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# *tej* Defines a Role for Poly(ADP-Ribosyl)ation in Establishing Period Length of the *Arabidopsis* Circadian Oscillator

**Satchidananda Panda,[1,2] Guy G. Poirier,[3] and Steve A. Kay[1,2,4]**
[1]Department of Cell Biology
The Scripps Research Institute
La Jolla, California 92037
[2]Genomics Institute of Novartis Research Foundation
John J. Hopkins Drive
San Diego, California 92121
[3]Health and Environment Unit
CHUL Research Center of CHUQ and Laval University
Sainte-Foy, Quebec G1V 4G2
Canada

## Summary

**In a genetic screen for altered circadian period length in *Arabidopsis*, we isolated a mutant with a long free-running period. The *tej* mutation acts independently of light quality and quantity. It affects clock-controlled transcription of genes in *Arabidopsis* and alters the timing of the photoperiod-dependent transition from vegetative growth to flowering. Map-based cloning of *TEJ* identified a poly(ADP-ribose) glycohydrolase (PARG). An inhibitor of poly(ADP-ribosyl)ation rescued the period phenotype of *tej* mutant and shortened the period length of wild-type plants. Posttranslational poly(ADP-ribosyl)ation of an oscillator component may contribute to setting the period length of the *Arabidopsis* central oscillator.**

## Introduction

The circadian clock is a timing mechanism by which organisms coordinate the temporal phases of physiological processes to the daily changes in their environment resulting from the earth's rotation. Heuristically, the circadian system has been envisioned to be composed of sensors of light and temperature that input timing information to the oscillator, which in turn regulates the phase and periodicity of multiple output processes. Interaction of the intrinsic oscillator with the gradual changes in the length and timing of the natural photoperiod also regulates seasonal biological processes such as breeding in animals and flowering in plants. Molecular genetic analysis of mutants with altered period lengths of circadian outputs has been successful in identifying oscillator components and understanding their function. In most organisms, different positive and negative regulators of transcription act in a transcription-translation negative feedback loop to generate a near 24 hr rhythm. This core clock mechanism is conserved in different organisms, although components of the oscillator are less conserved (Harmer et al., 2001).

[4]Correspondence: stevek@scripps.edu

It is quite obvious that several ancillary proteins must operate at different steps of this core oscillator to generate and sustain a near 24 hr rhythm from such a simple feedback loop. The best-understood mechanism involved in setting the period length is progressive phosphorylation of an oscillator component, which in many species triggers its degradation. Mutations attenuating phosphorylation of a negative component of the oscillator delays its disappearance, and thereby lengthens the period length. For example, Doubletime (Dbt) in *Drosophila* is a caseine kinase that phosphorylates the negative element Per and may regulate its stability (Price et al., 1998). Different mutant alleles of *dbt* either hypo- or hyperphosphorylate Per, and produce a long- or short-period phenotype, respectively. Mutation of the hamster caseine kinase 1$\epsilon$ (CK1$\epsilon$) or a CK1$\epsilon$ phosphorylation site in human *hPer2* may possibly lead to hypophosphorylation and stability of Per protein. A more stable Per protein may further promote activity of the positive factor Bmal1, and thereby result in a short-period rhythm in hamster and a comparable sleeping disorder in human (Lowrey et al., 2000; Toh et al., 2001). Many phosphorylated proteins are subsequently ubiquitinated for proteosomal degradation. The *Arabidopsis* protein ZTL—a putative FMN binding protein with PAS-LOV and KELCH repeats—is proposed to mediate degradation of an oscillator component in a light-dependent manner, thereby helping to set the pace of the oscillator (Somers et al., 2000). While these genes act on the clock proteins, some may modulate transcription to set the period length. For example, constitutive overexpression of *vrille* (*vri*) in *Drosophila* suppresses Per and Tim expression and abolishes behavioral rhythms. This and other observations suggest a transcriptional role of Vri in *Drosophila* clock (Blau and Young, 1999). In the mouse, the clock-regulated transcription factor DBP also contributes to setting the period of the oscillator, probably by some transcriptional control (Yamaguchi et al., 2000).

We are beginning to understand the clock function in plants. Clocks cease to operate either in constant light (*Drosophila*, rodents, and *Neurospora*) or in constant dark (cyanobacteria), while plant oscillators function under constant conditions of light and dark (Millar et al., 1992). Furthermore, under constant illumination the pace of the plant oscillator is fine-tuned by both quality (wavelength) and fluence rate (intensity) of incident light (Somers et al., 1998b). Molecular genetic analysis of period-altering mutants in *Arabidopsis* has begun to elucidate the plant clock function (reviewed in Young and Kay, 2001). In *Arabidopsis*, two single myb domain-containing transcription factors, CCA1 and LHY, negatively regulate transcription from *TOC1* promoter, and constitute the negative limb of the oscillator (Alabadi et al., 2001; Schaffer et al., 1998; Wang and Tobin, 1998). TOC1, in turn, acts as a positive element by promoting transcription of *CCA1* and *LHY* (Alabadi et al., 2001; Strayer et al., 2000). Genetic alteration of any of these three clock components produces light-independent

period length defects. Many light-dependent period-altering mutants in *Arabidopsis* have been characterized and have been ascribed a function in the interaction between light and the oscillator. Mutations in circadian photoreceptors (phytochromes and cryptochromes) affect period length only under specific light regimes of blue or red light (Millar et al., 1995b; Somers et al., 1998a). Loss-of-function mutation *elf3* causes arhythmicity under most light conditions, but exhibits wild-type rhythms under constant darkness (Hicks et al., 1996). Elf3 may act downstream of both red and blue light photoreceptors and help progression of the clock under light (Covington et al., 2001; Hicks et al., 2001). Another clock mutant, *ztl*, exhibits period lengthening in a fluence rate-dependent manner. Its protein product contains features suggestive of its role in regulating protein turnover in a light-dependent manner (Somers et al., 2000). The late-flowering mutant *gigantia* (*gi*) affects clock outputs, although no mechanism has been proposed for its role (Park et al., 1999). A dozen additional period-affecting mutants in *Arabidopsis* have been isolated (Millar et al., 1995a). Their roles in the oscillator will begin to emerge once they are cloned.

The *Arabidopsis tej* mutant exhibits longer circadian period length in a light-independent manner. We took a position-based cloning approach and complementation test to clone the *TEJ* gene. The *TEJ* gene codes for a poly-(ADP-ribose) glycohydrolase (PARG). PARG is involved in regulation of the poly(ADP-ribosyl)ation state of proteins, which may alter activities of transcription factors. *TEJ* defines a key role of poly(ADP-ribosyl)ation in setting the pace of the *Arabidopsis* circadian oscillator.

## Results

### Mutation at the *TEJ* Locus Affects a Wide Range of Clock Outputs

A cycling bioluminescence reporter phenotype was used to discover circadian period-altering mutants in *Arabidopsis thaliana*. Seeds transgenic for the morning-phased circadian-controlled *cab2* promoter fused to the firefly luciferase (*luc*) gene were mutagenized with EMS, and the M2 seedlings were screened for altered free-running period length of cycling bioluminescence under constant white light (Millar et al., 1995a). *tej* was originally isolated as a recessive, long-period mutant with a period length 2 hr longer than the wild-type (Figures 1A and 1B). The mutant plants exhibit no apparent growth phenotype, and they flower and set seed normally (data not shown). However, the mutant exhibits higher amplitude of cycling bioluminescence compared to wild-type ("*TEJ*" in Sanskrit means "bright").

We assayed other cycling genes to determine whether they were also affected by this mutation. *CCA1* and *CCR2* are two *Arabidopsis* genes whose steady-state message levels are under circadian regulation. CCA1 is a core clock component that binds to a target binding site or evening element shared by many *Arabidopsis* promoters, including *Cab2* and *CCR2* (Harmer et al., 2000; Wang et al., 1997). Interestingly, CCA1 behaves as a positive regulator of *Cab2*, but a suppressor of *CCR2*; transcript levels of *CCA1* cycle with a phase earlier than that of Cab2, whereas the phase of CCR2 is almost opposite to that of Cab2. We assayed the effect of the *tej* mutation on cycling bioluminescence from *cca1::luc* and *ccr2::luc* transgenes. For both reporters, the mutant exhibited longer period length of bioluminescence rhythms under constant white light (Figures 1C and 1D).

To assess whether the long-period phenotype of the mutant is also evident in the endogenous message levels of three clock-controlled genes, we performed Northern blot analyses. In all three cases, the cycling endogenous message levels exhibited a long-period phenotype (Figures 1E–1G), consistent with the period phenotypes observed with *luc* reporters. However, the mutant did not show any significant change in amplitude of oscillation of cycling endogenous messages. Therefore, the high-amplitude cycling of bioluminescence from the *luc* reporter construct is a reporter-specific phenotype.

Several plant genes regulating water and solute transport across membranes and genes involved in cell wall expansion exhibit a circadian rhythm of message accumulation (Harmer et al., 2000). Their concerted action may produce rhythmic changes in cell volume, which may underlie rhythmic movement of plant organs such as cotyledons and leaves. We examined the free-running period length of cotyledon and leaf movement rhythm in *Arabidopsis* seedlings under constant white light. The mutant exhibited a free-running period length 2 hr longer than wild-type (Figure 2A).

Various genetic and physiological studies have firmly established a role of the circadian oscillator in the photoperiodic control of flowering (Samach and Coupland, 2000). *Arabidopsis* is a facultative long-day plant that flowers earlier in long days (16 hr light:8 hr dark) than in short days (8 hr light:16 hr dark). *tej* plants always flower earlier compared to wild-type in both long- and short-day conditions (Figure 2B). Together, this result with the above-mentioned transcriptional effects on clock-regulated genes established that the *tej* mutation affects several clock outputs.

### Period Phenotype in *tej* Is Independent of Light Quality and Fluence Rate

In *Arabidopsis*, red/far-red photoreceptors, phytochromes, and blue light photoreceptors, cryptochromes, act as circadian photoreceptors (Devlin and Kay, 2001). Mutations in the photoreceptors or in any downstream signaling component can alter the free-running period length under a specific light quality and/or fluence rates. Fluence rate response curves (FRC) constructed under either blue or red light fluences have been useful in identifying components of circadian light input pathways (Somers et al., 1998a). We constructed FRCs for the wild-type (WT) and *tej* under different fluence rates of red and blue light. The mutant exhibited a constitutively lengthened period over a wide range of fluence rates (Figures 3A and 3B), over a 300-fold change in red light fluence rates, and over a 50-fold change in blue light fluence rates. Therefore, *tej* does not define a component of either the red or blue light input pathway to the oscillator.

The mutant may still act in the light input downstream

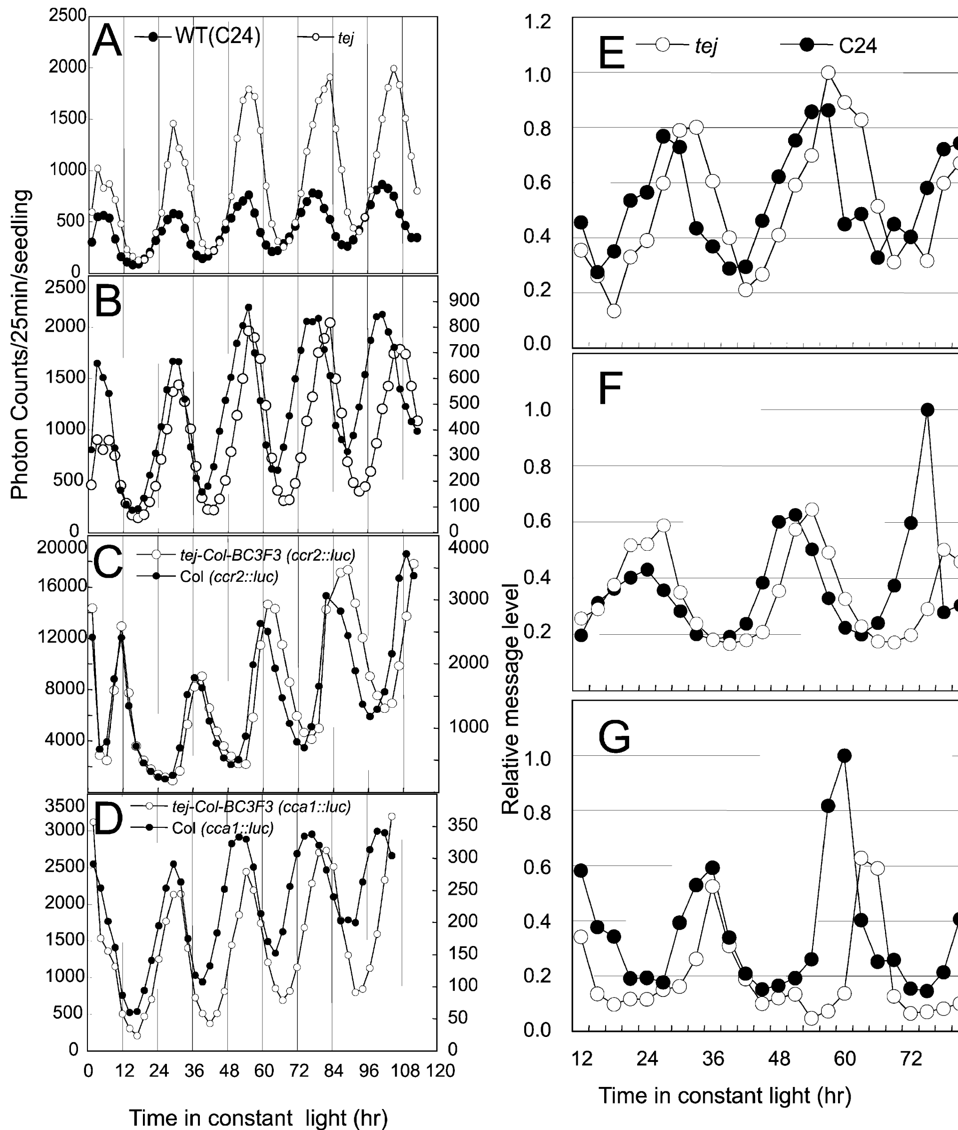


**Figure 1.** *tej* Is a Long-Period and High-Luminescence Mutant

(A) Mean luminescence traces (*cab2::luc* reporter) from *tej* (F3 progeny from backcross 3; [BC3]F3) and wild-type *C24* seedlings (n = 12). Period length estimates (mean ± SEM) are *tej* = 26.43 ± 0.2 hr and *C24* = 24.35 ± 0.14 hr.

(B–D) The same data plotted with WT values in the secondary x axis. The mutation similarly affects period length and luminescence levels from circadian reporters *ccr2::luc* (C) and *cca1::luc* (D) in *Col* ecotypic background. *cca1::luc* period estimates are 25.28 ± 0.08 hr (*tej*) and 23.85 ± 0.08 hr (*Col*), *ccr2::luc* period estimates are 25.31 ± 0.08 hr (*tej*) and 24.2 ± 0.07 hr (*Col*). The variations in period length estimates and in the magnitude of period lengthening by the mutation may be due to differences in ecotypic backgrounds.

(E–G) Endogenous transcripts of these three genes exhibit long periods of cycling, but no change in expression level. Wild-type (*C24*) and *tej* plants were entrained to cycles of 12 hr white light and 12 hr dark (LD cycles), allowed to free run in white light (50–70 $\mu$E m$^{-2}$ s$^{-1}$) and harvested at 3 hr intervals over the time course indicated. RNA blots were hybridized with *CAB2* (E), *CCA1* (F), or *CCR2* (G) DNA probes and quantitated relative to rRNA levels using an rDNA probe. By the end of 72 hr of free running under constant light, the phase of peak or trough levels of oscillation differed between WT and mutant by 3–6 hr (one to two time points), consistent with a period length difference of 1.5–2 hr.

of both blue and red light signaling pathways. Such a role of a mutant can only be revealed in the absence of light input to the clock. The *CCR2* mRNA exhibits robust cycling that, unlike *Cab2* mRNA, still persists in continuous darkness (Kreps and Simon, 1997). For the reporter, the mutant still exhibited a longer free-running period length compared to the WT in absence of any light input to the clock (Figure 3C), conclusively establishing that the effect of *tej* mutation on circadian period length in *Arabidopsis* is independent of light input to the clock. The pervasive period-lengthening effect of the mutation on various circadian outputs and light-independent action of *tej* firmly established it as a component acting close to the central oscillator.

### Positional Cloning of *TEJ* Identified *aPARG*

The *TEJ* locus was mapped to the bottom of chromosome 2 using the publicly available polymorphic markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993). Additional CAPS markers were developed using the

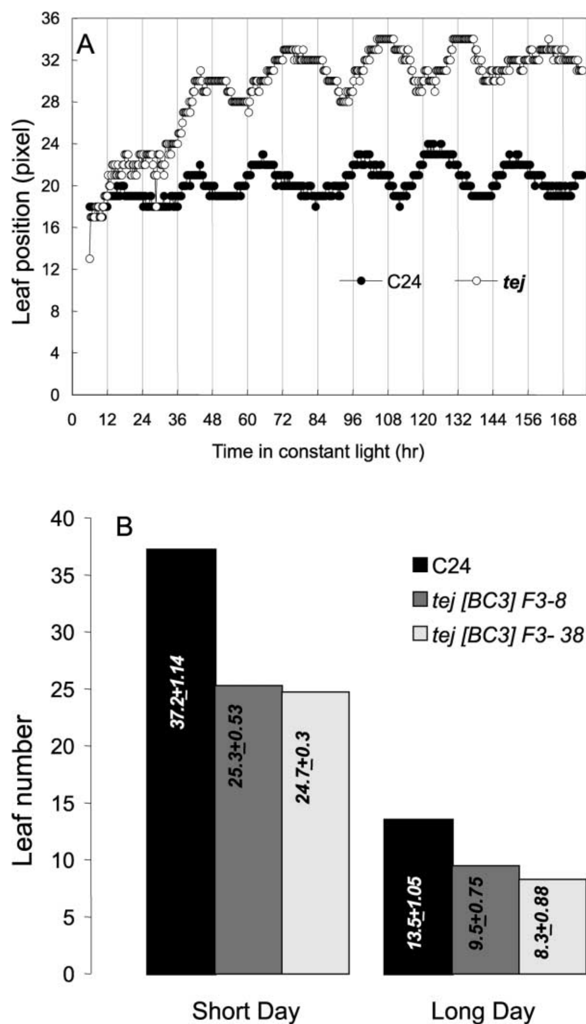


Figure 2. *tej* Mutation Affects Circadian Leaf Movement Rhythm and Photoperiodic Induction of Flowering

(A) *tej* lengthens free-running period length of the leaf movement rhythm. Seedlings were entrained for 7–9 days and then shifted to continuous white light (17–22 μmol $m^{-2}$ $s^{-1}$). Representative pixel positions of one leaf each of wild-type *C24* and *tej* seedling are shown. Mean period length estimates (±SEM) from 120 to 168 hr of pixel position recordings were 27.94 ± 0.19 hr (*C24*) and 30.44 ± 0.1 hr (*tej*).

(B) *tej* plants flower early relative to wild-type parents under both long (16 hr light and 8 hr dark) and short days (8 hr light and 16 hr dark). Mean (±SEM) number of total leaves at bolting from two different F3 lines of *tej* (from third back cross to the parental C24 ecotype) and from wild-type *C24* is plotted (n = 12–17 plants).

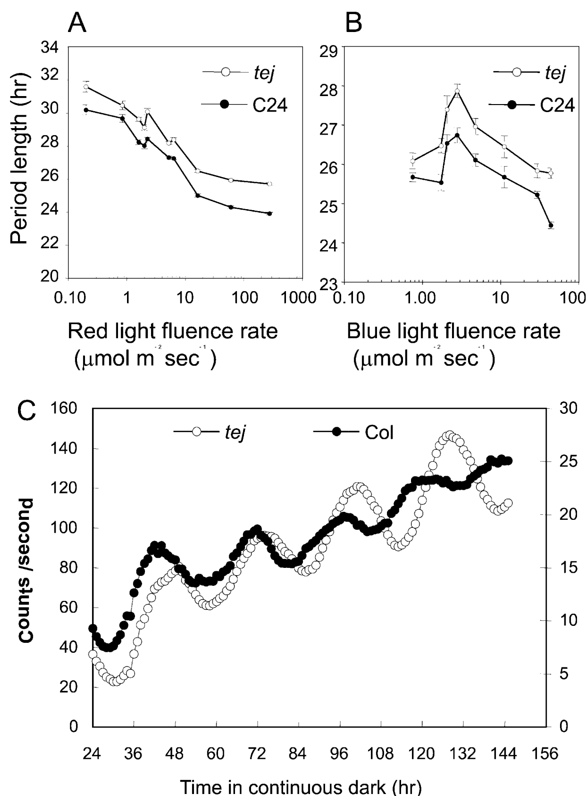


Figure 3. Period Length Defect in *tej* Is Light Independent

(A and B) Fluence rate response curves under different fluences of red (A) or blue (B) light showing constitutive period-lengthening effect of the mutation under different light qualities and fluence rates. Mutant or wild-type *C24* seedlings containing the *cab2::luc* reporter transgene were entrained as described in Experimental Procedures, and then released into constant red or blue light of different intensities. Mean period estimates (±SEM) from 100 hr of free running are plotted against fluence rates (n = 11–18 seedlings). The representative data from three to five experiments are shown.

(C) Period-lengthening effect of *tej* is also observed in bioluminescence rhythm from *ccr2::luc* reporter under constant darkness. Seedlings entrained to 5 days of LD cycles were released into constant darkness at ZT12 (lights off), and bioluminescence measurements were taken for 20 min every hour. Mean period estimates (±SEM) from 120 hr of free running are 27.44 ± 0.10 hr (*tej*) and 26.17 ± 0.20 hr (*Col*) (n = 25 [*tej*]; n = 19 [*Col*]).

publicly available genomic sequence (Lin et al., 1999). By scoring 2158 seedlings with newly developed CAPS markers, *TEJ* was finally delimited to a 110 kb region represented by a single BAC, F20M17. The BAC was randomly subcloned into a subgenomic library of 10–20 kb cosmid clones. These clones were fingerprinted and end sequenced to tile the 110 kb interval with cosmid clones overlapping each other by 2–5 kb (Figure 4A). Multiple T2 lines from independent T1 transformants of each clone were assayed for rescue of the long-period phenotype. Since the *tej* mutation is recessive in nature, and a majority of T-DNA insertions are single-locus insertions, we expected to see a Mendelian segregation of period length (in 3:1 wild-type:long-period) in T2 progenies from transformation with the clone carrying the wild-type *TEJ* gene. Independent T2 families segregating for the cosmid clone 4G6 showed the expected period length segregation (Figures 4B–4E) with the wild-type period length cosegregating with the cosmid marker (data not shown). Multipass sequencing of the 15 kb physical interval defined by the rescuing cosmid clone identified a single base substitution in a putative poly(ADP-ribose) glycohydrolase (PARG). Both complementation test and sequence identification of the mutation finally established the *TEJ* gene to be a putative PARG.

We performed 3′- and 5′-RACE and spliced the RACE products to obtain a 2.1 kb full-length cDNA sequence coding for a 548 amino acid protein (Figure 5C), which closely corresponds to a 2.3 kb band detected on poly($A^+$) RNA blot using a portion of the clone as a probe (data

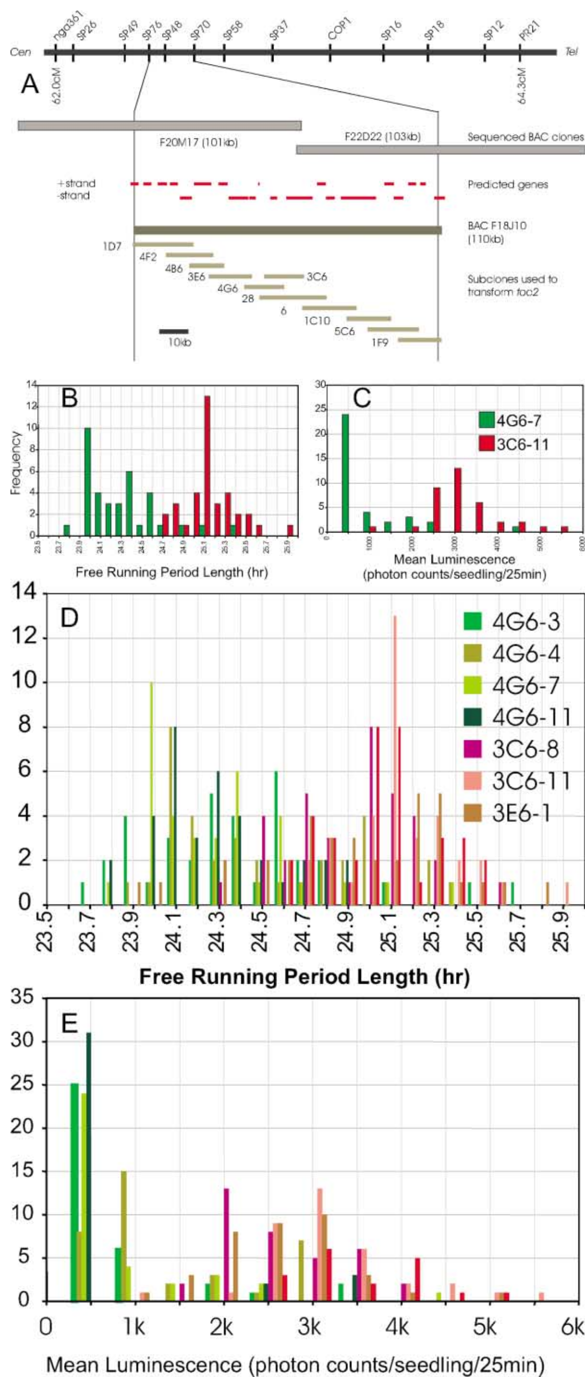


Figure 4. Position-Based Cloning of *TEJ*

(A) *TEJ* was mapped to the bottom of chromosome 2 between markers nga361 and PR21. Fine physical mapping delimited *TEJ* locus to a 110 kb interval represented by the BAC F18J10, which spans two sequenced BACs, F22D22 and F20M17. Eleven overlapping cosmid clones, generated from F18J10 and used for complementation, are shown.

(B–E) Frequency distributions of period length or mean luminescence in T2 families (n = 36) are shown. All seedlings were of the homozygous mutant type at the *TEJ* locus. A T2 family segregating for the clone 4G6 shows a shift in period length to wild-type value, while the clone 3C6 that partially overlaps with 4G6 failed to rescue the period phenotype (B). The same clone 4G6 also rescued the luminescence phenotype (C), while 3C6 failed to do so. Several T2 families, possibly representing independent insertion sites of the transgene 4G6, rescued both period (D) and luminescence (E) phenotypes. Clones 3E6 and 3C6, which partially overlap with 4G6, failed to rescue either phenotype.

not shown). Constitutive overexpression of this cDNA from a *CAMV35S* promoter rescued both the period (Figure 5D) and luminescence (data not shown) phenotypes of the *tej* plants, similar to rescue by the genomic clone. The mutation changes the glycine residue at position 262 to a glutamic acid residue (Figure 5A). Database searches with this predicted protein sequence identified PARGs from human, rodents, *Bos taurus*, *Drosophila*, and *C. elegans*. The *Arabidopsis* PARG shares extensive amino acid sequence similarity with the C-terminal ~360 amino acid segment of mammalian PARGs (36% identity and 55% similarity). The mutant seedlings accumulate over 20 times more pADPr polymers than the wild-type (Figure 5B). This increased polymer level is most likely due to a slow turnover of the pADPr polymers.

### Inhibitor of Poly(ADP-Ribosyl)ation Rescues the *tej* Phenotype

PARG degrades the pADPr polymers produced by poly (ADP-ribose) polymerase (PARP; Ueda et al., 1972). PARP produces pADPr polymers by using NAD as a substrate and liberating nicotinamide. Structural analogs of nicotinamides are common inhibitors of PARP (Banasik et al., 1992; Banasik and Ueda, 1994). Among them, 3-aminobenzamide (3-AB) is a specific inhibitor that phenocopies the dominant-negative PARP and PARP knockout phenotypes in animal cell lines. Although it is a specific inhibitor, it does not completely inhibit PARP activity (Banasik et al., 1992). Therefore, by slowing down the rate of synthesis of pADPr polymers, 3-AB may shift the equilibrium to wild-type in PARG-deficient seedlings. In the presence of 3-AB, *tej* seedlings exhibited a wild-type period length of bioluminescence rhythm from the *cab2::luc* reporter (Figure 6A). In addition, wild-type luminescence levels were also recovered in the 3-AB-treated seedlings (Figure 6B). Significantly, wild-type seedlings showed period shortening in the presence of the PARP inhibitor. Similar effects of the inhibitor were also observed for the *ccr2::luc* rhythm under constant darkness (data not shown). Conversely, 3-aminobenzoic acid, a structural analog of 3-AB that does not inhibit PARP but does inhibit other NAD-consuming enzymes (Hunting et al., 1985; Szabo et al., 1997), does not have any effect on period length of WT or the mutant (Figure 6C).

### *tej* Affects Period Length of All Known Clock-Controlled Genes

We used high-density oligonucleotide microarrays to examine the temporal gene expression profile in *tej* and WT *Arabidopsis* seedlings. The seedlings were entrained and then free run for 3 days under constant light prior to sampling so that the phases of clock-regulated transcripts in WT and *tej* would differ by more than the sampling interval of 4 hr. Therefore, the period length differences can be easily viewed as phase difference between the two genotypes. Expression profiles of *CCA1*, *CAB2*, and *CCR2* showed a difference of about 8 hr between their peak levels of expression in WT and

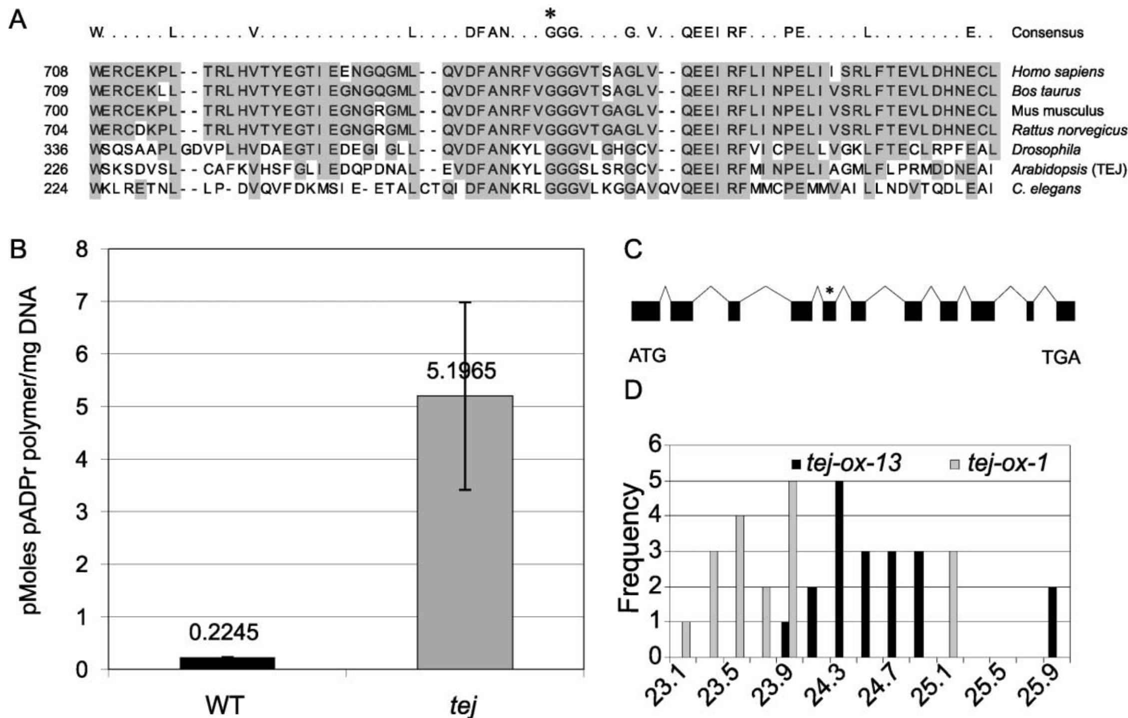


Figure 5. *TEJ* Is a Poly(ADP-Ribose) Glycohydrolase (PARG)

(A) The *tej* mutation changes an invariant glycine to a glutamic acid residue in a region of the protein that is highly conserved from *Arabidopsis* to human. The region of the TEJ protein from amino acid position 226 to 301 and the corresponding regions from other PARGs as detected by the psi-BLAST program with BLOSSUM 62 matrix are shown.

(B and C) The mutation may affect the catalytic site as *tej* seedlings accumulate higher levels of pADPr polymers as compared to wild-type *C24* parents. Representative estimates from five different sets of measurements from light-grown seedlings are shown. Mean $\pm$ SD ($n = 2$ groups of seedlings) are plotted. The 1644-nucleotide long TEJ open reading frame (C) was driven under a CaMV35S promoter and transformed into *tej* mutant plants.

(D) This ORF rescued the long-period phenotype of the *tej* mutant in a predicted 3:1 (wild-type:mutant period length) ratio. Period length frequency distribution of two T2 families segregating for the transgene are shown ($n = 18$).

*tej* (data not shown), validating our experimental methods. We examined the expression profile of nearly 450 clock-controlled genes described by Harmer et al. (2000), all of which showed phase differences of 4–8 hr (Figure 7A), consistent with an increase in their period length by 1.5–2 hr in the mutant. We also performed a direct comparison between WT and the mutant for all 8200 genes for possible changes in their level of expression by using the ANOVA test ($p < 0.001$). Among probe sets with average signal strength of 100 or more in at least one genotype, none of the genes showed more than a 3-fold change in mean expression level. Therefore, a PARG deficiency affects the period length of all known clock-controlled genes suggesting a general clock defect, but is not pleiotropic for expression of most genes.

## Discussion

### *TEJ* Plays a Role in the Plant Circadian Oscillator throughout Development

The *tej* mutation lengthens the free-running period length of multiple clock outputs under constant light. Cycling transcription, reported by bioluminescence rhythms from *luc* reporters, as well as steady-state message level of multiple clock-regulated genes, are equally lengthened in the mutant. Clock-controlled physiological processes such as leaf and cotyledon movement rhythms in young seedlings also exhibit lengthened free-running periods in the mutant. These cycling transcripts with different phases of expression may represent different clock output pathways within the same or different cell types. Such pervasive effect of the mutation on multiple clock outputs is reminiscent of other *Arabidopsis* circadian rhythm mutants, such as *toc1*, *elf3*, *lhy*, *cca1*, *gi*, and *ztl* (reviewed in McClung, 2001).

However the *tej* mutant, unlike *ztl* and *elf3-ox* seedlings (Covington et al., 2001; Somers et al., 2000), exhibits a constitutive period defect over a wide range of fluence rates (Figures 3A and 3B) of blue and red light. *ztl* and *elf3-ox* seedlings exhibit fluence rate-dependent period changes (characteristic of photoreceptor mutants in *Arabidopsis*), and have been implicated in light-dependent clock function. Similar to the *cca1*, *lhy*, and *toc1* mutations (Schaffer et al., 1998; Somers et al., 1998b; Wang and Tobin, 1998), the *tej* mutation affects clock function during light-independent seedling growth (Figure 3C), which conclusively establishes a light-independent role of *TEJ* in the plant oscillator.

The photoperiod-dependent induction of flowering is an indirect measurement of clock function beyond the seedling stage of plant growth. *tej* mutants exhibit a

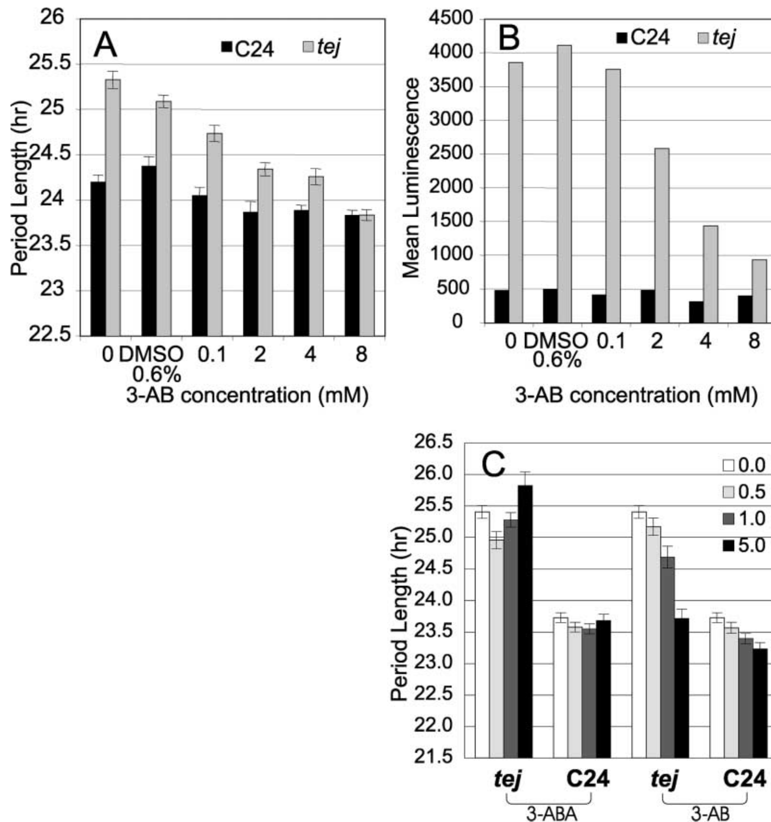


Figure 6. A PARP Inhibitor Affects Circadian Period Length in *Arabidopsis*

(A) The PARP inhibitor 3-aminobenzamide (3-AB) shortens period length in a dose-dependent manner and rescues the period length phenotype of *tej* seedlings. Mean period length estimates (±SEM; n = 9–18) of bioluminescence rhythm are from wild-type *C24* and *tej* seedlings containing *cab2::luc* reporter transgene. Seedlings entrained to LD cycles were sprayed once with the indicated concentration of 3-AB (average 25 μl/seedling) in 6% DMSO 1 day before imaging started.

(B) The same inhibitor also reduces the mean luminescence level of *tej* seedlings in a dose-dependent manner. Average of luminescence values (photon counts/seedling/25 min) collected once every 2.5 hr are shown. Counts are from the same seedlings as in (A).

(C) 3-AB, but not a closely related compound 3-aminobenzoic acid (3-ABA), shortens period length both in *tej* and wild-type *C24* seedlings. Seedlings were either treated with 3-AB or a related compound 3-ABA (which does not inhibit PARP). Period estimates are from 90 hr of free running under constant white light (50 $\mu$E m$^{-2}$ s$^{-1}$). Representative estimates from one of three trials are shown.

defect in flowering time (Figure 2B), suggesting an effect of the mutation on clock-controlled processes in later stages of plant development. The *tej* mutant, therefore, affects oscillator function throughout plant development in diverse tissue types.

Finally, genome-wide expression profiling of the mutant demonstrated its pleiotropic effect on almost all known clock-controlled genes (Figure 7A). Similar systematic gene expression analysis of a loss-of-function circadian mutant in flies has recently demonstrated the central role of a single oscillator in controlling all cycling genes in *Drosophila* (McDonald and Rosbash, 2001). Period lengthening of all cycling transcripts in *tej* implies a master oscillator in *Arabidopsis* regulating all circadian outputs, and that *TEJ* functions in this master oscillator.

### *TEJ* Encodes a Functional PARG

*TEJ* gene codes for a predicted protein that shares sequence similarity with the catalytically active C-terminal portion of PARGs from animals. The *tej* mutation *G262E* is in a highly conserved portion of the protein, and therefore may affect its catalytic activity. Two glycine residues at positions 262 and 263 of TEJ are invariant in all mammalian PARGs, while a third glycine at position 264 is well conserved in PARGs from different species (Figure 5A). Additionally, the high degree of amino acid sequence conservation from human to *Arabidopsis* in the immediate neighborhood of this GGG motif suggests that this region of the protein may have a critical role in catalytic activity. A significantly higher level of pADPr polymers in the mutant compared to their WT parents (Figure 5B) supports TEJ being a functional aPARG, and that the mutation affects its catalytic activity.

### PARG Deficiency Affects All Clock-Controlled Genes

Poly(ADP-ribosyl)ation is a posttranslational modification of mostly nuclear proteins. PARP is acutely induced under genotoxic stress, and its activity plays a critical role in modulating cellular response to stress. The range of targets and the extent of poly(ADP-ribosyl)ation correlates with the severity of stress and determines the type of cellular responses, from necrosis/apoptosis under severe stress to DNA repair under moderate stress, and to cellular defense/proinflammatory response via transcriptional changes under mild stress (Burkle, 2001). PARP automodifies itself, and in absence of stress, only a few DNA binding proteins including PARP remain poly(ADP-ribosyl)ated. In both plants and animals, though altered poly(ADP-ribosyl)ation modulates stress response, it does not affect any normal cellular physiology, implying a very limited role of poly(ADP-ribosyl)ation in the absence of stress (Burkle, 2001). The period phenotype of the *tej* mutant, therefore, establishes a role for poly(ADP-ribosyl)ation in homeostatic cellular functions.

### Poly(ADP-Ribosyl)ation Plays a Role in Establishing Circadian Period Length in *Arabidopsis*

The light-independent effect of *tej* on circadian regulation implies its function in the oscillator itself, or very close to it. The specific effect of the mutation on period

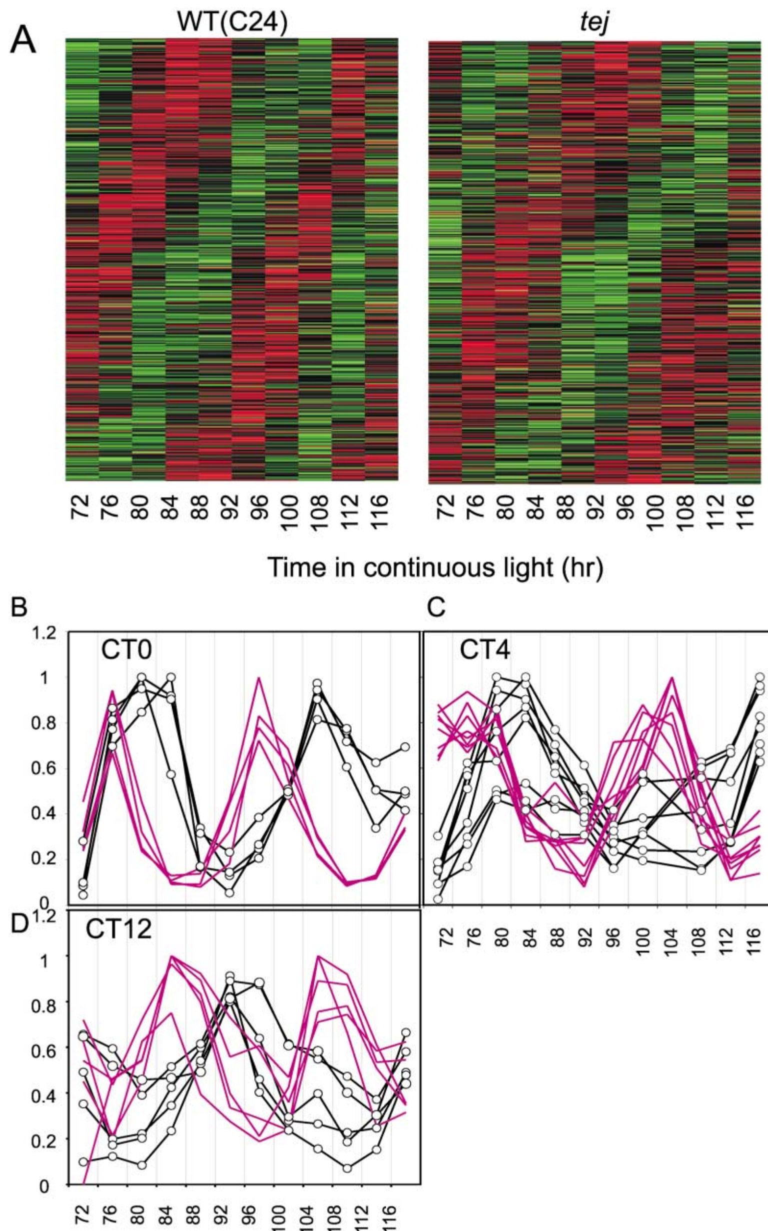


Figure 7. The *tej* Mutation Affects Transcription of Clock-Regulated Genes

Complementary RNA samples were prepared and hybridized to oligonucleotide-based microarrays as described in Harmer et al. (2001). Raw average difference values corresponding to cycling genes identified in Harmer et al. (2001) for both the C24 wild-type strain and for the *tej* mutant strain were extracted using a relational database. Genes (probe sets) were ordered according to phase values returned by the Corcos algorithm, normalized using Cluster, and visualized using Treeview (M. Eisen, Stanford University).

(B–D) For a given probe set, data were normalized to the peak value over time points (both wild-type and mutant). Representative traces from three different phases are shown. Data for the 104 hr time point are missing for some probe sets. Pink traces are from the WT (C24), and black traces are from the mutant. GenBank accession numbers corresponding to probe sets are indicated. Identities of proteins for some GenBank accessions are mentioned in parentheses. (B) CT0: T02684 (CCA1), AAD23680 (CONSTANS-like), CAA07004 (LHY), AAD32887 (GST). (C) CT4: T05496 (DNA J homolog), AAD15394, AAC35230, AAC42256 (putative CCCH-type Zn finger), T04438, AAD21702, AAC41679 (thionin), S71287 (Myb-related TF). (D) CT12: Q03250 (CCR2), S43320, CAA66408, AAB95293, T01857.

lengthening of clock-controlled genes without an appreciable change in steady-state message levels of any other gene further supports this conclusion. PARG deficiency in the *tej* mutant slows down turnover of poly (ADP-ribose) polymers on target proteins and results in period lengthening. 3-AB inhibits PARP-mediated poly (ADP-ribosyl)ation, and leads to period shortening (Figures 6A and 6C). This is analogous to hyper- and hypophosphorylating alleles of the *Drosophila* mutant *dbt* producing short- and long-period phenotypes (Price et al., 1998).

Both the genetic and pharmacological results suggest active participation of both PARP and PARG in period length determination. The *tej* mutation is completely recessive, and the *tej*/+ seedlings are phenotypically similar to the wild-type plants (+/+). This would suggest PARG activity is not limiting, and a single functional copy of the gene is sufficient enough to perform its circadian function in the heterozygous (*tej*/+) seedlings. Furthermore, either *TEJ* genomic cosmid clones (which are known to integrate into the genome in multiple copies in tandem and reverse tandem repeats) or constitutive overexpression of the TEJ cDNA in the mutant plants (Figure 5D) rescued the period phenotype to the WT values, and not to short-period values. Importantly, partial inhibition of PARP activity by 3-AB could shorten the period length in the WT seedlings. Therefore, we would expect a short-period or arrhythmic phenotype in the loss-of-function mutant of PARP. Taken together, the concerted action of both PARP and PARG determine the extent of poly(ADP-ribosyl)ation on target proteins, and determine the circadian period length.

Steady-state message levels of *PARP* and *TEJ* do not cycle in *Arabidopsis* (Harmer et al., 2000; Schaffer et al.,

2001 and data not shown), and therefore, poly(ADP-ribosyl)ation may not be a cycling element of the clock (although activity of the enzymes may still cycle). The circadian role of poly(ADP-ribosyl)ation may be mediated by automodification of PARP itself or by modification of a target transcription factor. The high-luminescence phenotype of *tej* plants (4- to 5-fold higher than the WT plants) may offer a clue to a possible mechanism of gene regulation by automodification of PARP. This phenotype is specific to the *luc* reporter transgene, as the endogenous mRNA levels of *Cab2*, *CCR2*, and *CCA1* do not exhibit any appreciable change from the WT levels (Figure 1). According to a model based on in vitro assays using mammalian PARP (Vispe et al., 2000), direct binding of PARP to a double-hairpin secondary structure in nascently transcribed *luc* RNA inhibits progression of the transcriptional machinery across the 3′ end of the *luc* gene. Automodification of PARP releases this repression, and transcription of *luc* message is enhanced. Deficiency of PARG activity, as in *tej* seedlings, would cause PARP to be perpetually automodified, thereby enhancing *luc* transcription. If a similar defect in gene regulation is implicated in the period alteration in *tej* plants, increased expression of an oscillator component or a factor acting close to the oscillator may affect clock function. However, none of the known genes affecting rhythms in *Arabidopsis*, nor genes represented on the DNA microarray, show at least a 3-fold increase in expression level in the mutant, although it is still possible that the mutation may affect transcript levels of genes not currently represented on the microarray. Furthermore, it is highly unlikely that a 4- to 5-fold increase in levels of a clock component would cause the observed modest period length change of 2 hr.

In many cases, association of an automodified PARP with a target transcription factor and/or modification of the target protein alter activities of the transcription factor (Akiyama et al., 2001; Burkle, 2001; Chang and Alvarez-Gonzalez, 2001; Mendoza-Alvarez and Alvarez-Gonzalez, 2001; Oei and Shi, 2001). Both the *tej* mutant and the effects of 3-AB demonstrate a direct correlation between poly(ADP-ribosyl)ation and period length. Although it is premature to unequivocally ascribe an effect of poly(ADP-ribosyl)ation on the activity of a target transcription factor, such modification may enhance transcription of a repressor or suppress transcription of a positive acting element of the clock leading to slowing of the oscillator. In either case, poly(ADP-ribosyl)ation would insert a delay mechanism in setting the pace of the oscillator.

At least three cycling transcription factors, CCA1, LHY, and TOC1, are part of the *Arabidopsis* circadian oscillator. Additionally, there is overwhelming support for participation of many additional transcription factors—for example, activator(s) of TOC1 transcription, and partner(s) of TOC1 (Young and Kay, 2001). Any of these proteins may be a target of poly(ADP-ribosyl)ation. Most of the proteins implicated in clock function in *Arabidopsis*, such as CCA1, LHY, ZTL, GI, and ELF3, are also key regulators of light signaling, and their respective mutants exhibit defects in photomorphogenesis. Given that the *tej* mutants do not exhibit any subtle light signaling or developmental defects, it is unlikely that TEJ acts upon any of these known clock proteins. Although the site of poly(ADP-ribosyl)ation is via the γ-carboxyl group of glutamic acid residues, lack of a consensus poly(ADP-ribosyl)ation signature motif leaves open the candidate oscillator target. Furthermore, we cannot rule out the possibility that PARP itself may interact with a core oscillator component and set the pace of the oscillator.

## Conclusion

The *tej* mutant acts similarly to *lhy*, *cca1*, and *toc1* mutants in affecting clock function in *Arabidopsis* in a light-independent fashion. The mutation affects period length of all known clock-controlled genes, demonstrating the existence of a single oscillator affecting multiple outputs or alternatively, suggesting a common component of multiple independent oscillators. The cloning and characterization of *TEJ* conclusively adds a novel component to the list of molecules that influence clock function and adds another mode of regulation for setting the period length of the oscillator. Generation of additional genetic and biochemical reagents will facilitate identification of PARP targets and elucidate its function in the circadian oscillator.

## Experimental Procedures

### Plant Growth Conditions and Period Length Assays

*tej* was isolated as a long-period mutant from an EMS-mutagenized population (C24 ecotype) carrying the *cab2::luc* reporter gene (Millar et al., 1995a). Period length assays for cycling bioluminescence under constant white light (50–60 $\mu$mol m$^{-2}$ s$^{-1}$), and under different fluence rates of blue or red lights, were conducted as previously described (Somers et al., 1998a, 1998b).

The mutant was introgressed three times to line (*Col* ecotype) carrying *ccr2::luc* or *cca1::luc* reporter transgene. These lines were germinated and bioluminescence rhythms were assayed as previously described (Millar et al., 1992). Lines containing *ccr2::luc* transgene were germinated under entraining conditions of 12 hr light and 12 hr dark for 5 days, sprayed with 5 mM luciferin solution in 0.01% Triton X-100, and monitored for bioluminescence levels for 20 min in every hour for 120–144 hr using a Night Owl imaging system and the WinLight software (Perkin Elmer) as previously described (Strayer et al., 2000). For leaf movement assays, plants were germinated under entraining condition for 5–6 days, transferred to continuous white light, and cotyledon position recorded every 20 min as described (Hicks et al., 1996). Period estimates were obtained according to Plautz et al. (1997) and Millar et al. (1995a).

### Genotyping of F2 Plants

To generate a mapping population, the *tej* (C24 ecotype) mutant was crossed to wild-type WS, and the F2 population from the cross was used for mapping. For preliminary mapping, long-period (>25 hr) seedlings were identified in the F2 population by estimating the free-running period of each seedling after 4 days in continuous white light. Published PCR markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993) were used to map *tej* to the bottom arm of chromosome 2. For subsequent fine mapping, individual F2 seedlings were first genotyped at the flanking polymorphic markers and only the seedlings with informative recombinations in the *TEJ* interval were selected for further analysis. Genotypes at the *TEJ* locus were determined in the F3 progeny. Publicly available *Arabidopsis* sequence and the *Arabidopsis* BAC fingerprint database were used to tile the *tej* interval with BACs and develop additional PCR-based markers (http://www.tigr.org/tdb/at/at.html, and http://www.arabidopsis.org).

### Complementation of the Mutant

A shotgun library of BAC F20M17 was constructed by a partial Sau3AI digestion yielding 15 and 20 kb long fragments. These were

ligated to the plant transformation vector pZP221, which had been cleaved with BamHI. The library was screened and restriction mapped to obtain a contig of overlapping clones covering the *tej* locus defined by genetic mapping. These clones were introduced into *tej* plants using *Agrobacterium*-mediated plant transformation. For each clone, at least five T2 families derived from independent T1 plants were imaged for 4 days under continuous light, and period length was estimated. TEJ ORF was cloned into a plant transformation vector containing CaMV35S promoter (Mas et al., 2000) and transformed into mutant *tej* seedlings. Subsequent analyses of the transformants were similar to the cosmid clone transgenics.

### Flowering Time Assay

Seeds were stratified on agar plates and grown in white fluorescent light under either long days (LD; 16 hr light, 8 hr dark; 100–120 $\mu$mol $m^{-2}$ $s^{-1}$) or short days (SD; 8 hr light, 16 hr dark; 100–120 $\mu$mol $m^{-2}$ $s^{-1}$) for 7–10 days, and subsequently transplanted to soil. The total number of rosette and cauline leaves were recorded as the measure of developmental time to flowering.

### RNA Gel Blot Analyses

Northern blot analyses were performed as previously described (Kreps and Simon, 1997; Wang and Tobin, 1998).

### Oligonucleotide Microarray Hybridization

Wild-type and *tej* (C24) seeds were stratified and germinated under entraining conditions for 6 days, and then allowed to free run under constant light for 3 days before collection of tissue samples for RNA extraction. Seedling growth condition, RNA extraction, cRNA probe preparation and hybridization, and normalization of fluorescence data were performed as described (Harmer et al., 2000). Normalized traces for each probe set representing a clock-controlled gene (453 genes in total, as listed in Harmer et al., 2000) were compared for phase changes.

### cDNA Isolation and Analyses

Total RNA was extracted from tissue samples of 6- to 7-day-old wild-type *C24* seedlings grown under entraining condition. RNA from six tissue samples collected at 4 hr intervals over one complete LD cycle were pooled and used as starting material to prepare a cDNA library using SMART cDNA Synthesis kit (Clontech). Sense and antisense oligonucleotides were designed near the mutation site and used as internal primers in separate PCR reactions to amplify the 5′ and the 3′ portions of the cDNA using the SMART RACE kit (Clontech). The resultant cDNA products were cloned into TOPO cloning vector, and ten independent clones from each reaction were sequenced.

### Estimation of Poly(ADP-Ribose) Levels

Seedlings of both *tej* and wild-type genotypes were grown under the conditions used for period length assays. Tissue from 8-day-old seedlings were harvested and pADPr polymer levels were estimated as described (Shah et al., 1995; Affar et al., 1999).

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## Accession Numbers

The GenBank accession number for the TEJ sequence is AF394690.