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

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Abstract

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 the 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, 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 the 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 the transcription of genes, but also affects their posttranscriptional regulation by influencing alternative splicing and alternative polyadenylation.

Introduction

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 the 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 transcriptional level, the core-clock genes are regulated at 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 the recruitment of splicing factors and heterogeneous nuclear ribonucleoproteins (hnRNPs), but other splicing regulators such as SR proteins and KH-domain RBPs are also involved in this process (8, 9). In addition to transcripts encoding proteins with different properties, AS leads to the production of transcripts with a premature termination codon (PTC) that marks them for degradation by the nonsense-medicated decay (NMD) 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 *PSEUDO-RESPONSE REGULATORs (PRRs)* and *REVEILLE 8 (RVE8)*, evening-phased *TIMING OF CAB EXPRESSION 1 (TOC1)*, *GIGANTEA (GI)*, *EARLY FLOWERING 3 (ELF3)* have been shown to be alternatively spliced (13–16). On the other hand, the loss of function of PROTEIN ARGININE METHYLTRANSFERASE5 (PRMT5), a splicing factor, and SPLICEOSOMAL TIMEKEEPER LOCUS1 (STIPL1), playing a role in spliceosome disassembly, lead to longer circadian period (17–19), indicating the role of splicing in *Arabidopsis* circadian clock. GEMIN2, required for the spliceosome assembly, has been shown to affect AS of core-clock genes and function as the link between temperature change and circadian clock (20).

Alternative polyadenylation (APA) is another posttranscriptional mechanism that generates multiple transcripts through the 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 the attachment of a poly(A) tail (21). APA can produce

isoforms that differ in their 3'-untranslated regions (UTRs) or proteins with differing functions, stability or localization (22–24). APA is widespread in plants and more than 75% of the mRNA transcripts in *Arabidopsis* are APA-regulated (25). APA has been reported to be involved in numerous biological processes, especially in flower development (26–29).

Herein, we examined the 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 either 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 transcription level, but also modulates gene expression at the posttranscriptional level by regulating AS and APA.

Results

Non-homogeneous 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-days-old *Arabidopsis* seedlings, grown under long day condition (16/8h light-dark), were transferred to continuous light or kept under the same light/dark condition for 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 rhythmicity of a gene based upon the total expression levels of all 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 are circadian- and diurnal-regulated, respectively, which is in line with previous microarray-based analyses (32–34). The comparison of oscillating circadian and diurnal genes revealed that 3104 genes show rhythms in both conditions, and 3575 genes showed diurnal-specific rhythmicity. In addition, a subset of circadian genes (2916 genes) oscillated only at constant light (Fig. 1A, Supplemental Fig. S1A), similar to the observation in lettuce (35). Our RT-qPCR analyses of LHCA6 and RBCS2B genes verified that these genes oscillate under continuous light, but not at light-dark condition. The loss of expression induction at dark phase presumably leads to non-rhythmicity at diurnal condition in these genes (Supplemental Fig. S1B,C). The phase value distribution of the 2916 exclusively circadian and the 3575 exclusively diurnal genes exhibited phases of maximum expression at late day (Circadian Time (CT)13) and at early dark (Zeitgeber Time (ZT)16-18), 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 both circadian and diurnal-controlled genes were circadian clock and photosynthesis. We detected more enriched GO terms for circadian genes than diurnal genes (Supplemental Fig. S2), suggesting that the diurnal genes are more diverse in terms of biological function compared to the circadian genes.

We next examined the oscillation in expression patterns at both gene level and transcript level. We found that many oscillating genes (42% of 6020 circadian genes and 43% of 6679 diurnal genes) possess only one transcript (single-transcript); however, a higher proportion of cycling genes (57.9% and 57.2% of circadian and diurnal genes, respectively) have more than one transcript (multi-transcript) (Fig. 1C). The proportion of oscillating multiple-transcript genes is significantly higher than the multi-transcript genes over the whole genome (39.2%, p-value of Fisher's s Exact Test < 2.2e-16). Interestingly, a comparison of oscillation profiles of multi-transcript genes and their transcripts showed the existence of cases in which 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 exhibit different oscillation patterns (Fig. 1E). As an example, while both isoforms of *4CL3* gene encoding a protein involved in phenylpropanoid pathway (36), exhibited the same oscillation pattern with their gene (Fig. 1F), a RING/Ubox superfamily gene and one of its isoforms showed anti-phase oscillation patterns (Fig. 1G). Overall, genome-wide transcriptome analysis of *Arabidopsis* throughout the day reveals 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 for the differences in oscillation behaviors 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 11603 and 12195 AS events, respectively, including the following types of AS: alternative 5' splice site (A5SS), alternative 3' splice site (A3SS), mutual exclusive exons (MXE), retained introns (RI) and skipped exons (SE). Our analysis revealed that all types of AS events occur in *Arabidopsis thaliana*; however, intron retention and exon skipping are the major AS events (Supplemental 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. 2A,B; Supplemental Fig. S3C), and the oscillation behaviors of AS events largely differ under the two light regimens (Supplemental Fig. S3B).

We identified 57 A3SS, 42 A5SS and 55 SE events that cycle 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 the diurnal AS events revealed that the enriched GO terms include regulation of vesicle fusion, abscisic acid-activated signaling pathway, response to cadmium ion, long-day photoperiodism (flowering), response to salt stress, positive regulation of transcription (DNA-templated and from RNA polymerase II promoter) and activation of GTPase activity (Supplemental Fig. S4). Among enriched GO terms for all rhythmic AS events, we identified circadian rhythm and negative regulation of circadian rhythm that support the link between alternative splicing and circadian clock (17).

The most prevalent oscillating AS type was intron retention with 203 circadian and 246 diurnal events (Supplemental Fig. S3A). Unlike the other types of rhythmic AS whose phase peaks were distributed over the entire cycle, the phases of cycling RI events peaked at late day (CT10-14 and ZT10-13) under both 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 the RI events with diurnal rhythms (Supplemental Fig. S5A,B). The significantly enriched GO terms for RI events that oscillate in a circadian manner included DNA unwinding involved in DNA replication, inositol phosphate dephosphorylation, phosphatidylcholine metabolic process and pollen germination (Fig. 2D). The GO terms for cycling RI events showed difference based on whether the growth regimen is circadian or diurnal (Supplemental Fig. S5C). We conclude that alternative splicing in *Arabidopsis* is under the control of circadian and diurnal regulation, and intron retention is the most prevalent cycling AS type.

Circadian clock regulates alternative polyadenylation

We explored rhythmicity of alternative polyadenylation from our transcriptome data and identified circadian and diurnal-regulated APA events by using the Metacycle software with a meta2d_p-value cutoff of 0.05. We detected 643 circadian APA events out of the total 25076 APA events identified in 597 genes and 1446 diurnal APA events out of the total 24036 APA events identified in 1312 genes. A low proportion of cycling APA events overlapped at both conditions, similar to our observation in our analyses at gene and AS level (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 approximately 60% of total APA events and 75% of oscillating APA events detected in our analysis have been previously identified (Fig. 3B), showing that our APA predictions using RNA-seq data is reliable. Most circadian APA events showed an oscillation trend with a decrease during the early day and peak at late day (CT11-17) (Fig. 3C, D, 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 (Supplemental Fig. S6C). Among genes with rhythmic APA regulation is *FIN4* which encodes aspartate oxidase, an enzyme involved in NAD biosynthesis (38). We found that APA generates two *FIN4* isoforms with different 3'UTR lengths and the proportion of long to short isoform exhibits circadian rhythmicity. The abundance of these isoforms displayed a trend such that the longer isoform predominates at subjective night, whereas the shorter isoform predominates during the subjective day (Fig. 3F). Overall, we detected a higher number of cycling APA events under diurnal growth condition compared to circadian growth condition (Fig. 3A). The phase distribution analysis of diurnal APA events revealed that these events peak at a later timepoint than circadian APA events (Fig 3D, E, Supplemental Fig. S6A). Unlike circadian events, diurnal APA events, whose enriched GO terms include response to cytokinin, photosynthesis, response to light, cadmium ion and salt stress (Supplemental Fig. S6D), showed two phase peaks at dawn and dusk (Fig. 3E, Supplemental Fig. S6B). To sum up, 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 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 the rhythmicity in the genes encoding these AS and APA factors. To test this hypothesis, we focused on daily expression patterns of the genes encoding the proteins playing role in AS and APA (AS- and APA-related genes) and evaluated their correlations with cycling AS and APA events. We first analyzed the 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). The circadian-regulated AS-related genes peaked at late day (CT13-14), whereas the AS-related genes with diurnal rhythms showed one main phase at early night (ZT16-17) (Fig. 4B). To explore the roles of cycling AS-related genes in AS rhythmicity, we performed correlation analysis and determined that oscillation patterns of the expressions of AS-related genes were highly correlated with the patterns of rhythmic AS events **(**Pearson's coefficient > 0.8) (Supplemental Fig. S7). As an example, the circadian expression pattern of *AtGRP8* gene encoding an RNA-binding protein involved in AS (40) showed a high correlation with circadian AS events **(**Pearson's coefficients 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 genes oscillated in both circadian and diurnal conditions. In contrast, CFIS1, PABS1, PABS2 and PABS4 showed circadian-specific rhythmicity; and PABN1, PABN2, PCFS5, CSTF50, CSTF64, CPSF100 and ESP5

oscillated only in the diurnal growth condition (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 (ZT15-16), respectively (Fig. 4D), which coincides with APA events (Fig. 3e). We identified that 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, diurnal-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 coefficients range: 0.80 - 0.88) (Supplemental 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 A3SS and A5SS events are mRNA splicing via spliceosome, spliceosomal complex assembly and RNA splicing (Supplemental 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 diurnal-regulated (Fig. 5A). Intron retention in *LSM1B* gene whose expression is non-rhythmic 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 usage that lead to the production of two different splice variants. Although the *RS2Z32* gene was expressed in a non-rhythmic manner, the splicing events exhibited similar diurnal oscillations (Fig. 5C, Supplemental Fig. S9A). Furthermore, we tested whether AS-related genes show APA rhythmicity as well. We found that the APA regulation of AS-related genes also shows circadian and diurnal rhythms (Fig. 5D, Supplemental Fig. S8B). For example, APA in splicing factor *SF3b14b gene* produces two isoforms whose proportion is rhythmic (Fig. 5E, Supplemental Fig. S9B). We have not detected 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 either 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 microarray-based approach, however, has limitations for transcript detection. In this work, we created genome-wide profiles of *Arabidopsis thaliana*'s oscillating transcriptome by performing strand-specific RNA-seq of seedlings collected at 3h intervals throughout a 24h period. These RNA-seq data were further analyzed using Metacycle to uncover underlying patterns. Our analysis with a strict oscillation criterion indicated that approximately 1/5 of genes exhibit diurnal or circadian rhythms and 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 with the seedlings grown under light/dark cycle. Genes exhibiting expression rhythms only under light/dark cycle

are considered as diurnal, and genes with oscillating expression at constant light are considered as circadian. It is expected that circadian-controlled genes also oscillate at 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 *LHCA6* and *RBCS2B* genes. The expression of these genes oscillates under continuous light, but they are not rhythmic at light-dark condition. And, the disruption of rhythmicity at light-dark condition results from the loss of increase in expression at dark phase, implying that these genes are regulated by another light-dependent pathway in addition to being regulated by core-clock mechanism, and this additional pathway disrupts rhythmicity. So, our results suggest that there are two subgroups of circadian genes: one group with oscillation behavior both at light-dark condition and continuous light are only regulated by core clock mechanism, while the other group with continuous light-specific oscillation are regulated by core clock mechanism and also by another pathway that is effective at light-dark condition. Our result is consistent with the conclusion of a previous study in which they 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 the oscillating AS and APA events: some events became rhythmic only under constant light, and the oscillation profiles of AS and APA events dramatically altered with the change of light regimen. All these suggest 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 the plants' adaptation to their environment should be explored by future studies.

Our analysis showed that approximately 50% of rhythmic genes have a single transcript, indicating that the circadian clock controls these genes' expression 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 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 that produces several transcripts with distinct properties. AS regulation of core-clock genes has been shown to be an important factor for the 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. While the link between the circadian clock and alternative splicing has been well-studied, the genome-wide control of AS by 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 to 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 shows a difference from other types and an AS factor specifically involved in RI is under the control of the circadian clock. Furthermore, we revealed 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, is conserved among nocturnal and diurnal organisms.

In addition to AS, we identified genome-wide rhythmicity of APA in *Arabidopsis*, that has been previously reported 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 affects AS also 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 belongs to AS-related genes since 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 association between 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 through controlling alternative splicing (AS) and alternative polyadenylation (APA). The clock controls AS and APA by regulating the expressions 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 the circadian clock influence on posttranscriptional regulation. We believe our work contributes to the understanding of the highly complex mechanism of circadian clock.

Experimental procedures

Plant materials and growth conditions

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

RNA-seq library preparation and data preprocessing

Total RNA from seedlings collected at different timepoints was isolated by RNeasy Plant Mini Kit (QIAGEN) and treated with 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 fastq_quality_filter program (49), 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). The 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 genome annotation file downloaded from Ensembl Genomes website (53). For consistency across different samples, all the genes and transcripts identified in any of the libraries were merged together using the merge function of StringTie.Expression abundance of each transcript was measured and normalized based on their library sizes by computing the number of fragments per kilobase of exon per million fragments mapped (FPKM), and gene-level expression abundance was computed by summing up the expression abundances of its transcripts.

RT-qPCR analysis

Total RNA from seedlings collected in 6-h intervals (ZT5, ZT11, ZT17, and ZT23) was isolated by RNeasy Plant Mini Kit (QIAGEN), treated with TURBO DNA-free Kit (Invitrogen) following the manufacturer's instructions, and

cDNA 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 Quantstudio™ 7 Flex Real-Time PCR instrument. The average of Ct values from two biological and three technical replicates obtained for each sample was used to determine relative expression levels by 2-ΔΔCT method.

Detection of alternative splicing

rMATS v. 4.0.2 was applied to determine five major types of alternative splicing (AS) events: alternative 5' splice sites (A5SS), alternative 3' splice sites (A3SS), mutual exclusive exons (MXE), retained introns (RI) and skipped exons (SE) (54). We created *unions* of potential splice sites by combining alternative splicing events from any time point(s). Specifically, we pooled together all the AS profiles within each condition (CT or ZT), and extracted the proportions of inclusion isoforms from each of the alternative splicing events that occurs in 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 was identified and characterized using APAtrap package (55). Specifically, sorted bam file of each time point was first converted to bedgraph format using the genomeCoverageBed function of bedtools (56), with the -bg option to report the mapping depth in the output bedgraph file. All the bedgraph files were taken as input to refine annotated 3'UTR and detect novel 3'UTR or 3'UTR extensions using 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 usage, of each gene were computed by predictAPA program. The distance between any of 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 gene, AS and APA levels

The oscillation queries at both transcriptional and post-transcriptional levels were performed using Metacycle software (30). Specifically, for expression data, we calculated the oscillation profile over a series of time points at both gene and transcript level, where the gene-level oscillation profiles reflect an accumulating result of all the expressed transcripts. For AS and APA events, we only performed transcript-level oscillation analysis. Rhythmic signals were examined using all the three methods ARS (ARSER), JTK (JTK_CYCLE) and LS (Lomb-Scargle). First, we detected rhythmic signals at both gene and transcript levels. The genes/isoforms were considered as significantly oscillating if meeting 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, while < -0.8 was considered the completely opposite pattern. At the post-transcriptional level, we performed 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_pvalue < 0.05. Since too few oscillating AS events were identified for MXE type, we only included the other four types in the downstream analysis. Gene ontology (GO) enrichment analysis was implemented for the significantly oscillating genes and isoforms using Bioinformatics Resources v. 6.8 (57). GO terms were of statistically significant enrichment if p-value < 0.05.

To assess the oscillating patterns of post-transcriptional events over time within each condition, trend analyses were performed for oscillating AS and APA events, respectively, using Short Timeseries Expression Miner (STEM) version 1.3.8.43 (58). All the events belonging to the same profile were proposed to have similar temporal oscillating pattern with each other. The profiles whose adjusted p-value < 0.05 (Bonferroni correction at the significant

level of p-value < 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 event. The events which have a Pearson's coefficient > 0.8 were considered as highly correlated. 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 (GEO; http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE137732.

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Figures

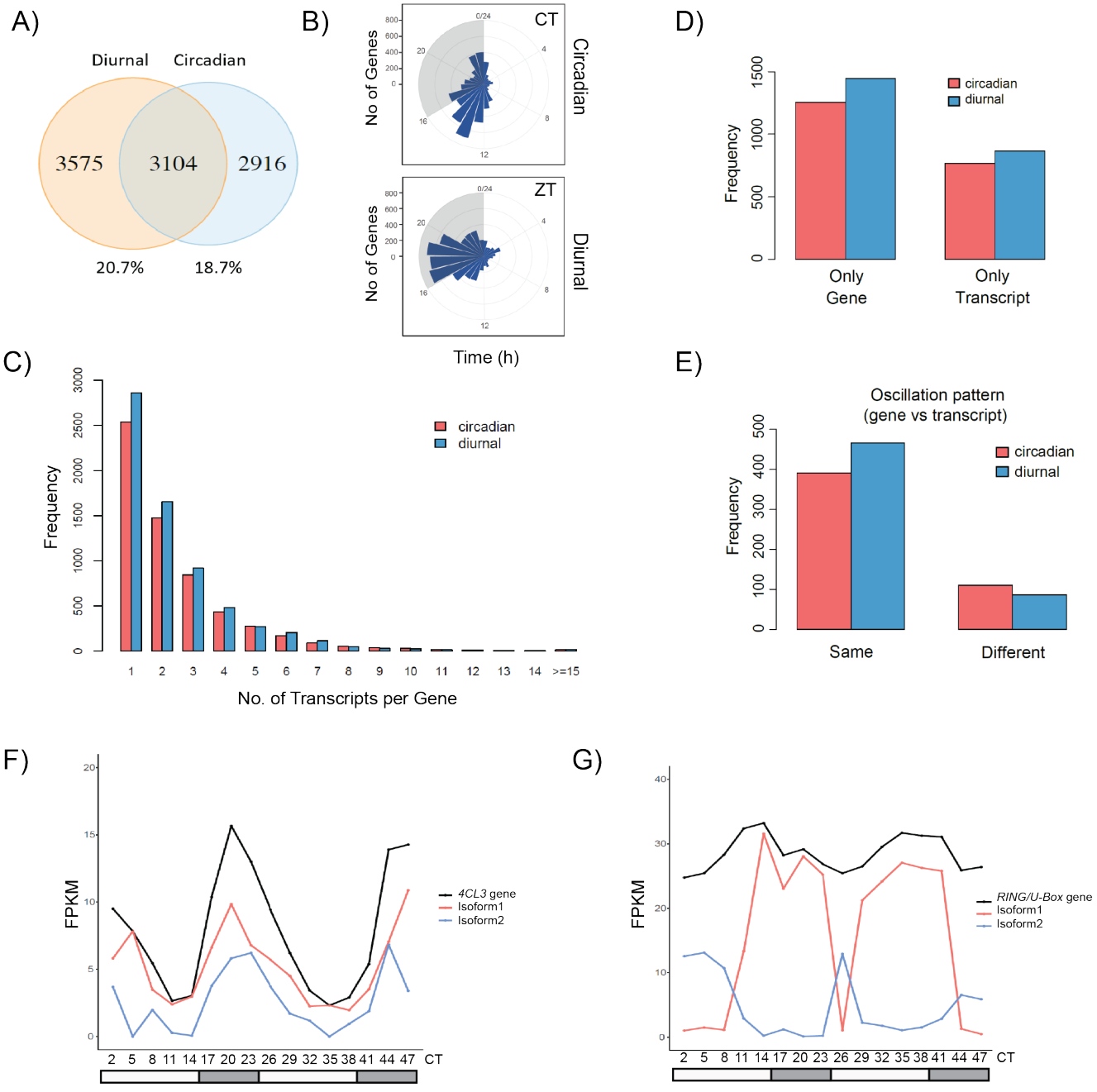

Figure 1. Multi-transcript genes and their transcripts reveal different circadian and diurnal oscillation patterns **A.** The percentage and number of circadian and diurnal oscillating genes (Metacycle, BH.Q <0.05). **B.** The phase value distribution of diurnal- and circadian-regulated genes. The gray shade represents the dark period. **C.** The distribution of transcript number for each oscillating gene. **D.** The number of genes oscillating only at gene or transcript level. **E.** The number of genes that show the same or different oscillation patterns with their transcripts. **F.** The circadian oscillation patterns of 4CL3 gene and its isoforms. **G.** The circadian oscillation patterns of a RING/U-box gene (At3G26730) and its isoforms.

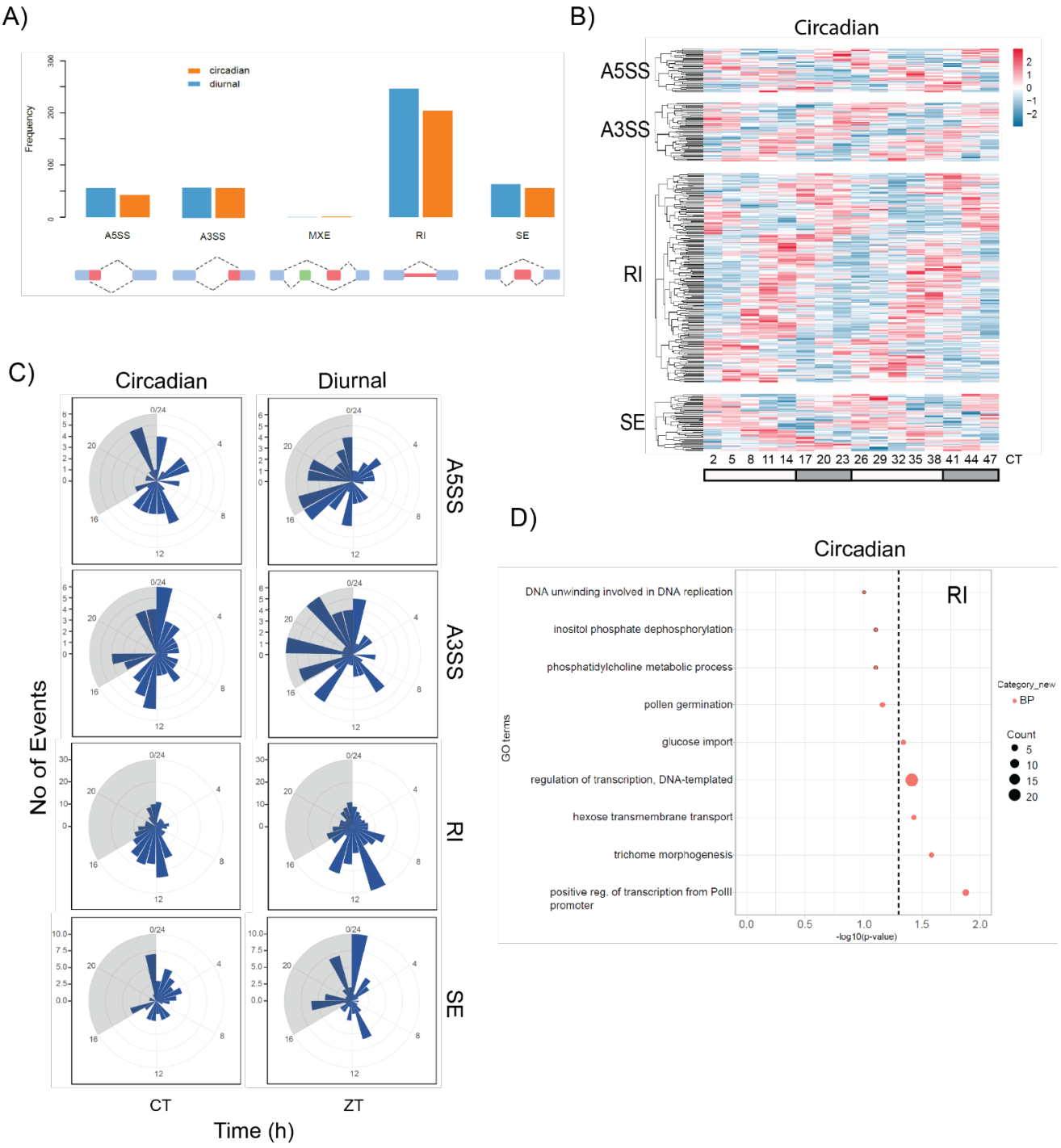

Figure 2. Intron retention is the major type of rhythmic alternative splicing event. **A.** The number of diurnal- and circadian-regulated AS types: alternative 5' splice sites (A5SS), alternative 3' splice sites (A3SS), mutual exclusive exons (MXE), retained introns (RI) and skipped exons (SE). **B.** Heat map 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.** The GO enrichment analysis of the genes that show circadian RI event. The vertical dashed line represents *p*-value = 0.05.

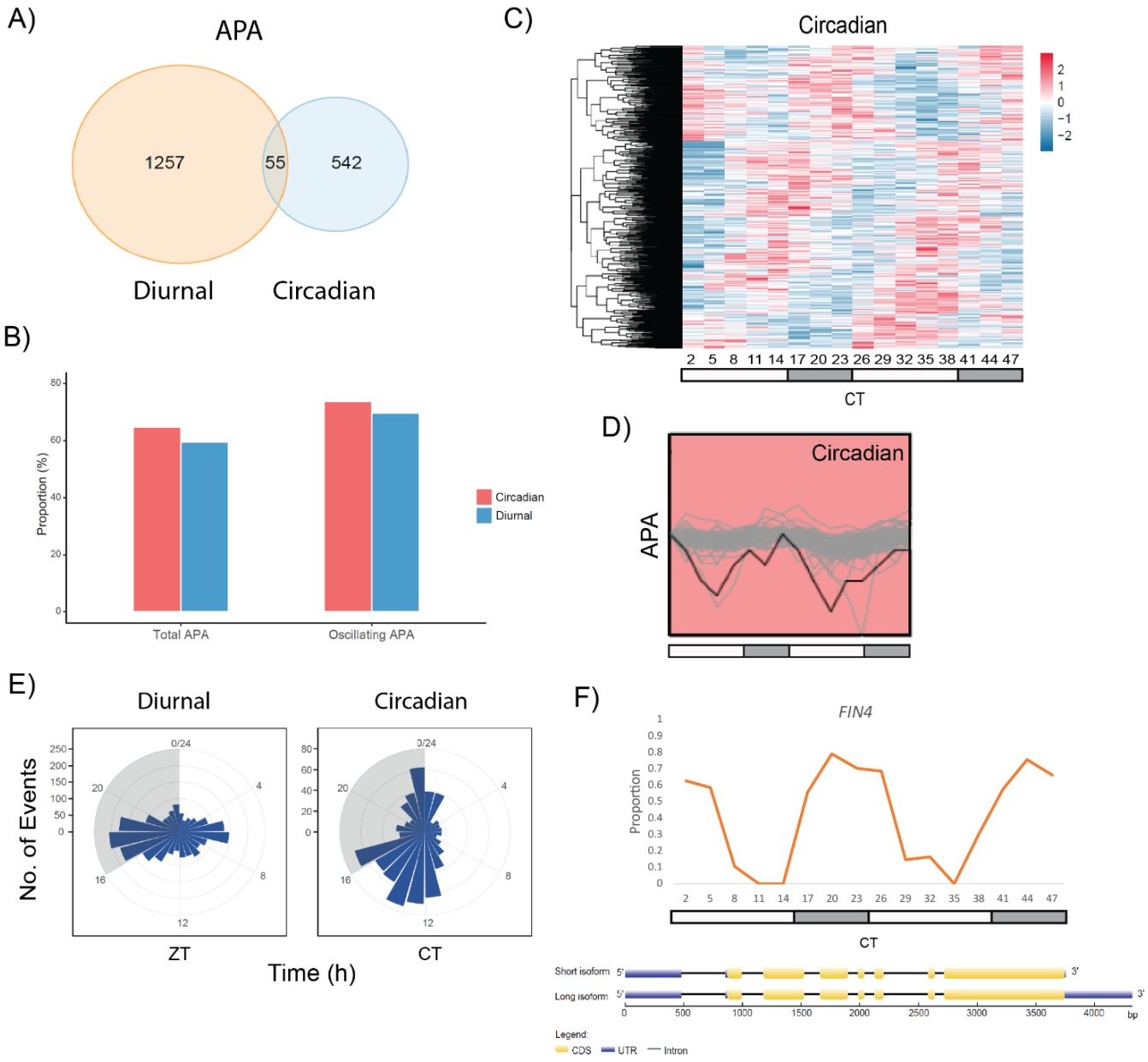

Figure 3. Circadian clock regulates alternative polyadenylation. **A.** The number of genes with circadian- and diurnal-regulated APA events. **B.** The proportion of total and oscillating APA events identified in our analysis to known APA events in public database. **C.** Heat map of APA events that have a circadian proportion of poly(A) usage. **D.** The oscillation trend of circadian poly(A) usage in APA events. Gray lines and black line represent the proportion pattern of each APA event and the proportion tendency of all events, respectively. **E.** Phase value distribution of circadian and diurnal APA events. The gray shade represents the dark period. **F.** Circadian oscillation of poly(A) usage in *FIN4* gene. "Proportion" means the proportion of short isoform with an indicated poly(A) site to total isoforms. The exon-intron structure for each isoform was displayed using Gene Structure Display Server (GSDS) v. 2.0.

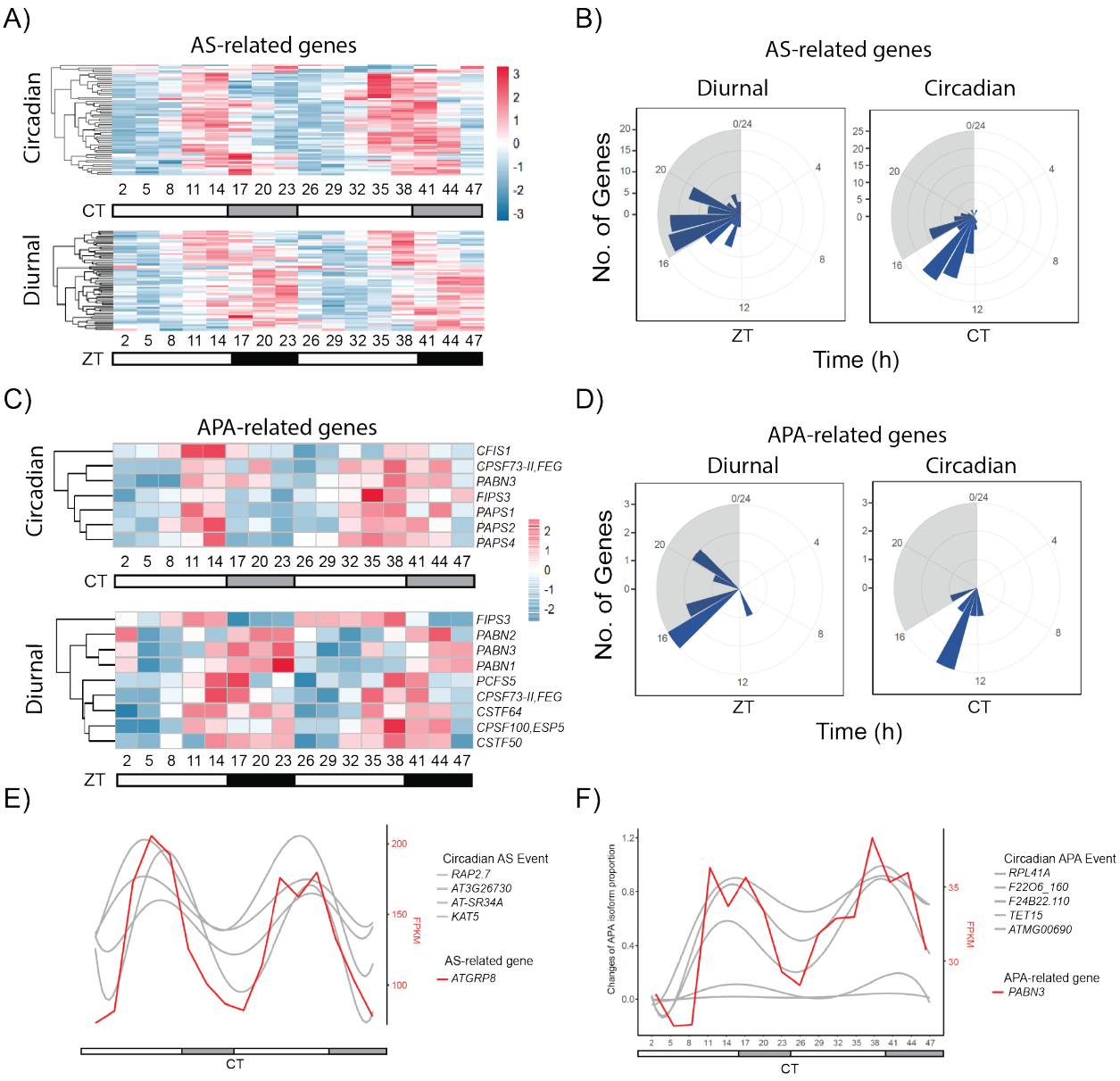

Figure 4. AS and APA factors exhibit circadian and diurnal rhythmicity at gene level. **A.** Heat map of the AS-related genes. **B.** Phase value distribution of the rhythmic AS-related genes. **C.** Heat map of the APA-related genes. **D.** Phase value distribution of the rhythmic APA-related genes. **E.** Circadian profiles of *AtGRP8* (red line) gene and the AS events highly correlated with *AtGRP8* expression (gray lines; Pearson's correlation > 0.8). **F.** Circadian profiles of *PABN*3 (red line) gene and the APA events highly correlated with *PABN3* expression (gray lines; Pearson's correlation > 0.8).

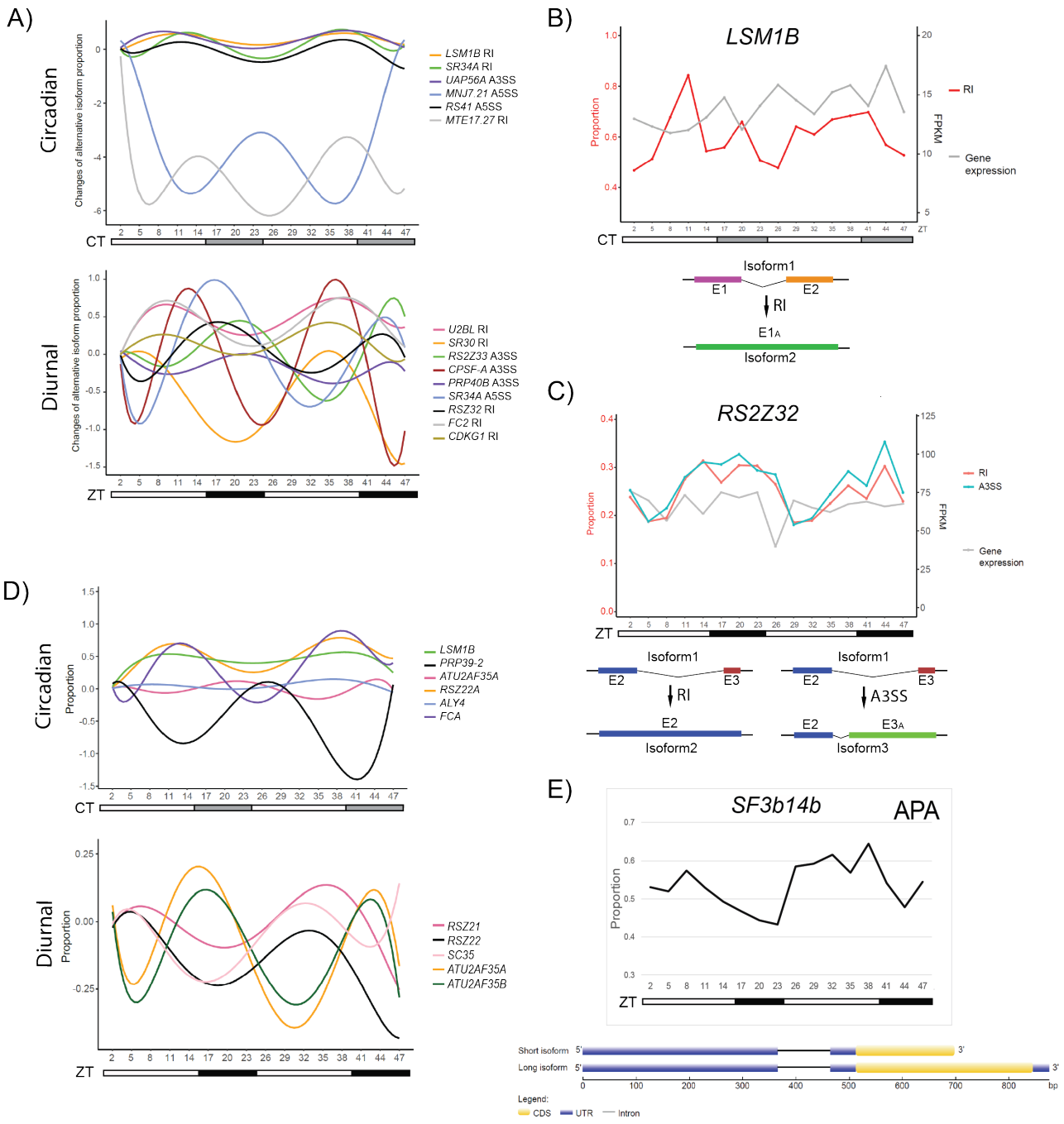

Figure 5. The splice variants of AS-related genes undergo rhythmic AS and APA. **A.** Diurnal and circadian AS profiles of AS-related genes. Each color represents an AS-related gene and its splicing type. **B.** Circadian profiles of *LSM1B* gene expression and the proportion of two isoforms generated by intron retention. **C.** Diurnal profiles of *RS2Z32* gene expression and the proportions of isoforms that are produced by intron retention and the alternative 3' splice site usage. **D.** Diurnal and circadian APA profiles of AS-related genes. **E.** Diurnal profile of poly(A) usage in *SF3b14b* gene. "Proportion" means the proportion of short isoform with an indicated poly(A) site to total isoforms. The exon-intron structure for each isoform was displayed using Gene Structure Display Server (GSDS) v. 2.0.

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

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