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.

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and found to bind a variety of DNA binding proteins (Anderson and Kay, 1995; Carré and Kay, 1995), including the critical clock protein CCA1 (Wang and Tobin, 1998).

Numerous lines of evidence, including the expression of certain genes such as CCR2/AtGRP7 (Heintzen et al., 1997; Strayer et al., 2000), indicate that central circadian oscillators continue to operate in plants in DD (Johnson et al., 1998; Johnson, 2001), yet most of the known outputs of plant circadian oscillators—including LHC gene expression—dampen in DD. We have discovered a new plant gene, which we call ZGT, from tobacco, whose overexpression maintains the expression of the LHCBI+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 LHCBI+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

In addition, putative evening elements AAAATATCT (EE1, /H11002 178 to /H11002 186) and AATATCT (EE2, /H11002 231 to /H11002 237) 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 /H11002 870 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 equivalently 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 **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 AGTGT 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 PZGT:\luc construct in transgenic seedlings damped in constant darkness (Figure 5A). After LL was restored, however, the rhythm of PZGT:\luc luminescence recovered immediately, indicating that a crucial light signal is involved in the expression of the ZGT gene. Because the luminescence of the PZGT:\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 photoreponsiveness 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 (Martinez-Garcia et al., 2000) and HY5 (Chattopadhyay et al., 1998).

Expression of ZGT Sustains Circadian Oscillations of LHC 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. (C) 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 vril (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 orientation in their promoters. Identical nucleotides in at least four of the eight flanking sequences are shaded in gray. (D) 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 elements 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 \( \text{P}_{\text{LHCB}}:\text{luc} \) 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 \( \text{P}_{\text{LHCB}}:\text{luc} \) reporter in seedlings between single transformants with \( \text{P}_{\text{LHCB}}:\text{luc} \) (wild type) and double transformants with \( \text{P}_{\text{LHCB}}:\text{luc} \) and \( \text{P}_{\text{35S}}:\text{ZGT} \) (OX/ZGT). \( \text{P}_{\text{LHCB}}:\text{luc} \) 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
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 $pZGT/Luc$ 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 $P_{LHC}$::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).
were calculated as the difference between the P
based on either peak or trough values as indicated. Phase differences
as by overexpression of
in LL but dampen in DD. Our data clearly show that under
Many plant circadian rhythms exhibit sustained oscillations

| Table 1. Comparison of the Luminescence Period, Amplitude, and Phase of Reporter Expression between P\textsubscript{ZGT:luc} and P\textsubscript{LHCB:luc} Transgenic Seedlings in LL |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Period (hr)a    | Phase Difference (hr)a |
| Promoter Peak   | Trough           | Amplitudeb Peak | Trough          |
| ZGT             | 24.9 ± 0.4       | 24.4 ± 0.5      | 1.4 ± 0.1       | 6.2 ± 0.6       | 7.0 ± 1.1 |
| LHCB            | 24.5 ± 0.3       | 24.6 ± 0.3      | 3.9 ± 0.3       |                  |                  |

a Periods and phases of the luminescence rhythms were determined based on either peak or trough values as indicated. Phase differences were calculated as the difference between the P\textsubscript{ZGT:luc} and the P\textsubscript{LHCB:luc} rhythms in hours. Values shown are averages ± sd (n = 6).
b Amplitude was evaluated as the ratio of the second peak value to the second trough value.

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\textsubscript{LHCB:luc} luminescence. It has been shown that overexpression of phytochrome and/or stimulation of phytochrome by brief red light pulses can help to sustain LHCB 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\textsubscript{LHCB:luc} luminescence (Millar et al., 1995). In addition, mutations in PHYA and CRY1 sustain CAT mRNA rhythms (Zhong et al., 1997). However, one difference between the results with OX/ZGT and the det1/cop1 mutants is that ZGT overexpression does not alter the circadian period as do the det1 and cop1 mutations. In addition to the presence of a G/E box in P\textsubscript{ZGT} that might be regulated by PIF3 and/or HY5, these correlations suggest that ZGT could interact with phototransduction pathways in plants. Aside from sustaining rhythms in DD, the other phenotype of ZGT overexpression that we have observed—is increased sensitivity to light-induced phase shifting (Figure 7)—suggests that ZGT is involved directly in phototransduction (Figure 8).

Why might plant output rhythms dampen in DD even though their central oscillator is running? Plant metabolism is absolutely dependent on light energy. Consequently, changes in light intensity profoundly alter the metabolic status of plant cells. However, for a timekeeping mechanism to be useful, it must be impervious to these changes in metabolism. Therefore, plants appear to have evolved a system in which the linkage between central oscillators and outputs is adjustable depending on metabolic status. The central clock oscillates under all conditions, as befits its role as an accurate timekeeper, but the degree of its coupling to outputs varies as a function of metabolism. For plants, light (and likely temperature as well) is probably an excellent predictor of metabolic capacity, so having the coupling between clock and outputs be dependent on light energy is logical. Thus, the amplitude of output rhythms is a function of light energy even while the underlying oscillator is relatively unaffected by light intensity (in wild-type plants). This hypothesis would be particularly applicable to plant output genes whose functions relate to the use of light energy, such as the LHCB 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-
Circadian Clock Coupling Factor

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
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;

Figure 6. Effect of Constitutive Expression of the ZGT Gene on Circadian Rhythms of the Clock-Controlled LHCB Gene in Transgenic Seedlings.

(A) Scheme of the ZGT expression cassette in the construct pOX/ZGT. Between the left and right border sequences (LB and RB) of the T-DNA, the ZGT coding region and its 3′ flanking sequence (from +69 to +1236 relative to the transcription start site) were fused to the cauliflower mosaic virus 35S promoter (35S) with a tobacco mosaic virus 5′ untranslated leader sequence (II) followed by the poly(A) addition sequence from the nopaline synthase gene (gray box). A plant selection marker (a gentamycin resistance cassette) was included. Arrows indicate the direction of transcription.

(B) Overproduction of ZGT transcripts in the OX/ZGT transgenic plants. Total RNA (20 μg/lane) was prepared at CT 0 from the leaves of 6-week-old wild-type plants (WT) or six different randomly chosen OX/ZGT transgenic plants (lanes 1 to 7). The blot was probed sequentially with the ZGT probe, stripped, and reprobed with an rDNA probe.

(C) and (D) Overexpression of the ZGT gene sustains the rhythmic expression of the clock- and light-regulated LHCB1*1 promoter in DD but has no significant effect on its rhythms in LL. Rhythmic P_{LHCB1*1}::luc luminescence in wild-type or OX/ZGT transgenic seedlings was measured continuously in white LL (C) or DD (D) after release from LD 16:8 cycles. Representative data are shown as normalized luminescence from at least three independent experiments; in each experiment, 10 channels (three seedlings per channel) were used for both wild-type and OX/ZGT lines.
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 LHCb rhythmicity? Let us start by mentioning what ZGT is not. First, it is not maintaining the 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. This possibility can be eliminated because of the evidence that the central oscillator does not dampen rapidly in DD (Johnson et al., 1998; Strayer et al., 2000; Johnson, 2001). Second, rhythmic expression of ZGT is not the clock-regulated gate of 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 LHCb promoter activity.

ZGT promoter activity appears to phase-lag that of 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 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 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 LHCb and possibly other genes. It is possible that ZGT is a link between PIF3/HY5/phytochrome (and other photoreceptors) and 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 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

![Figure 7. Phase Resetting by Light Pulses in Wild-Type and OX/ZGT Transgenic Seedlings.](image-url)
Cloning of the ZGT Gene

The ZGT gene was originally isolated from a tobacco (*Nicotiana tabacum*) genomic DNA/Charon 35 library using a probe containing the cyanobacterial *kai*ABC gene cluster (Ishiiura 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 assays, a HindIII fragment (1.2 kb), a BamHI fragment (2.5 kb), and a BamHI-SacI fragment (6.0 kb) containing ZGT DNA sequences from pSac8.5 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 dyeode 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 used to design primers to determine the complete genomic or cDNA sequence of the ZGT gene along with other subclones. The DNA and protein sequences were analyzed using GCG software (Genetics Computer Group, Madison, WI). The GenBank database was searched with the putative amino acid sequence of ZGT, using the BLAST (Altschul et al., 1997) and FASTA (Pearson and Lipman, 1998) algorithms.

3’ and 5’ RACE Assay

The 3’- and 5’-ends of cDNA sequences of the ZGT gene were determined by 3’- or 5’-rapid amplification of cDNA ends (RACE), according to the manufacturer’s directions (Life Technologies, Rockville, MD). Total RNA for RACE analysis was isolated from leaf tissues of 45-day-old tobacco plants 8 hr into the light/dark cycle. The 5’-RACE of ZGT cDNA was performed using the first and nested gene-specific primers 5’-TCTTCAGCTCCACCTGC-3’, 5’-TCTACCGCCTAAGCG-ACCG-3’, and 5’-TCTTCAGCTCCACCTGC-3’. The 3’-RACE was conducted with the nested gene-specific primers 5’-TGATAACGGCGTTAACAGAC-3’, 5’-TGATCCGTGTGAGCATC-3’, and 5’-ACGGAGCTTGTTGGCTG-3’. All of the 3’- and 5’-RACE fragments obtained were cloned into pBluescript II KS+ and then sequenced as described above.

Recombinant Plasmid Construction

A 1168-bp DNA fragment at positions +69 to +1236 containing the entire ZGT coding region and part of its 3’ flanking sequence was cloned into the Smal site of pBluescript II KS+, and the insert was confirmed by sequence analysis. The Sall-Sacl fragment containing a structural gene for firefly luciferase (*luc*) in pJD301 (Luehrsen et al., 1992), which was kindly provided by Dr. Virginia Walbot (Stanford University, Stanford, CA), was replaced with the ZGT coding sequence to produce pUC/35S::ZGT. The SphI (end-blunted)-BgIII fragment from pUC/35S::ZGT was inserted into the Smal-BamHI site of the binary vector pPZP122 (Hajdukiewicz et al., 1994), which was kindly provided by Dr. Pat Maliga (Rutgers, State University of New Jersey, Piscataway, NJ), to make pOX/ZGT. The BamHI-Ncol fragment in pJD301 was removed and then replaced with the 5’-flanking polymerase chain reaction fragment (~950 to +73) of the ZGT gene to create the pZGT::luc fusion gene. The BamHI-BglII fragment containing pZGT::luc from the resulting plasmid pUC/ZGT::luc was cloned into the BamHI site of the binary vector pPZP112 (Hajdukiewicz et al., 1994) to produce the plasmid pZGT/luc. Sequence-confirmed recombinant plasmids pOX/ZGT and pZGT/luc were introduced independently into competent cells of the host *Agrobacterium tumefaciens* LBA4404 (Life Technologies).

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 the fusion of the promoter for the *LHCBI*m gene from *Arabidopsis thaliana* and the firefly luciferase gene (*P*luc::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 $P_{ZGT}\text{luc}$ 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 $P_{UCB}\text{luc}$ 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[CT 19]). 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.

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