The circadian clock shapes the *Arabidopsis* transcriptome by regulating alternative splicing and alternative polyadenylation

The circadian clock in plants temporally coordinates biological processes throughout the day, synchronizing gene expression with diurnal environmental changes. Circadian oscillator proteins are known to regulate the expression of clock-controlled plant genes by controlling their transcription. Here, using a high-throughput RNA-Seq approach, we examined genome-wide circadian and diurnal control of the *Arabidopsis* transcriptome, finding that the oscillation patterns of different transcripts of multitranscript genes can exhibit substantial differences and demonstrating that the circadian clock affects posttranscriptional regulation. In parallel, we found that two major posttranscriptional mechanisms, alternative splicing (AS; especially intron retention) and alternative polyadenylation (APA), display circadian rhythmicity resulting from oscillation in the genes involved in AS and APA. Moreover, AS-related genes exhibited rhythmic AS and APA regulation, adding another layer of complexity to circadian regulation of gene expression. We conclude that the *Arabidopsis* circadian clock not only controls transcription of genes but also affects their posttranscriptional regulation by influencing alternative splicing and alternative polyadenylation.

The plant circadian clock, entrained by environmental stimuli, coordinates biological processes on a daily basis and enables proper growth and development (1–3). The circadian oscillator consists of multiple interlocked transcriptional feedback loops formed by sequential induction of core clock genes acting as reciprocal transcriptional repressors throughout the day (4). Through this regulatory system, about 30% of genes exhibit circadian oscillation in *Arabidopsis thaliana*, based on microarray analyses (5).

In addition to the transcriptional level, the core clock genes are regulated at the posttranscriptional level, adding another layer of complexity to shape the circadian-controlled gene network. Among the posttranscriptional mechanisms influencing the circadian clock is alternative splicing (AS), which generates multiple transcripts from the same gene (6, 7). Precursor mRNA (pre-mRNA) possesses 5′ and 3′ donor splice sites at the exon–intron boundaries. The spliceosome, a protein complex consisting of five small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6), and other auxiliary RNA-binding proteins bind to splice sites and remove introns from pre-mRNA. The choice of splice sites mainly depends on recruitment of splicing factors and heterogeneous nuclear ribonucleoproteins, but other splicing regulators, such as serine and arginine-rich proteins and K homology domain RNA-binding proteins are also involved in this process (8, 9). In addition to transcripts encoding proteins with different properties, AS leads to production of transcripts with a premature termination codon that marks them for degradation by the nonsense-mediated decay mechanism (10–12). In *Arabidopsis*, 61% of genes, including the components of the circadian oscillator, such as morning-phased *CIRCADIAN CLOCK ASSOCIATED 1* (CCA1), *LATE ELONGATED HYPOCOTYL* (LHY), day-phased *PSUEDO-RESPONSE REGULATORS* (PPRs), *REVEILLE 8* (RVE8), evening-phased *TIMING OF CAB EXPRESSION 1* (TOC1), *GIANTANEA* (GI), and *EARLY FLOWERING 3* (ELF3), have been shown to be alternatively spliced (13–16). On the other hand, loss of function of PROTEIN ARGinine METHYLTRANSFERASE5 (PRMT5), a splicing factor, and *SPLICESOMAL TIMEKEEPER LOCUS1* (STIPL1), which plays a role in spliceosome disassembly, lead to longer circadian periods (17–19), indicating a role of splicing in the *Arabidopsis* circadian clock. GEMIN2, required for spliceosome assembly, has been shown to affect AS of core clock genes and to function as the link between temperature change and the circadian clock (20).

Alternative polyadenylation (APA) is another posttranscriptional mechanism that generates multiple transcripts through use of different potential polyadenylation (poly(A)) sites. Cleavage and poly(A) factors are recruited to the poly(A) site and form a multiprotein complex to cleave pre-mRNA, followed by attachment of a poly(A) tail (21). APA can produce isoforms that differ in their 3′ UTRs or proteins with differing functions,

---

3 The abbreviations used are: AS, alternative splicing; pre-mRNA, precursor mRNA; APA, alternative polyadenylation; poly(A), polyadenylation; CT, circadian time; ZT, zeitgeber time; GO, Gene Ontology; AS55, alternative 5′ splice site; AS35, alternative 3′ splice site; MXE, mutually exclusive exon; RI, retained intron; SE, skipped exon; CPSF, subunit of cleavage and polyadenylation factor; RT-qPCR, quantitative real-time PCR.

This work was supported by National Institutes of Health Grants ES027255 and GM118102 (to A.S.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S9 and Tables S1–S6.

1 To whom correspondence may be addressed. E-mail: aziz_sancar@med.unc.edu.

2 To whom correspondence may be addressed. E-mail: ooztas@med.unc.edu.
stability, or localization (22–24). APA is widespread in plants, and more than 75% of the miRNA transcripts in Arabidopsis are APA-regulated (25). APA has been reported to be involved in numerous biological processes, especially flower development (26–29).

Here we examined genome-wide circadian and diurnal control of the Arabidopsis transcriptome using a high-throughput RNA-Seq approach. We found that alternative transcripts of many genes can show different oscillation patterns because of the rhythmicity in two major posttranscriptional mechanisms: AS and APA. These rhythmic profiles result from rhythmic expression of genes involved in AS and APA or from rhythmic AS and APA regulation of AS-related genes. Our results show that the plant circadian clock controls gene expression not only at the transcription level but also modulates gene expression at the posttranscriptional level by regulating AS and APA.

**Results**

**Nonhomogeneous circadian and diurnal oscillation patterns among alternative transcripts**

To explore daily expression dynamics of Arabidopsis genes and their transcripts, we performed strand-specific RNA-Seq at 3-h intervals throughout the day. Eight-day-old Arabidopsis seedlings, grown under long-day conditions (16/8 h light/dark), were transferred to continuous light or kept under the same light/dark conditions for an additional 2 days to determine circadian and diurnal transcriptomes, respectively. We determined the oscillation profiles of each transcript of a gene (transcript-level oscillation) and assessed the rhythmicity of a gene based on the total expression levels of all of their transcripts as a function of time (gene-level oscillation). We detected rhythms of genes and their individual transcripts using Metacycle software with a strict criterion of false discovery rate (meta2d_BH.Q < 0.05 (30, 31)). We found that 18.7% (6020 genes) and 20.7% (6679 genes) of total genes were circadianally and diurnally regulated, respectively, which is in line with previous microarray-based analyses (32–34). Comparison of oscillating circadian and diurnal genes revealed that 3104 genes show rhythms under both conditions, and 3575 genes showed diurnally specific rhythmicity. In addition, a subset of circadian genes (2916 genes) oscillated only at constant light (Fig. 1A and Fig. S1A), similar to the observation in lettuce (35). Our RT-qPCR analyses of the LHCA6 and RBCS2B genes verified that these genes oscillate under continuous light but not under light/dark conditions. Loss of expression induction at the dark phase presumably leads to nonrhythmicity under diurnal conditions in these genes (Fig. S1, B and C). The phase value distribution of the 2916 exclusively circadian and 3575 exclusively diurnal genes exhibited phases of maximum expression at late day (circadian time 13 (CT13)) and at early dark (zeitgeber time 16 (ZT16)—ZT18), respectively (Fig. 1B). To analyze the biological processes of the cycling genes, we performed Gene Ontology (GO) enrichment analysis. The most highly enriched GO terms for circadianally and diurnally controlled genes were circadian clock and photosynthesis. We detected more enriched GO terms for circadian genes than diurnal genes (Fig. S2), suggesting that diurnal genes are more diverse in terms of biological function compared with circadian genes.

We next examined the oscillation in expression patterns at the gene and transcript levels. We found that many oscillating genes (42% of 6020 circadian genes and 43% of 6679 diurnal genes) possessed only one transcript (single-transcript); however, a higher proportion of cycling genes (57.9% and 57.2% of circadian and diurnal genes, respectively) had more than one transcript (multi-transcript) (Fig. 1C). The proportion of oscillating multiple-transcript genes is significantly higher than multi-transcript genes over the whole genome (39.2%, Fisher’s exact test \( p < 2.2e−16 \)). Interestingly, a comparison of oscillation profiles of multi-transcript genes and their transcripts showed the existence of cases in which a gene oscillates but not its transcripts (1257 circadian and 1446 diurnal genes) or vice versa (764 circadian and 864 diurnal transcripts) (Fig. 1D). Furthermore, in 110 circadian and 86 diurnal cases, genes and their transcripts exhibited different oscillation patterns (Fig. 1E). As an example, although both isoforms of the 4CL3 gene, encoding a protein involved in phenylpropanoid pathway (36), exhibited the same oscillation pattern as their gene (Fig. 1F), a RING/Ubox superfamily gene and one of its isoforms showed antiphase oscillation patterns (Fig. 1G). Overall, genome-wide transcriptome analysis of Arabidopsis throughout the day revealed a difference in the oscillation profiles of genes and their transcripts, implying an effect of the circadian clock on posttranscriptional regulation in addition to transcription. To understand the basis of the differences in oscillation behavior of genes and their transcripts, we analyzed circadian clock regulation of two major posttranscriptional mechanisms: alternative splicing and alternative polyadenylation.

**Intron retention is the major type of rhythmic alternative splicing**

Using our circadian and diurnal RNA-Seq datasets, we identified 11,603 and 12,195 AS events, respectively, including the following types of AS: alternative 5’ splice site (A5SS), alternative 3’ splice site (A3SS), mutually exclusive exon (MXE), retained intron (RI), and skipped exon (SE). Our analysis revealed that all types of AS events occur in A. thaliana; however, intron retention and exon skipping are the major AS events (Fig. S3A), consistent with a previous report (13). We further performed Metacycle software analysis, applying a meta2d_p value cutoff of 0.05 to determine circadian and diurnal AS events. We found that all types of AS events except MXE exhibit circadian or diurnal rhythms (Fig. 2, A and B, and Fig. S3C), and that the oscillation behaviors of AS events largely differ under the two light regimens (Fig. S3B).

We identified 57 A3SS, 42 A5SS, and 55 SE events that cycled in a circadian manner. The corresponding enriched GO terms for these circadian events were cellular response to DNA damage stimulus, sucrose biosynthetic process and response to sucrose, fatty acid biosynthetic process, protein ubiquitination, and regulation of transcription (DNA-templated). Over a diurnal cycle, 58 A3SS, 55 A5SS, and 63 SE events showed rhythms. Our GO analysis of diurnal AS events revealed that the enriched GO terms included regulation of vesicle fusion, abscisic acid–activated signaling pathway, response to cadmium ion, long-
The most prevalent oscillating AS type was intron retention with 203 circadian and 246 diurnal events (Fig. S3A). Unlike the other types of rhythmic ASs, whose phase peaks were distributed across the day, the circadian oscillation of intron retention was strongly associated with the dark period (Fig. S4).

Among enriched GO terms for all rhythmic AS events, we identified circadian rhythm and negative regulation of transcription (DNA-templated and from RNA polymerase II promoter), and activation of GTPase activity (Fig. S4). The most prevalent oscillating AS type was intron retention with 203 circadian and 246 diurnal events (Fig. S3A). Unlike the other types of rhythmic ASs, whose phase peaks were distrib-
uted over the entire cycle, the phases of cycling RI events peaked at late day (CT10–CT14 and ZT10–ZT13) under circadian and diurnal growth conditions (Fig. 2C). Consistent with these results, circadian RI events showed only one highly enriched oscillation pattern that peaked at dawn, which was also observed for diurnal RI events. However, an additional enriched oscillation pattern with a peak at dusk existed for RI events with diurnal rhythms (Fig. S5, A and B). The significantly enriched GO terms for RI events that oscillate in a circadian manner included DNA unwinding involved in DNA replication.

Figure 2. Intron retention is the major type of rhythmic alternative splicing event. A, the number of diurnally and circadially regulated AS types: A5SS, A3SS, MXE, RI, and SE. B, heatmap of circadian AS events for each AS type. Each row represents the change in the proportion of a splice variant. C, phase value distribution of oscillating AS events. The gray shade represents the dark period. D, GO enrichment analysis of genes that show circadian RI events. The vertical dashed line represents \( p = 0.05 \).
We detected 643 circadian APA events of the total 25,076 APA events identified in 597 genes and 1446 diurnal APA events of the total 24,036 APA events identified in 1312 genes. A low proportion of cycling APA events overlapped under both conditions, similar to our observation in our analyses at the gene and AS levels (Fig. 3A). To test the accuracy of our APA prediction, we compared the APA events identified in our study with those in a public database (37). We found that 60% of total APA events and 75% of oscillating APA events detected in our analysis were identified previously (Fig. 3B), showing that our APA prediction is accurate.

**The circadian clock regulates alternative polyadenylation**

We explored the rhythmicity of alternative polyadenylation from our transcriptome data and identified circadially and diurnally regulated APA events by using the Metacycle software with a meta2d_p value cutoff of 0.05. We detected 643 circadian APA events of the total 25,076 APA events identified in 597 genes and 1446 diurnal APA events of the total 24,036 APA events identified in 1312 genes. A low proportion of cycling APA events overlapped under both conditions, similar to our observation in our analyses at the gene and AS levels (Fig. 3A). To test the accuracy of our APA prediction, we compared the APA events identified in our study with those in a public database (37). We found that ~60% of total APA events and 75% of oscillating APA events detected in our analysis were identified previously (Fig. 3B), showing that our APA predic-
tions using RNA-Seq data are reliable. Most circadian APA events showed an oscillation trend with a decrease during the early day and peak at late day (CT11–CT17) (Fig. 3, C–E). The biological processes significantly enriched for these events were flavonoid biosynthetic process and glucuronidation, response to UV-B, metabolic process, and cellular response to ethylene stimulus (Fig. S6C). Among the genes with rhythmic APA regulation is F1N4, which encodes aspartate oxidase, an enzyme involved in NAD biosynthesis (38). We found that APA generates two F1N4 isoforms with different 3’ UTR lengths and that the proportion of long to short isoform exhibits circadian rhythmicity. The abundance of these isoforms displayed a trend where the longer isoform predominates at subjective night, whereas the shorter isoform predominates during subjective day (Fig. 3F). Overall, we detected a higher number of cycling APA events under diurnal growth conditions compared with circadian growth conditions (Fig. 3A). The phase distribution analysis of diurnal APA events revealed that these events peaked at a later time point than circadian APA events (Fig. 3, D and E, and Fig. S6A). Unlike circadian events, diurnal APA events, whose enriched GO terms include response to cytokinin, photosynthesis, response to light, and cadmium ion and salt stress (Fig. S6D), showed two phase peaks at dawn and dusk (Fig. 3E and Fig. S6B). In summary, our APA analysis indicates genome-wide regulation of alternative polyadenylation by the circadian clock and by rhythmic light exposure in Arabidopsis.

**Alternative splicing and alternative polyadenylation factors exhibit circadian and diurnal rhythmicity at the gene level**

The reason why alternative splicing and alternative polyadenylation oscillate is presumably the time-of-day-dependent changes in the abundance of AS and APA factors that result from rhythmicity in the genes encoding these AS and APA factors. To test this hypothesis, we focused on daily expression patterns of genes encoding proteins playing role in AS and APA (AS- and APA-related genes) and evaluated their correlation with cycling AS and APA events. We first analyzed gene-level oscillation of AS-related genes listed in a publicly available database (39) and found that 95 and 96 of these AS-related genes oscillate in a circadian and diurnal manner, respectively (Fig. 4A). Circadian-regulated AS-related genes peaked at late day (CT13–CT14), whereas AS-related genes with diurnal rhythms showed one main phase at early night (ZT16–ZT17) (Fig. 4B). To explore the roles of cycling AS-related genes in AS rhythmicity, we performed a correlation analysis and determined that the oscillation patterns of the expression of AS-related genes were highly correlated with the patterns of rhythmic AS events (Pearson’s coefficient > 0.8) (Fig. S7). As an example, the circadian expression pattern of the AtGRP8 gene encoding an RNA-binding protein involved in AS (40) showed a high correlation with circadian AS events (Pearson’s coefficient range, 0.80–0.86) (Fig. 4E).

We further explored rhythmicity in the expressions of known APA-related genes in Arabidopsis (27). We found that, among these known genes, CPSF73-II, FEG, PABN3, and FIPS3 oscillated under circadian and diurnal conditions. In contrast, CFIS1, PAB51, PAB52, and PAB54 showed circadianly specific rhythmicity, and PABN1, PABN2, PCFF5, CSTF50, CSTF64, CPSF100, and ESP5 oscillated only under diurnal growth conditions (Fig. 4C). As in rhythmic AS-related genes, the phases of circadian and diurnal APA-related genes were at late day (CT13) and at early night (ZT15–ZT16), respectively (Fig. 4D), which coincides with APA events (Fig. 3E). We identified that the PABN3 gene encoding a poly(A) binding protein (27, 41) shows a circadian expression pattern with a maximum phase at dusk and highly correlates with the rhythmic trends of circadian APA events (Fig. 4F). Similarly, diurnally regulated CPSF100, one of the subunits of cleavage and polyadenylation factor (CPSF) complex (42, 43), also showed a peak of expression at dusk and was highly correlated with diurnal APA events (Pearson’s coefficient range, 0.80–0.88) (Fig. S8A). Our analyses suggest that rhythmic patterns of AS and APA in Arabidopsis are due to gene-level oscillations of AS and APA factors.

**Alternative splicing–related genes undergo rhythmic alternative splicing and alternative polyadenylation**

Our GO enrichment analysis indicated that the significantly enriched GO terms for diurnal ASSS and ASSS events are mRNA splicing via spliceosome, spliceosomal complex assembly, and RNA splicing (Fig. S4), suggesting that splicing events of AS-related genes oscillate. We therefore explored whether splicing events of AS-related genes are rhythmic and found that many splicing events of AS-related genes are circadian or diurnally regulated (Fig. 5A). Intron retention in the LSM1B gene, whose expression is nonrhythmic, generates two splice variants whose proportions oscillate over a circadian cycle (Fig. 5B). In addition, splicing factor RS2Z32 undergoes intron retention and alternative 3’ splice site use that leads to production of two different splice variants. Although the RS2Z32 gene was expressed in a nonrhythmic manner, the splicing events exhibited similar diurnal oscillations (Fig. 5C and Fig. S9A). Furthermore, we tested whether AS-related genes show APA rhythmicity as well. We found that APA regulation of AS-related genes also shows circadian and diurnal rhythms (Fig. 5D and Fig. S8B). For example, APA in the splicing factor SF3b14b gene produces two isoforms whose proportion is rhythmic (Fig. 5E and Fig. S9B). We did not detect any APA-related genes with rhythmic AS or APA regulation. Together, our results show that, in addition to their gene-level oscillation, genes involved in alternative splicing undergo rhythmic AS and APA regulation.

**Discussion**

The plant circadian clock maintains daily synchronization of biological processes through several interlinked transcriptional/translational feedback loops. This complex network, entrained by environmental signals, orchestrates gene expression by regulating transcription. Our results indicate that the plant circadian clock also affects posttranscriptional regulation by regulating alternative splicing and alternative polyadenylation, leading to differences in oscillation patterns of genes and their transcripts. The circadian clock can regulate AS and APA by controlling gene expression of AS- and APA-related genes or by controlling AS and APA regulation of AS-related genes.

Our knowledge of clock-controlled genes in plants is mainly based on microarray analyses. Such a microarray-
them with seedlings grown under a light/dark cycle. Genes exhibiting expression rhythms only under a light/dark cycle are considered diurnal, and genes with oscillating expression at constant light are considered circadian. It is expected that circadianly controlled genes also oscillate under diurnal conditions. Interestingly, we detected a subset of genes that oscillate only under continuous light. By RT-qPCR, we experimentally validated continuous light–specific oscillation in the \textit{LHCA6} and \textit{RBCS2B} genes. Expression of these genes oscillates under continuous light, but they are not rhythmic under light/dark conditions. The disruption of rhythmicity under light/dark condition results from a loss of increase in expression at the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{AS and APA factors exhibit circadian and diurnal rhythmicity at the gene level. \textbf{A}, heatmap of AS-related genes. \textbf{B}, phase value distribution of rhythmic AS-related genes. \textbf{C}, heatmap of APA-related genes. \textbf{D}, phase value distribution of rhythmic APA-related genes. \textbf{E}, circadian profiles of the \textit{AtGRP8} (red line) gene and the AS events highly correlated with \textit{AtGRP8} expression (gray lines; Pearson’s correlation $> 0.8$). \textbf{F}, circadian profiles of the \textit{PABN3} (red line) gene and the APA events highly correlated with \textit{PABN3} expression (gray lines; Pearson’s correlation $> 0.8$).}
\end{figure}

based approach, however, has limitations for transcript detection. In this work, we created genome-wide profiles of \textit{A. thaliana}’s oscillating transcriptome by performing strand-specific RNA-Seq of seedlings collected at 3-h intervals throughout a 24-h period. These RNA-Seq data were further analyzed using Metacycle to uncover underlying patterns. Our analysis with a strict oscillation criterion indicated that $\sim \frac{1}{2}$ of genes exhibit diurnal or circadian rhythms and that the percentage of rhythmic genes can change according to the oscillation criterion (data not shown). To distinguish light-regulated and core clock–regulated transcriptomes, we transferred light/dark cycle–entrained seedlings to constant light and compared them with seedlings grown under a light/dark cycle. Genes exhibiting expression rhythms only under a light/dark cycle are considered diurnal, and genes with oscillating expression at constant light are considered circadian. It is expected that circadianly controlled genes also oscillate under diurnal conditions. Interestingly, we detected a subset of genes that oscillate only under continuous light. By RT-qPCR, we experimentally validated continuous light–specific oscillation in the \textit{LHCA6} and \textit{RBCS2B} genes. Expression of these genes oscillates under continuous light, but they are not rhythmic under light/dark conditions. The disruption of rhythmicity under light/dark condition results from a loss of increase in expression at the
dark phase, implying that these genes are regulated by another light-dependent pathway in addition to being regulated by the core clock mechanism, and that this additional pathway disrupts rhythmicity. Our results suggest that there are two subgroups of circadian genes. One group with oscillation behavior under light/dark conditions and continuous light is only regulated by the core clock mechanism, whereas the other group with continuous light-specific oscillation is regulated by the core clock mechanism and also by another pathway that is effective under light/dark conditions. Our result is consistent with the conclusion of a previous study that adopted a different approach for rhythmicity analysis (35) and also with the finding...
of a recent study showing continuous-dark–specific oscillation in the liver of mice (44). We also observed the same phenomenon with oscillating AS and APA events; some events became rhythmic only under constant light, and the oscillation profiles of AS and APA events were altered dramatically with the change in light regimen. These findings show that plants organize genome-wide transcriptional and posttranscriptional oscillation according to the timing and duration of light and dark periods. The importance of this phenomenon for plants’ adaptation to their environment should be explored in future studies.

Our analysis showed that ~50% of rhythmic genes have a single transcript, indicating that the circadian clock controls expression of these genes during transcription. We also identified a similar number of oscillating genes with more than one transcript. Comparison of the oscillation profiles of these genes and their transcripts revealed that some genes can show different expression patterns from their individual transcripts, indicating the effect of the circadian clock on posttranscriptional regulation. Among posttranscriptional mechanisms, alternative splicing is widespread in a variety of organisms, including plants. In Arabidopsis, a high proportion of genes undergo AS, which produces several transcripts with distinct properties. AS regulation of core clock genes has been shown to be an important factor for generation of circadian timing, and this is supported by our finding that the GO terms enriched for rhythmic AS events are circadian rhythm and negative regulation of circadian rhythm. Although the link between the circadian clock and alternative splicing has been well-studied, genome-wide control of AS by the circadian clock was unknown. In this study, we identified the circadian clock’s genome-wide influence on AS in Arabidopsis and found that the clock synchronizes A5SS, A3SS, SE, and RI. The only AS type that does not show rhythmicity was MXE, which is also much less common in plants compared with other AS types. Although we detected a similar number of RI and SE events, the most abundant rhythmic AS type was RI, suggesting that the molecular mechanism of RI is different from other types and that an AS factor specifically involved in RI is under control of the circadian clock. Furthermore, we revealed that many AS-related genes with rhythmic expression patterns correlated with rhythmic AS events. Rhythmicity in AS-related genes has been also shown in human colorectal cancer cells (45), Drosophila neurons (46), and mouse liver (47). Interestingly, AS-related genes mainly peaked at late day in human colorectal cancer cells and during the day in mouse liver, in line with the late-day phases of AS-related Arabidopsis genes. This suggests that the oscillation patterns of AS-related genes, so presumably AS, are conserved among nocturnal and diurnal organisms.

In addition to AS, we identified genome-wide rhythmicity of APA in Arabidopsis, which has been reported previously in mouse liver (48). Interestingly, APA-related and AS-related genes oscillated in a similar pattern, implying that the factors of two different posttranscriptional mechanisms are synchronized by the circadian clock in a similar way. Furthermore, we identified that AS-related genes undergo rhythmic APA events, implying that the circadian clock also affects AS through APA regulation of AS-related genes. The fact that AS-related genes also undergo rhythmic AS regulation implies a feedback mechanism. Rhythmic posttranscriptional regulation only applies to AS-related genes because we did not observe AS and APA oscillation in APA-related genes. Most interestingly, APA-related gene oscillation peaks were associated with corresponding peaks in APA events; however, the oscillation peaks of AS-related genes and AS events were clearly not correlated in many cases. In these cases, it is likely that rhythmicity was strongly influenced by the posttranscriptional oscillation of AS-related genes.

The Arabidopsis circadian clock synchronizes posttranscriptional regulation by controlling AS and APA. The clock controls AS and APA by regulating the expression of AS- and APA-related genes and also by controlling AS and APA regulation of AS-related genes. Our study paves the way to illuminate the detailed molecular mechanism for circadian clock influence on posttranscriptional regulation. We believe that our work contributes to understanding the highly complex mechanism of the circadian clock.

**Experimental procedures**

**Plant materials and growth conditions**

Seeds of A. thaliana ecotype Columbia (Col-0) were surface-sterilized, stratified for 2 days at 4 °C, and then planted on a Murashige and Skoog plate. The seedlings were grown under long-day conditions (16 h light/8 h dark) with a cool white fluorescent light at 24 °C for 8 days. Then they were transferred to continuous light or kept under long-day conditions at 24 °C for an additional 2 days to explore circadian and diurnal regulation. To determine the oscillating transcriptome, about 30 seedlings were collected at 3-h intervals (ZT2, ZT5, ZT8, ZT11, ZT14, ZT17, ZT20, and ZT23). Two biological replicates at each time point were utilized.

**RNA-Seq library preparation and data preprocessing**

Total RNA from seedlings collected at different time points was isolated using the RNeasy Plant Mini Kit (Qiagen) and treated with the Turbo DNA-free Kit (Invitrogen) following the manufacturer’s instructions. Library preparation and strand-specific paired-end sequencing (2 × 150 bp) on a HiSeq 4000 platform (Illumina) were performed by Novogene.

Low-quality reads, which have less than 60% of bases with a quality score greater than 20, were first filtered out for each sample using the fastq_quality_filter program (RRID: SCR_005534), and only paired-end reads were retained for further analysis. Hisat2 was employed to align the clean reads onto the Arabidopsis reference genome (TAIR10) using the default parameters (50). Reads that were uniquely mapped to only one genomic region were retained and sorted according to their positions in the reference genome using SAMTools (51). These sorted alignments were then assembled into transcripts using StringTie (52) with the guidance of a genome annotation file downloaded from the Ensembl Genomes website (53). For consistency across different samples, all genes and transcripts identified in any of the libraries were merged using the merge function of StringTie. The expression abundance of each transcript was measured and normalized based on its library size by computing the number of fragments per kilobase of exon per million fragments mapped, and gene-level expression abundance.
was computed by summing up the expression abundance of its transcripts.

**RT-qPCR analysis**

Total RNA from seedlings collected at 6-h intervals (ZT5, ZT11, ZT17, and ZT23) was isolated using the RNeasy Plant Mini Kit (Qiagen) and treated with the Turbo DNA-free Kit (Invitrogen) following the manufacturer’s instructions. Complementary DNA was synthesized using Superscript™II reverse transcriptase (Invitrogen). PCR reaction mixtures were prepared using iTaq Universal SYBR Green Mix (Bio-Rad) according to the manufacturer’s instructions and run on a Quantstudio™7 Flex Real-Time PCR instrument. The average of cycle threshold values from two biological and three technical replicates obtained for each sample was used to determine relative expression levels by the 2-ΔΔCT method.

**Detection of alternative splicing**

rMATS v. 4.0.2 was applied to determine the five major types of AS events: A3SS, A5SS, MXE, RI, and SE (54). We created unions of potential splice sites by combining alternative splicing events from any time point(s). Specifically, we pooled all AS profiles within each condition (CT or ZT) and extracted the proportions of inclusion isoforms from each of the alternative splicing events that occur at least one time point for downstream analysis, effectively creating a union of splice sites for each condition.

**Characterization of alternative polyadenylation dynamics**

Alternative polyadenylation dynamics across different time points were identified and characterized using the APAtrap package (55). Specifically, a sorted bam file of each time point was first converted to bedgraph format using the genome-CoverageBed function of BEDTools (56), with the -bg option to report the mapping depth in the output bedgraph file. All bedgraph files were taken as input to refine the annotated 3’ UTR and detect novel 3’ UTR or 3’ UTR extensions using the identifyDistal3UTR program with a window size of 50 bp and a pre-extension size of each 3’ UTR of 5000 bp. Potential APA sites, as well as their use, of each gene were computed by the predictAPA program. The distance between two close APA sites is required to be no less than 50 bp. Then, for each gene, the proportions of the isoforms using different APA sites were calculated for each time points.

**Oscillation analysis at the gene, AS, and APA levels**

The oscillation queries at transcriptional and posttranscriptional levels were performed using Metacycle software (30). Specifically, for expression data, we calculated the oscillation profile over a series of time points at the gene and transcript levels, where gene-level oscillation profiles reflect an accumulating result of all expressed transcripts. For AS and APA events, we only performed transcript-level oscillation analysis. Rhythmic signals were examined using all three methods: ARS (ARSER), JTK (JTK_CYCLE) and LS (Lomb-Scargle). First, we detected rhythmic signals at the gene and transcript levels. The genes/isoforms were considered to be significantly oscillating when the criterion of meta2d_BH.Q (false discovery rate based on the integrated p values) < 0.05. For each gene, Pearson’s correlation was computed between the temporal expression profiles of the gene and each of its isoforms. Pearson’s coefficient > 0.8 indicated the same oscillation pattern between gene and transcript, whereas Pearson’s coefficient < −0.8 was considered the completely opposite pattern. At the posttranscriptional level, we performed an analysis for the proportion of inclusion isoforms for each AS event and the proportion of isoforms for each APA event with a criterion of meta2d_p < 0.05. Because too few oscillating AS events were identified for the MXE type, we only included the other four types in the downstream analysis. GO enrichment analysis was implemented for significantly oscillating genes and isoforms using Bioinformatics Resources v. 6.8 (57). GO terms were of statistically significant enrichment when p < 0.05.

To assess the oscillating patterns of posttranscriptional events over time within each condition, trend analyses were performed for oscillating AS and APA events, respectively, using Short Timeseries Expression Miner version 1.3.8.43 (49). All events belonging to the same profile were proposed to have a similar temporal oscillating pattern with each other. Profiles whose adjusted p < 0.05 (Bonferroni correction at the significant level of p < 0.05) were considered significantly overrepresented for these events.

For each of the AS–related genes, we computed the Pearson’s correlations between its temporal expression profile and the inclusion proportions of each of the oscillating AS events. Events that have a Pearson’s coefficient > 0.8 were considered highly correlated. A similar analysis was also applied to APA-related genes and oscillating events.

**Data availability**

All data are included in this article and supplementary files. All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus under accession number GSE137732.

**Author contributions**—Y. Y. and Y. L. software; Y. Y. and O. O. formal analysis; Y. Y., Y. L., and O. O. validation; Y. Y. and O. O. investigation; Y. Y., Y. L., and O. O. methodology; Y. Y. and O. O. writing-original draft; Y. Y., Y. L., A. S., and O. O. writing-review and editing; A. S. resources; A. S. and O. O. supervision; A. S. funding acquisition; A. S. and O. O. project administration; O. O. conceptualization; O. O. data curation; O. O. visualization.

**Acknowledgments**—We thank Dr. Christopher P. Selby and Dr. Laura Lindsay-Boltz for critical and helpful comments on the manuscript.

**References**

1. Hsu, P. Y., and Harmer, S. L. (2014) Wheels within wheels: the plant circadian system. *Trends Plant Sci.* 19, 240–249 CrossRef Medline
2. Sanchez, S. E., and Kay, S. A. (2016) The plant circadian clock: from a simple timekeeper to a complex developmental manager. *Cold Spring Harb. Perspect. Biol.* 8, pii: a027748 CrossRef Medline
3. Oztas, O., Selby, C. P., Sancar, A., and Adebali, O. (2018) Genome-wide excision repair in *Arabidopsis* is coupled to transcription and reflects circadian gene expression patterns. *Nat. Commun.* 9, 1503 CrossRef Medline
4. McClung, C. R. (2019) The plant circadian oscillator. *Biology* 8, pii: E14 CrossRef Medline
43. Lin, J., Xu, R., Wu, X., Shen, Y., and Li, Q. Q. (2017) Role of cleavage and polyadenylation specificity factor 100: anchoring poly(A) sites and modulating transcription termination. *Plant J.* 91, 829–839 CrossRef Medline

44. Li, H., Zhang, S., Zhang, W., Chen, S., Rabearivony, A., Shi, Y., Liu, J., Corton, C. J., and Liu, C. (2020) Endogenous circadian time genes expressions in the liver of mice under constant darkness. *BMC Genomics* 21, 224 CrossRef Medline

45. El-Athman, R., Fuhr, L., and Relógio, A. (2018) A systems-level analysis reveals circadian regulation of splicing in colorectal cancer. *EBioMedicine* 33, 68–81 CrossRef Medline

46. Wang, Q., Abruzzi, K. C., Rosbash, M., and Rio, D. C. (2018) Striking circadian neuron diversity and cycling of *Drosophila* alternative splicing. *eLife* 7, pii: e35618 CrossRef Medline

47. McGlincy, N. J., Valomon, A., Chesham, J. E., Maywood, E. S., Hastings, M. H., and Ule, J. (2012) Regulation of alternative splicing by the circadian clock and food related cues. *Genome Biol.* 13, R54 CrossRef Medline

48. Gendreau, K. L., Unruh, B. A., Zhou, C., and Kojima, S. (2018) Identification and characterization of transcripts regulated by circadian alternative polyadenylation in mouse liver. *G3* 8, 3539–3548 CrossRef Medline

49. Ernst, J., and Bar-Joseph, Z. (2006) STEM: a tool for the analysis of short time series gene expression data. *BMC Bioinformatics* 7, 191 CrossRef Medline

50. Kim, D., Pagii, J. M., Park, C., Bennett, C., and Salzberg, S. L. (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* 37, 907–915 CrossRef Medline

51. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., and 1000 Genome Project Data Processing Subgroup (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* 25, 2078–2079 CrossRef Medline

52. Pertea, M., Pertea, G. M., Antonescu, C. M., Chang, T.-C., Mendell, J. T., and Salzberg, S. L. (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 33, 290–295 CrossRef Medline

53. Kersey, P. J., Allen, J. E., Allot, A., Barba, M., Boddu, S., Bolt, B. J., Carvalho-Silva, D., Christensen, M., Davis, P., Grabmueller, C., Kumar, N., Liu, Z., Maurel, T., Moore, B., McDowall, M. D., et al. (2018) Ensembl Genomes 2018: an integrated omics infrastructure for non-vertebrate species. *Nucleic Acids Res.* 46, D802–D808 CrossRef Medline

54. Park, J. W., Tokheim, C., Shen, S., and Xing, Y. (2013) Identifying differential alternative splicing events from RNA sequencing data using RNASeq-MATS. *Methods Mol. Biol.* 1038, 171–179 CrossRef Medline

55. Ye, C., Long, Y., Ji, G., Li, Q. Q., and Wu, X. (2018) APAtrap: identification and quantification of alternative polyadenylation sites from RNA-seq data. *Bioinformatics* 34, 1841–1849 CrossRef Medline

56. Quinlan, A. R., and Hall, I. M. (2010) BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842 CrossRef Medline

57. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 4, 44–57 CrossRef Medline