
</tr>
<tr>
<td>ZGT</td>
<td>24.9 ± 0.4</td>
</tr>
<tr>
<td>LHC</td>
<td>24.5 ± 0.3</td>
</tr>
</tbody>
</table>

DISCUSSION

Plant Circadian Oscillators Can Run in DD

Many plant circadian rhythms exhibit sustained oscillations in LL but dampen in DD. Our data clearly show that under the appropriate conditions, these rhythms can be sustained, as by overexpression of ZGT. This result strongly suggests that the dramatic damping of rhythms in DD is not due to a concomitant damping of the oscillator itself but to reduction of the coupling between oscillator and rhythm. There is other evidence for the continued oscillation of central plant clockworks in DD. For example, another gene (CCR2) is expressed rhythmically for many cycles in DD (Strayer et al., 2000), and several other lines of evidence support the same conclusion (summarized in Johnson et al., 1998 and Johnson, 2001). Overexpression of ZGT can restore this coupling between clockwork and rhythm and allow sustained rhythms of P_{LHC}:luc luminescence. It has been shown that overexpression of phytochrome and/or stimulation of phytochrome by brief red light pulses can help to sustain LHC expression rhythms (Kay and Millar, 1992; Johnson, 2001). Mutations in genes involved in phototransduction pathways, especially det1 and cop1, also can allow sustained rhythmicity of P_{LHC}:luc luminescence (Millar et al., 1995). In addition, mutations in PHYA and CRY1 sustain CAt gene family. DD is an extreme case of low metabolic capacity that is rarely or never seen in nature (at least not after plants have passed the seedling stage); when light energy is absent, output gene expression becomes uncoupled from clock control. These speculations further imply that photoreceptive pathways might have evolved in such close conjunction with plant circadian oscillators that they became intrinsically linked in terms of expression of the output rhythms (Johnson, 1994; Johnson, 2001).

Light- and Clock-Regulated Motifs in the ZGT Promoter

As shown in Figure 5, we found that ZGT is robustly activated by light. This light regulation is likely to be mediated at least in part by the G/E-box motifs in the ZGT promoter. Martínez-García et al. (2000) demonstrated that PIF3, a nucleus-localized basic helix-loop-helix transcription factor that interacts with phytochrome, binds specifically to G-box–containing promoter sequences from various light-regulated plant genes, such as Arabidopsis CCA1, LHY, SPA1, and RBCS-1A. Phytochrome B binds reversibly to G-box–bound PIF3 upon light-triggered conversion of phytochrome B to its biologically active conformer. A similar story has been reported for the bZIP protein factor, HY5 (Chattopadhyay et al., 1998). The presence in the ZGT promoter of this core G-box element for light signaling is consistent with a role in the light regulation of ZGT by PIF3 and/or HY5. It is fascinating that this same motif functions in animal circadian feedback loops (there called an E-box element), where it is thought to be crucially important for rhythmic transcriptional regu-
We also found evening element (EE) motifs in the ZGT promoter. This EE was recently identified in Arabidopsis to be a motif that regulates circadianly controlled promoters for peak expression near dusk (Harmer et al., 2000). Although the EE motives were found in an antisense orientation in both tobacco ZGT and Arabidopsis pZGT gene promoters, it is possible that they function as circadian enhancers partially mediating rhythmic expression of ZGT. The G/E and EE sequence information provide clues that can direct future research in analyzing the regulation of ZGT expression by light and the circadian clock.

The Role of ZGT in Plant Circadian Systems

Our evidence for a role of ZGT in plant circadian systems comes from experiments in which ZGT was overexpressed (Figures 6 and 7). Our results clearly show that when ZGT is expressed constitutively, the circadian oscillator that controls LHCB expression is “unmasked” and shown to be running in DD. We believe that ZGT plays a role in the endogenous circadian system as well. Although it is possible that the results of this overexpression provoke ZGT’s gene products into performing unusual functions, we postulate that its light and circadian regulation (Figures 1 and 5) argue for a physiological role. Ultimately, this question can

Figure 5. ZGT Gene Expression Is Light Inducible.

(A) The rhythmicity of ZGT promoter activity is light dependent. Reporter expression of the fusion gene P_{ZGT}::luc in transgenic seedlings that had been grown under LD 16:8 for 1 week showed significant damping of the circadian rhythm after the seedlings were placed in DD. The expression of P_{ZGT}::luc luminescence rapidly recovered stable rhythmicity upon transfer to white LL after 6 days in DD (at time 144 hr).

(B) Acute induction of P_{ZGT}::luc reporter expression by brief light pulses. One-week-old transgenic P_{ZGT}::luc seedlings grown under LD 16:8 cycles were transferred to DD, and the luminescence was measured by an automated photomultiplier apparatus (one seedling per channel). After the seedlings had been maintained in DD for 80 hr, they were irradiated with white light pulses (50 μE m^{-2} sec^{-1}; LP) for 5, 30, or 60 min (triplicates for each). Controls were seedlings that did not receive a light pulse (No LP). After the light pulses, seedlings were returned to DD, and luminescence was measured. The basal levels were normalized to correspond with those of the control. Data for two representative seedlings are shown for each treatment.

(C) Light-induced accumulation of the ZGT transcripts. Wild-type seedlings were grown under LD 12:12 cycles for 3 weeks and then transferred to DD. After 56 hr in DD, the seedlings (five seedlings in each group) were irradiated or not irradiated with a 1-hr white light pulse (50 μE m^{-2} sec^{-1}). One hour after the light pulse had ended, seedlings were harvested for isolation of total RNA. Analysis of the RNA gel blot (20 μg/lane) was as described in Figure 1.
be answered by knocking out ZGT and determining whether expression of light-dependent cycling genes is drastically altered. Our data indicate that in nature, the LD cycle and the circadian clock cooperate to cause a robust rhythm of ZGT mRNA during the daily cycle. Assuming that the ZGT protein is the relevant active gene product and that it has a turnover rate that allows significant accumulation, we postulate that the rhythmic abundance of ZGT plays a role in coupling central circadian oscillators to output rhythms and possibly amplifies light input signals into the clockwork. Although we have tested only the LHCB output rhythm, the pattern of many circadian outputs is similar to that of LHCB;
therefore, it is reasonable to postulate that ZGT expression may affect other outputs.

What might the role of ZGT be in the maintenance of robust \textit{LHCB} rhythmicity? Let us start by mentioning what ZGT is not. First, it is not maintaining the \textit{LHCB} rhythm in DD by feeding back to the central oscillator ([i.e., output feedback, as in other systems [Mrosovsky, 1996]] and thereby maintaining the oscillation of a central clockwork that normally damps in DD (Johnson et al., 1998; Strayer et al., 2000; Johnson, 2001). Second, rhythmic expression of ZGT is not the clock-regulated gate of \textit{LHCB} expression postulated by Nagy, Kay, and co-workers (Kay and Millar, 1992; Fejes and Nagy, 1998), because constitutive expression of ZGT does not lead to constitutive expression of \textit{LHCB} promoter activity.

ZGT promoter activity appears to phase-lag that of \textit{LHCB} promoter activity (Figure 4C). On the basis of relatively infrequent sampling, ZGT mRNA abundance (Figure 1B) appears to follow ZGT promoter activity relatively closely in LL (Figure 4C). We have no information about the abundance of ZGT protein, but assuming that its half-life is a few hours, then it is unlikely that a rhythm of ZGT expression directly turns on and off the rhythm of \textit{LHCB} promoter activity. Our working model postulates that a rhythm of ZGT abundance in natural LD cycles establishes a state during the light interval such that the coupling of the \textit{LHCB} gene (and other genes) is maintained through the next cycle (Figure 8). This role of ZGT might be likened to the clutch of a vehicle: when engaged (by light-dependent expression of ZGT in LD and LL), clock and outputs are linked; when disengaged (by low expression of ZGT in DD), clock and outputs are unlinked. Thus, in this model, ZGT becomes the sensor of illumination that determines whether the clock gates the rhythmic expression of \textit{LHCB} and possibly other genes. It is possible that ZGT is a link between PIF3/HY5/phytochrome (and other photoreceptors) and \textit{LHCB} expression; possibly phytochrome overexpression and/or stimulation by brief red light pulses (Kay and Millar, 1992) leads to a concomitant increase in ZGT expression that maintains \textit{LHCB} rhythms. In addition to its direct control by light, ZGT also is controlled by the central oscillator, as demonstrated by its expression patterns in LL (Figures 1 and 4).

On the basis of the results depicted in Figure 7, it is possible as well that ZGT acts as an amplifier of the circadian phototransduction pathway (Figure 8). The data in Figure 7 show that ZGT expression can increase significantly the sensitivity of the clock to light pulses, but the times at which light pulses are most effective in shifting phase are when ZGT mRNA levels are very low, namely, around dawn and dusk. Therefore, whether ZGT can act in the entrainment path of the circadian system in natural conditions depends largely on the turnover rate of the ZGT protein, which is unknown at present. If the turnover of ZGT is slow enough that significant levels are present at dawn and/or dusk, then it could play a significant role in the input signal to the clock. Until more is known about ZGT protein expression, however, we favor the interpretation that the primary effect of ZGT is in the coupling of clock to outputs. Nevertheless, the data shown in Figures 5 and 7 suggest that ZGT could function as a component in plant...
Cloning of the ZGT Gene

The ZGT gene was originally isolated from a tobacco (*Nicotiana tabacum*) genomic DNA/λCharon 35 library using a probe containing the cyanobacterial kaiABC gene cluster (Ishii et al., 1998) under conditions of low stringency. To clone the ZGT gene, we inserted ~8.5 kb of SacI-digested genomic DNA harboring the ZGT fragment into pBluescript II KS+ (Stratagene, La Jolla, CA) to produce pSac8.5. On the basis of restriction analyses and DNA gel blot analyses, a HindIII fragment (1.2 kb), a BamHI fragment (2.5 kb), and a BamHI-SacI fragment (6.0 kb) containing ZGT sequence were then subcloned into pBluescript II KS+ or pGEM-7 (Promega, Madison, WI), yielding pHind1.2, pBam2.5, and pBS6.0, respectively.

Nucleotide sequencing of both DNA strands in the subclones was determined by the dideoxy chain termination method (Del Sal et al., 1989) or using an ABI automated sequencer (PE Applied Biosystems, Foster City, CA). The initial sequence information from pHind1.2 was determined by the dideoxy chain termination method (Del Sal et al., 1989) or using an ABI automated sequencer (PE Applied Biosystems, Foster City, CA), yielding pHind1.2, pBam2.5, and pBS6.0, respectively.

Generation of Transgenic Plants

Transgenic tobacco plants were obtained by Agrobacterium-mediated transformation of leaf discs with the binary constructs pOX/ZGT and pZGT/luc (Horsch et al., 1985). Tobacco (var Xanthi) expressing a fusion of the promoter for the LHCB1'1 gene from *Arabidopsis thaliana* and the firefly luciferase gene (P_{LHCB1'1}-luc), which was kindly provided by Drs. Shawn Anderson and Steve Kay (Anderson and Kay, 1995; Dr. Kay is presently at Scripps Research Institute, La Jolla, CA), was transformed by Agrobacterium harboring pOX/ZGT with a gentamycin-resistant selection marker. Tobacco (var NC
Three-week-old transgenic seedlings expressing the fusion gene P_{ZGT}/luc by Dr. Takao Kondo (Nagoya University, Nagoya, Japan) and the CHRONO program (Roenneberg and Taylor, 1999).

Antibiotic resistance of transgenic seedlings was tested by germinating P_{ZGT} transgenic tobacco seed in 10 \mu g/mL gentamycin sulfate (for OX/ZGT transformants) or kanamycin sulfate (for ZGT/luc transformants). Homozygous transformants were used in all experiments; these were determined by assaying transgene expression and by confirming nonsegregation of corresponding antibiotic resistance in the T2 or T3 seedling populations.

Seedling Growth Conditions

Seed were sterilized in 20% bleach (Clorox) containing 0.1% SDS for 15 min and washed three times with sterilized water. After the sterilized seed were soaked in 10 \mu M gibberellic acid overnight, they were germinated on 0.8% agar-solidified half-strength MS medium and grown in an incubator at 26\degree C under light/dark (LD) cycles of cool-white fluorescent light (50 \mu E m^{-2} sec^{-1}) and darkness (either LD 12:12 or LD 16:8) for 1 week in the case of bioluminescence assays or for 3 to 4 weeks in the case of RNA isolation. In some experiments, the seedlings were then transferred to constant light (LL) or constant darkness (DD) as specified in the figure legends.

RNA Gel Blot Analysis

Total RNA was prepared from transgenic or wild-type tobacco (var Xanthi) plants and analyzed in RNA gel blots as described by Xu et al. (1996). An \alpha-^{32}P-dCTP–labeled 1.2-kb HindIII fragment from the ZGT gene was used as a probe for RNA gel blot assays. RNA gel blots were then stripped and rehybridized with a 2.5-kb EcoRI fragment probe containing an Arabidopsis rRNA gene for normalization of the RNA loading in each lane (Xu et al., 1996). Quantification of RNA gel blots was performed with an IS-1000 digital imaging system using Spot Density Tool software (Alpha Innotech, San Leandro, CA).

Analysis of Luminescence Rhythms in Transgenic Plants

Seedlings expressing firefly luciferase were germinated on half-strength MS agar medium and grown at 26\degree C in LD 16:8. Before lights off in the last LD cycle, individual 7-day-old seedlings were transplanted into a small scintillation vial filled with 0.4 mL of liquid MS medium containing 200 \mu M luciferin (Promega). For luminescence measurement in DD, the assay medium was supplemented with 10 \mu M ATP. This vial was then placed inside a 20-mL glass scintillation vial. After the dark interval was over, the vials were transferred to an automated multichannel photomultiplier tube apparatus (Johnson et al., 1995) for recording luminescence in either LL or DD. Periods and phases of the luminescence rhythms were determined based on the trough and/or peak values using LVA software supplied by Dr. Takao Kondo (Nagoya University, Nagoya, Japan) and the CHRONO program (Roenneberg and Taylor, 1999).

Imaging of Luminescent Seedlings

Three-week-old transgenic seedlings expressing the fusion gene P_{ZGT}/luc that had been grown in LD 12:12 cycles were transferred into a 100-mm-diameter Petri dish filled with 15 mL of liquid MS medium supplemented with 200 \mu M luciferin and incubated in continuous light. After 24 hr in LL, luminescent seedling images were captured with 80-sec exposures every 4 hr for 20 hr. Images were captured with a cooled charge-coupled device camera (TE/CCD512BKS; Princeton Instruments, Trenton, NJ) under the control of and analyzed by custom software designed by Dr. Takao Kondo (Kondo et al., 1994).

Assay of Phase Shifting

Seedlings of the P_{VIC8}:luc reporter in either the wild-type or the OX/ZGT background were germinated and grown as described above. At dusk of the final LD cycle, seedlings were placed into darkness for 32 hr. For the phase response experiment (data not shown), seedlings were exposed to 30 min of white light pulses (50 \mu E m^{-2} sec^{-1}) at different phases during the dark treatment (three samples for each phase and/or treatment). At the end of the 32 hr of darkness, seedlings were placed in constant red light (20 \mu E m^{-2} sec^{-1}), and their luminescence rhythms were monitored as described above. For the experiments shown in Figure 7, seedlings were exposed to white light pulses (50 \mu E m^{-2} sec^{-1}) of the indicated durations starting at hr 27 in DD (circadian time 19\text{CT} 19\text{J}). After completion of the light pulse, seedlings were returned to DD until hr 32 and then transferred to constant red light, and their luminescence rhythms were measured. Periods and phases of the luminescence rhythms were determined based on the phases of the peaks and/or troughs.

ACKNOWLEDGMENTS

We are grateful to many colleagues for their assistance in this study: to Drs. Takao Kondo and Masahiro Ishiura for sharing the kai DNA before publication of its sequence for use as a hybridization probe; to Drs. Matt Olson and Mark Stokes for assistance with statistical analyses; to Dr. Jiqing Sai for sharing his phase-shifting protocol (Figure 7) before publication; to Drs. Steve Kay and Shawn Anderson for generously providing P_{VIC8}:luc transgenic tobacco seed; to Dr. Robert Hayes for the tobacco genomic library; to Dr. Virginia Walbot for the firefly luciferase (luc) construct; to Dr. Pat Maliga for binary vectors; to Dr. Judith Thomas for NC TG22 (Maryland Mammoth) tobacco seed; and to Dr. Takao Kondo for software for the acquisition and analysis of luminescence rhythms. This research was supported by grants from the National Institute of Mental Health (Nos. MH43836 and MH01179).

Received December 22, 2000; accepted March 26, 2001.

REFERENCES


A Clock- and Light-Regulated Gene That Links the Circadian Oscillator to LHCB Gene Expression
Yao Xu and Carl Hirschie Johnson
*Plant Cell* 2001;13;1411-1426
DOI 10.1105/TPC.000545

This information is current as of December 29, 2017

<table>
<thead>
<tr>
<th>Section</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>This article cites 38 articles, 19 of which can be accessed free at:</td>
</tr>
<tr>
<td></td>
<td>/content/13/6/1411.full.html#ref-list-1</td>
</tr>
<tr>
<td>eTOCs</td>
<td>Sign up for eTOCs at:</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>CiteTrack Alerts</td>
<td>Sign up for CiteTrack Alerts at:</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.plantcell.org/cgi/alerts/ctmain">http://www.plantcell.org/cgi/alerts/ctmain</a></td>
</tr>
<tr>
<td>Subscription Information</td>
<td>Subscription Information for <em>The Plant Cell</em> and <em>Plant Physiology</em> is available at:</td>
</tr>
<tr>
<td></td>
<td><a href="http://www.aspb.org/publications/subscriptions.cfm">http://www.aspb.org/publications/subscriptions.cfm</a></td>
</tr>
</tbody>
</table>