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Acute Effects of Light on Alternative Splicing in Light-Grown Plants†

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ABSTRACT

Light modulates plant growth and development to a great extent by regulating gene expression programs. Here, we evaluated the effect of light on alternative splicing (AS) in light-grown *Arabidopsis thaliana* plants using high-throughput RNA sequencing (RNA-seq). We found that an acute light pulse given in the middle of the night, a treatment that simulates photoperiod lengthening, affected AS events corresponding to 382 genes. Some of these AS events were associated with genes involved in primary metabolism and stress responses, which may help to adjust metabolic and physiological responses to seasonal changes. We also found that several core clock genes showed changes in AS in response to the light treatment, suggesting that light regulation of AS may play a role in clock entrainment. Finally, we found that many light-regulated AS events were associated with genes encoding RNA processing proteins and splicing factors, supporting the idea that light regulates this posttranscriptional regulatory layer through AS regulation of splicing factors. Interestingly, the effect of a red-light pulse on AS of a gene encoding a splicing factor was not impaired in a quintuple phytochrome mutant, providing unequivocal evidence that nonphotosensory photoreceptors control AS in light-grown plants.

INTRODUCTION

Plants have evolved the ability to perceive changes in light quantity, quality, direction and duration, and use this information to adjust their growth and development to the prevailing environmental conditions (1). Seedlings growing in the dark, because they have not emerged from the soil yet, have elongated hypocotyls, closed cotyledons and nonphotosynthetic etioplasts. Once the seedlings are exposed to light, they undergo a series of physiological changes that include hypocotyl growth inhibition, cotyledon expansion and chloroplast development, all of which contribute to the switch from heterotrophic to autotrophic growth (1). Light-grown plants, in turn, detect changes in the red/far-red ratio of the incoming light caused by the selective transmission and/or reflection of these wavelengths in the leaves of nearby plants, and respond to this signal with a series of physiological responses that minimize the chances of being shaded (2). Finally, mature plants perceive changes in day-length and use this information to adjust the timing of the floral transition to the most favorable season of the year (3).

Light signals are perceived through different families of photoreceptors. Particularly in *Arabidopsis thaliana* plants, the known photosensory photoreceptors include five phytochromes (phys), which mostly absorb red and far-red light; two cryptochromes (crys), two phototropins and three members of the zeitlupe protein family, which absorb blue and UV-A light, and the more recently characterized UV-B photoreceptors (4).

Upon light activation, most of these photoreceptors control multiple physiological processes by regulating gene expression (4,5). Light-activated phytochromes, in particular, directly interact with transcription factors of the bHLH protein family, controlling their turnover (6). Phytochromes also control gene expression by inactivating the E3 ubiquitin ligase, *CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)*, which targets several regulatory proteins for degradation, such as the bZIP transcription factor *ELONGATED HYPOCOTYL 5 (HY5)* (6). All these phytochrome-regulated transcription factors then control the expression of thousands of genes by activating their transcription (7).

There is also increasing evidence that light regulates other aspects of the gene expression regulatory network, such as mRNA stability, translation and alternative splicing (AS), the process by which a single gene can give rise to different mRNA isoforms (5,7–17) (Fig. 1).

For example, a short exposure to red light in etiolated seedlings regulates the AS patterns of hundreds of genes, including splicing factors themselves. These effects are mediated by the most abundant phytochromes, phyA and phyB, at least in etiolated seedlings (13). Similar results revealing phytochrome control of AS were obtained in a recent study conducted in *Physcomitrella patens* (16). Light also modulates AS in light-grown plants. At this developmental stage, however, most of the effect of light on AS appears to be mediated through a retrograde signaling pathway involving an intermediate molecule generated in the chloroplast during the photosynthetic process, rather than through photosensory photoreceptors (12).

Although the effect of light on AS has been evaluated at a genome-wide level in etiolated seedlings, its effect on AS in light-grown plants has only been investigated for a subset of genes. Here, we used RNA-seq to perform a genome-wide

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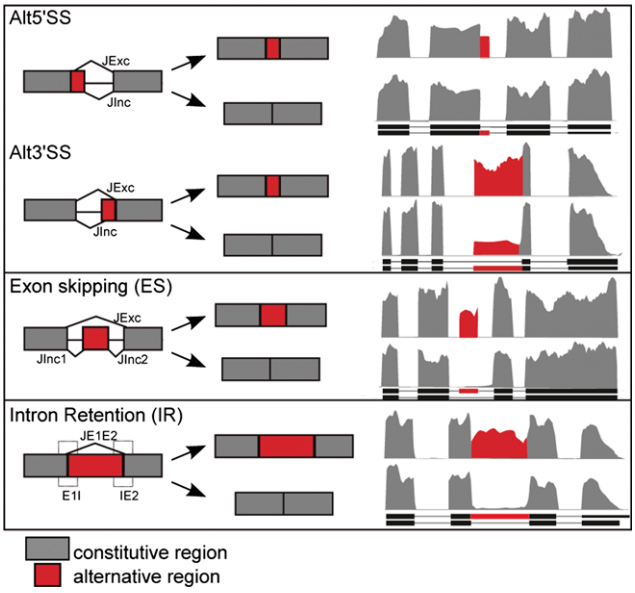

Figure 1. Scheme of the four main types of AS events and their visualization in RNA-seq data. Constitutive regions are shown in grey and alternative regions in red. Final transcripts including or not including alternative regions are shown. Partial gene models are indicated below the RNA-seq coverage plots.

analysis of the effects of a brief light pulse given in the middle of the night on AS in 12 h light: 12 h dark-grown *Arabidopsis thaliana* plants. This experimental protocol is aimed to simulate earlier dawn or later dusk signals associated with photoperiod lengthening, and could allow us to identify posttranscriptional mechanisms involved in the regulation of clock function and/or the floral transition by light.

MATERIALS AND METHODS

Plant material and light pulse treatment. Three biological replicates with fifty seeds of the wild-type Columbia ecotype were sown onto Murashige and Skoog medium containing 0.8% agar, stratified for 4 days in the dark at 4°C and then, transferred to 12 h light: 12 h dark cycles at 22°C (50 μmol m^{-2} s^{-1} of white light provided by fluorescent tubes). After 9 days, the seedlings were irradiated in the middle of the night (ZT18) with either a 2 h white or red light pulse (depending on the experiment), or kept in darkness as controls. The white-light pulse (50 μmol m^{-2} s^{-1}) was provided with fluorescent tubes (Sylvania Standard F18W/T8/154), whereas the red-light pulse (80 μmol m^{-2} s^{-1}) was provided with light emitting diodes (Kingbright Super Bright Leds, L934-SRC, $\lambda = 660 \pm 20$ nm). Samples of the light treated and dark control groups were harvested at the same time and flash frozen in liquid nitrogen. A brief summary of the experimental protocol is described in Fig. 2.

cDNA library preparation and high-throughput sequencing. Total RNA was extracted with RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's protocols. To estimate the concentration and quality of the samples, NanoDrop 2000c (Thermo Scientific) and the Agilent 2100 Bioanalyzer (Agilent Technologies) with the Agilent RNA 6000 NanoKit were used, respectively. Libraries were prepared using 3 μg of total RNA following the TruSeq RNA Sample Preparation Guidelines (Illumina), except for the enrichment step, where only 12 cycles of PCR were performed. Library validation included size and purity assessment with the Agilent 2100 Bioanalyzer and the Agilent DNA 1000 kit (Agilent Technologies). Samples were pooled to create 6 multiplexed DNA libraries, which were single-end sequenced with an Illumina Genome Analyzer II kit on the Illumina GAIIx platform, providing 100-bp single-end reads. On average 11.4 million 100-nt single-end reads (22,9X) were obtained for each sample (Table S1a).

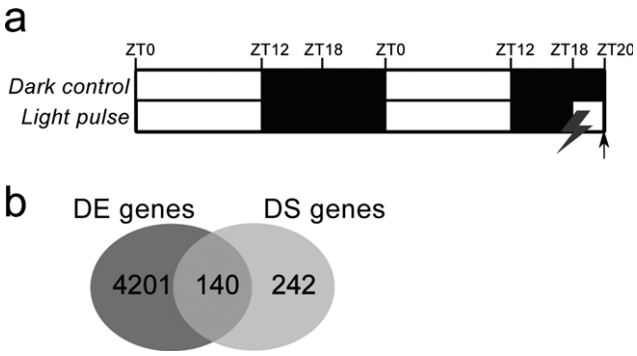

Figure 2. (a) Scheme of the experimental design. After 9 days of growth under 12 h light: 12 h dark cycles, half of the plants were exposed to a 2 h white light treatment in the middle of the night (ZT18), while the other half remained in darkness as controls. (b) Overlap between genes differentially affected by light at the expression level (DE genes) and genes regulated by light at the AS level (DS genes).

Processing of RNA sequencing reads. Sequence reads were aligned against the *Arabidopsis thaliana* genome (TAIR10) with TopHat v2.0.9 (18,19) with default parameters, except in the case of the maximum intron length parameter, which was set at 5000. Count tables for the different feature levels were obtained from bam files using custom R scripts and considering the TAIR10 transcriptome. Count tables at the gene level presented a good correlation overall between replicates and samples (Table S1b). Raw sequences (fastq files) and count tables at gene, exon, intron, AS bin and junction levels used in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE68560).

Differential gene expression analysis. Differential gene expression analysis was conducted for 15 925 genes whose expression was above a minimum threshold level (> 10 reads and a read density > 0.05) in at least one experimental condition. Read density (RD) was computed as the number of reads in each gene divided by its effective width. The term effective width corresponds to the sum of the length of all the exons of a given gene. Differential gene expression was estimated using the edgeR package version 3.4.2 (20), and resulting *P*-values were adjusted using a false discovery rate (FDR) criterion. Genes with FDR values lower than 0.05 and an absolute fold change > 1.5 were considered to be differentially expressed. This dataset was labeled as DE Genes (Table S2).

Differential AS analysis. For the analysis of differential AS, multiexonic genes were partitioned into features defined as "bins", corresponding to exonic regions, intronic regions and alternatively spliced regions. We labeled these three kinds of bins as exon-bins, intron-bins or AS-bins, respectively. In addition, AS-bins were further classified as Exon Skipping (ES), Alternative 5′ Splice Site (Alt5′SS), Alternative 3′ Splice Site (Alt3′SS) and Intron Retention (IR). Bins with three or more different AS events in the same subgenic region were labeled as Multiple. Read summarization was performed at those three levels: exon, intron and AS bins. These datasets were then filtered according to several criteria applied at the gene and bin level. First, defined subgenic regions (i.e. bins) were considered for differential AS analysis only if the genes with which they are associated were expressed above a minimum threshold level (more than 10 reads per gene and RD > 0.05) in all experimental conditions. Next, bins were considered for differential AS analysis only if they had more than 5 reads and a RD bin/RD gene ratio > 0.05, in at least one experimental condition. After applying these filters, reads summarized at the bin level were normalized to the read counts of their corresponding gene. This was done to avoid the influence of changes in gene expression on the differential AS analysis at the bin level. Then, similarly to the approach used for the differential expression analysis, differential AS analysis was conducted at the bin levels using the edgeR package version 3.4.2 (20). Bins with FDR values lower than 0.15 were considered to undergo differential AS. Finally, we restricted the selection of AS-bins to those bins for which differential AS analysis was supported by expected changes in the numbers of splice junctions. In order to do this, we obtained information on the number of reads associated with each splice junction, both

annotated as well as novel. Junction coordinates were extracted from gap containing aligned reads. Junctions with fewer than 5 reads were discarded. We then computed the metrics PSI (percent spliced-in) and PIR (percent intron retention), which were used as a final filtering criteria for the AS analysis. PSI was defined as the percentage of the number of junction reads supporting bin inclusion relative to the combined number of reads supporting inclusion and exclusion (21). PSI values were computed for ES, Alt5′SS and Alt3′SS. PIR values, calculated as previously described (22), were used for the IR analysis. Briefly, PIR is defined for each experimental condition as the percentage of the number of reads supporting intron retention (E1I + IE2) relative to the combined number of reads supporting intron retention and exclusion (E1I + IE2 + 2 Exclusion junction [JE1E2]) (See Fig. 1). AS, exon and intron bins were considered to be differentially spliced if, in addition to fulfilling the filtering criteria described above, the difference in PSI or PIR between experimental conditions was > 5%. Bins corresponding to alternatively spliced regions identified through novel splice junctions were considered to be differentially alternatively spliced if there was a difference in the PSI value larger than 10% between experimental conditions. This dataset was labeled as Differentially Spliced (DS) genes (Table S3).

Semi-quantitative RT-PCR. Three biological replicates with fifty seeds of *Arabidopsis thaliana* plants from the Col-0 accession, *phyA-211;phyB-9* (*phyAB*), *phyA-211;phyB-9;phyC-2;phyD-201;phyE-201* (*phyABCDE*) (23) or *cry1-304;cry2-1* (*cry1;cry2*) (24) mutants were sown in MS-agar plates, cold stratified in darkness for 5 days and grown for 8 days under 12 h:12 h light:dark conditions. On the 9th day plants were subjected to a 2 h light pulse at ZT18 or kept under dark conditions and after 2 h all the plants were harvested and immediately flash frozen in liquid nitrogen. RNA was extracted by the Trizol method and reverse transcription was performed using SuperScript II reverse transcriptase (Life Technologies), in accordance with the manufacturer instructions. cDNA was then used for PCR amplification. Amplification of cDNA was carried out for 30 cycles to measure splicing at the linear phase. RT-PCR products were electrophoresed in agarose 3% (wt/vol) and detected with Sybr Green. The sequences of the primers used in the RT-PCR experiments are described in Table S4. All reagents were purchase from Life Technologies unless otherwise specified. RT-PCR products of AS variants were quantified as previously described (25).

Gene Onthology (GO) analysis. GO terms assignment for the DE genes and DS genes datasets were obtained using the BioMaps tool from the virtual plant software (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb). An enrichment test was performed for the following categories: BP (biological process), MF (molecular function) and CC (cellular component). *P*-values were obtained using the Fisher Exact Test and corrected for multiple testing using FDR. The enrichment factor (EF) was estimated as the ratio between the proportions of genes associated with a particular GO category present in the dataset under analysis, relative to the proportion of the number of genes in this category in the whole genome (Table S5). Bubble plots were generated, using a custom script written in R language, for all those categories for which the adjusted p-value was lower than 0.01 in at least one dataset.

RESULTS

To investigate the effect of light on AS at a genome-wide level in light-grown plants, *Arabidopsis thaliana* seedlings of the Columbia ecotype were grown under 12 h light: 12 h dark cycles for 8 days. On the 9th day, half of the plants were exposed to a 2-h pulse of white light given in the middle of the night (ZT 18), whereas the other half were kept in darkness as controls (Fig. 2a). Both groups of plants were harvested at ZT 20 and light effects on mRNA levels and AS were analyzed through RNA-seq. In order to accomplish this, we sequenced cDNA libraries prepared from three independent biological replicates of each group of plants. Then, we mapped the resulting reads to the *Arabidopsis thaliana* genome (TAIR10 version), and evaluated changes in gene expression and splicing of annotated, as well as novel, AS events associated with genes expressed above a predefined threshold level (See Tables S2 and S3).

First, we identified a total of 4341 genes whose mRNA levels significantly increased or decreased more than 1.5-fold (FDR < 0.05) in response to the 2 h light pulse (Table S2 and Fig. 2b). This group was enriched in genes associated with chloroplast components, primary and secondary metabolism, oxidative stress and abiotic stress responses (Fig. 3, Tables S2 and S5). We also observed enrichment in genes associated with the circadian rhythms category, supporting the idea that light effects on the circadian network are mediated to a great extent through its effect on the mRNA levels of several core clock genes (Fig. 3, Tables S2 and S5).

We then evaluated the effects of light on AS by characterizing light effects on both annotated as well as novel AS events (see Materials and Methods). We identified a total of 382 genes with AS events that were regulated by the light pulse treatment (Fig. 2b, Table S3). Interestingly, less than half of the genes whose AS patterns were affected by light showed alterations at the total mRNA level, suggesting that light affects the AS of these genes through mechanism(s) that are distinct from those

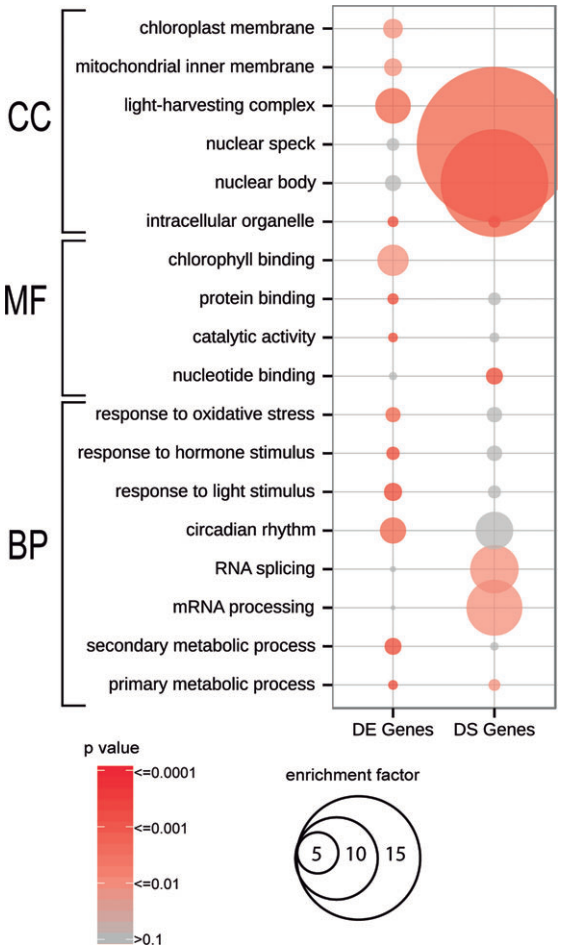

Figure 3. Analysis of Gene Ontology (GO) enrichment comparing differentially expressed (DE) genes and differentially spliced (DS) genes. GO enrichment was evaluated at three different levels: BP: Biological Processes, MF: Molecular Function and CC: Cellular component. The color gradient represents adjusted *P*-values and the differences in bubble size correlate with the enrichment factor. Only those categories showing a statistically significant enrichment at either the gene expression or AS level are depicted.

through which light affects gene expression at the transcriptional level (Fig. 2b).

As previously reported for light effects on AS in etiolated seedlings (13), or in response to prolonged light/dark transitions (12), we also observed a strong enrichment in GO categories associated with RNA processing and AS among the genes whose AS patterns were affected by a brief light pulse in light-grown plants (Fig. 3, Tables S3 and S5). These categories were not enriched among the genes whose mRNA levels rather than AS patterns were affected by light. This observation supports the idea that light regulates AS patterns mostly through its effect on AS of splicing factors themselves.

In a genome-wide study analyzing light effects on AS in *Physcomitrella patens* (16), the authors found that light promoted intron retention in most genes. Interestingly, we also found that intron retention was the most frequent AS event affected by light (Table S6), but we also observed that light promoted intron retention of some genes, whereas it enhanced intron exclusion in others (Fig. 4). A similar observation was made at the exon level, with some genes showing enhanced exclusion whereas others showed increased inclusion of specific exons (Fig. 4). These observations indicate that in light-grown *Arabidopsis thaliana* plants light acts modulating AS rather than affecting the splicing process itself.

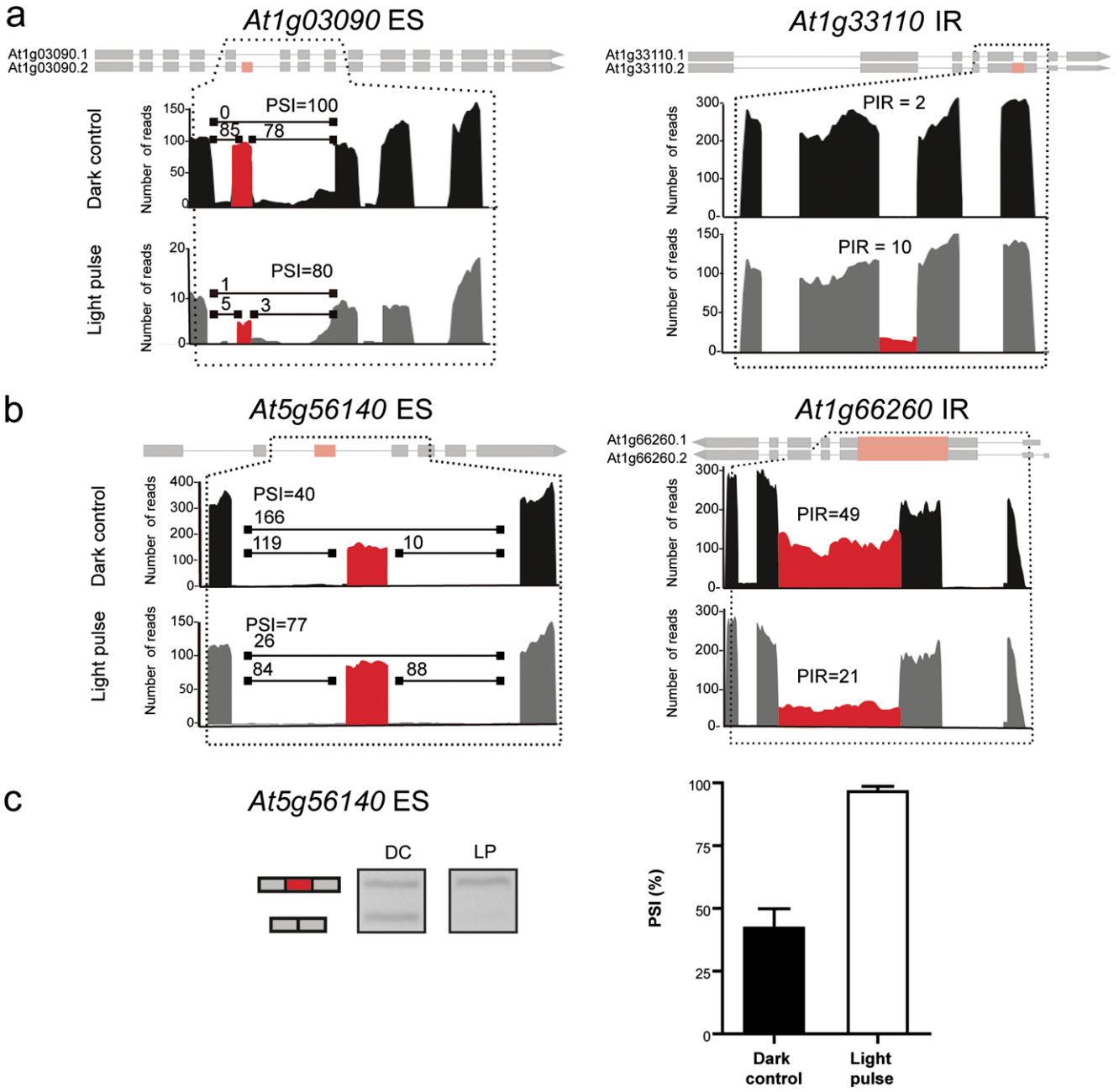

Figure 4. RNA-seq results of representative genes showing changes in AS at the intron or exon level. Coverage plot areas and associated gene models are shown for a selected group of genes. (a) Examples of two annotated AS events (ES and IR). (b) Examples of two novel AS events (ES and IR). (c) Validation of the RNA-seq result for a novel ES event by RT-PCR. The graph represents the average of three independent biological replicates and error bars represent the standard error. Alternative regions are highlighted in red.

We then focused our analysis on genes associated with the circadian network. Although the category circadian rhythm was not enriched among the subset of AS analyzed, we found light-regulated AS events associated with the clock genes *REVEILLE 8* (*RVE8/LCL5,* AT3G09600*)*, *JUMONJI DOMAIN CONTAINING 5* (*JMJD5,* AT3G20810), *LATE ELONGATED HYPOCOTYL* (*LHY,* AT1G01060), *TIME FOR COFFEE* (*TIC,* AT3G22380) and *CASEIN KINASE II BETA CHAIN 3* (*CKB3,* AT3G60250) (Table S3). Some of these light-regulated pre-mRNA splicing events were already annotated as AS events, whereas others, such as the usage of a novel Alt 3′SS in the *JMD5* gene, were novel (Fig. 5). Whether any of these light-regulated AS events associated with clock genes play a role in the modulation of clock entrainment remains to be determined.

Interestingly, among the genes showing light-regulated AS events we found several encoding serine rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP), which are known to modulate the recruitment of spliceosomal snRNPs to donor and acceptor splice sites (26) (Fig. 6 and Table S3). Most of these genes have already been shown to be regulated at the AS level by light in response to more prolonged exposure to light in light-grown plants (12), or to an acute treatment with red light in etiolated seedlings (13). Here, we show that light also induces rapid changes in AS of these splicing factors in light-grown plants.

Interestingly, the effect of prolonged light exposures on AS of light-grown plants was shown not to depend on photosensory photoreceptors such as phytochromes or cryptochromes, and to be mediated mostly by light effects on the photosynthetic process (12). In contrast, a 3 h red light exposure perceived through phytochromes A (phyA) and B (phyB) was recently shown to regulate the AS of several genes, including those encoding splicing factors, in etiolated seedlings (13). Here, we first evaluated the acute effect of white light on AS of gene encoding the splicing factor *SERINE/ARGININE-RICH PROTEIN SPLICING FACTOR 30* (*SR30*, AT1G09140) in light-grown plants in *phyAB* and *cryptochrome 1* (*cry1*); *cry2* double mutants. We found that, similarly to what was reported for prolonged effects of light in light-grown plants, the acute effects of light on AS of *SR30* was still present in *phyAB* as well as in *cry1;cry2* double mutant backgrounds, suggesting that a significant part of the light effect may be modulated by an intermediate molecule generated during the photosynthetic process, rather than by traditional photosensory photoreceptors (Fig. 7). However, the persistence of an effect of white light on AS of *SR30* in *phyAB* and *cry1;cry2* mutants could still be due to the action of additional photosensory photoreceptors belonging either to the same or different families of photoreceptors. We therefore evaluated the effect of red-light on AS of *SR30* in the quintuple *phyABCDE* mutant background, which is completely blind to red-light signals that control plant development (23). Interestingly, we found a strong effect of a 2 h red-light pulse on AS of *SR30*, which was of similar magnitude in wild-type and *phyABCDE* mutant plants.

DISCUSSION

Accumulating evidence indicates that light modulates plant growth and development by controlling different steps of the gene expression process. While genome-wide studies performed over the last two decades have revealed light regulation of the mRNA levels of thousands of genes, global studies describing light effects on AS have only recently started to appear. The first study evaluating light effects on AS at a global level was conducted in rice and identified 10 genes with AS patterns regulated by light (27). Most of the effects involved changes in the UTR rather than coding regions, suggesting they may regulate gene expression by controlling mRNA stability or translatability. The small number of AS events identified was likely the result of using gene expression arrays, which are not an ideal tool to characterize changes in AS. Two recent studies have evaluated light effects on AS at a global level in *Physcomytrella patens* and in etiolated *Arabidopsis thaliana* seedlings using RNA-seq (13,16). Those studies found several hundreds of light-regulated AS events, many of which were associated with genes encoding splicing factors and light signaling components. Similar findings were obtained in a recent study that characterized AS in light-grown *Arabidopsis thaliana* plants using a high-resolution RT-PCR panel, where prolonged exposure to light/dark transitions were shown to modulate AS of approximately 50% of the events evaluated, most of which encoded splicing factors (12). Here, we expanded the knowledge of light effects on AS in light-grown *Arabidopsis thaliana* plants by performing a

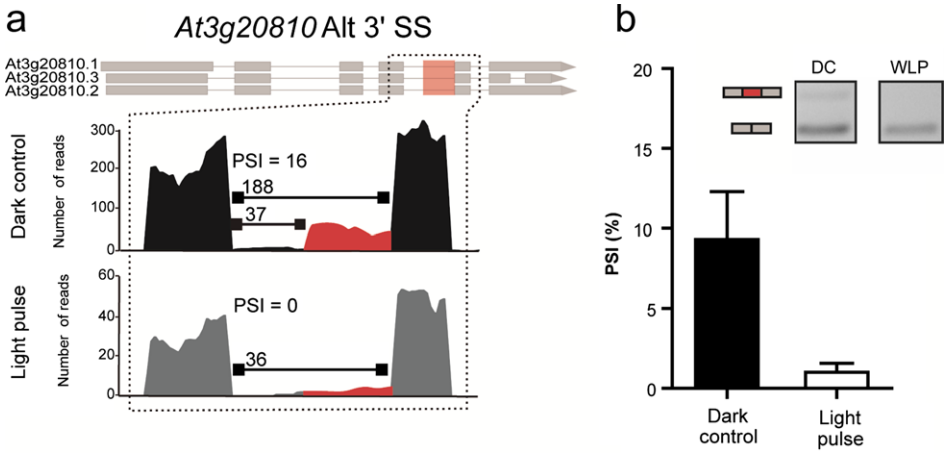

Figure 5. Light regulates AS of the clock gene *JMJD5*. (a) Coverage plot areas and associated gene model are shown. (b) Validation of the RNA-seq result by RT-PCR. The graph represents the average of three independent biological replicates and error bars represent the standard error. Alternative regions are highlighted in red.

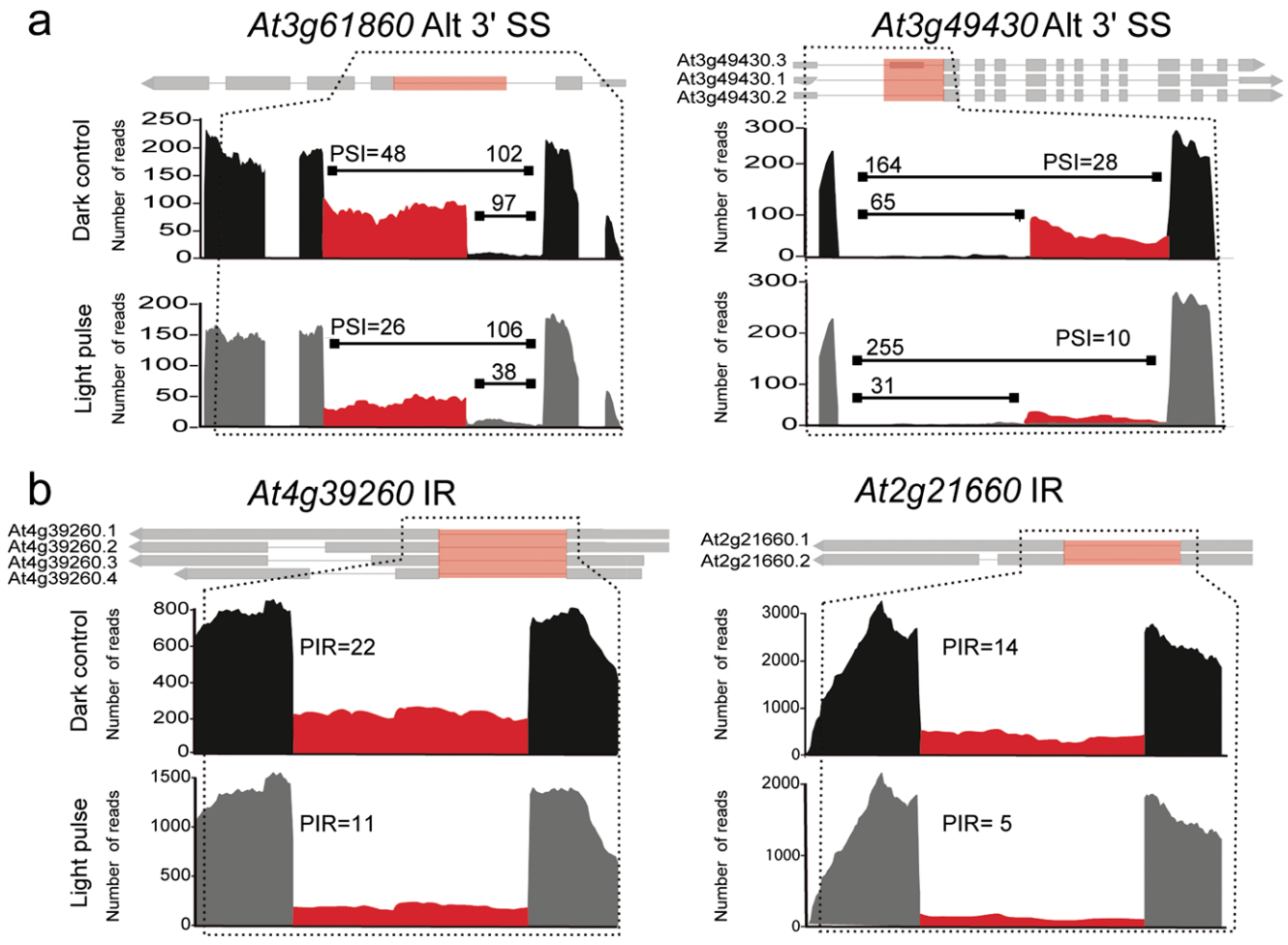

Figure 6. Coverage plot area and associated gene models of representative light regulated AS events associated with genes encoding splicing factors. (a) AS events associated with genes encoding serine rich proteins (SR). (b) AS events associated with genes encoding heterogeneous nuclear ribonucleoproteins (hnRNPs). Alternative regions are highlighted in red.

genome-wide characterization of the effect of a brief light pulse given in the middle of the night, a treatment that simulates photoperiod lengthening. In agreement with all the previous studies, we found that a brief light pulse had significant effects on AS of a large subset of genes, preferentially those encoding splicing factors themselves. Altogether, these findings reveal that light controls plant growth and development to a significant extent modulating the AS of splicing factors and light signaling components, in addition to the well-established effects that light produces on many transcription factors at the mRNA and protein levels. These effects of light on AS take place across distant plant species, developmental stages and in response to either brief or prolonged exposures to the light signal, indicating that it is an ancient and widespread mechanism through which light regulates gene expression programs.

Interestingly, in both etiolated *Arabidopsis thaliana* seedlings as well as in *Physcomytrella patens*, the effects of brief light treatments on AS were modulated to a great extent, although not exclusively, by members of the phytochrome family of photosensory photoreceptors (13,16). In contrast, the effects of prolonged exposures to light or dark conditions in light-grown *Arabidopsis thaliana* plants appeared to operate largely independently of the most abundant phytochrome and cryptochrome photoreceptors and, instead, were mediated by a retrograde signaling circuit connecting photosynthetic activity in the chloroplast to the regulation of AS in the nucleus. Here, we found that the effect of a brief light pulse on AS of *SR30* was almost as strong in the double *phyAB* and *cry1;cry2* mutants as in wild-type plants. Furthermore, we observed that the strong effect of a 2 h red-light pulse on AS of *SR30* was not impaired in the quintuple *phyABCDE* mutant. Therefore, although we do not rule out that photosensory photoreceptors might have some role in the control of AS in light-grown plants, it is clear that, at least in the AS evaluated here, most of the effect of light does not appear to operate through the traditional photosensory photoreceptor pathways. The differences in the main phototransduction pathways mediating light regulation of AS in etiolated seedlings and light-grown plants might be associated to the fact that etiolated seedlings lack fully developed chloroplasts, which might be required for the generation of a retrograde signal controlling AS in the nucleus.

Regardless of the photoreceptors that mediate the effect of light on AS, an important question that remains unanswered is how light modulates AS. Splicing is catalyzed by the spliceosome, a large and dynamic RNA-protein complex (26). Core spliceosomal components interact with auxiliary splicing factors, such as SR and hnRNP proteins, which contribute to the recognition and/or selection of

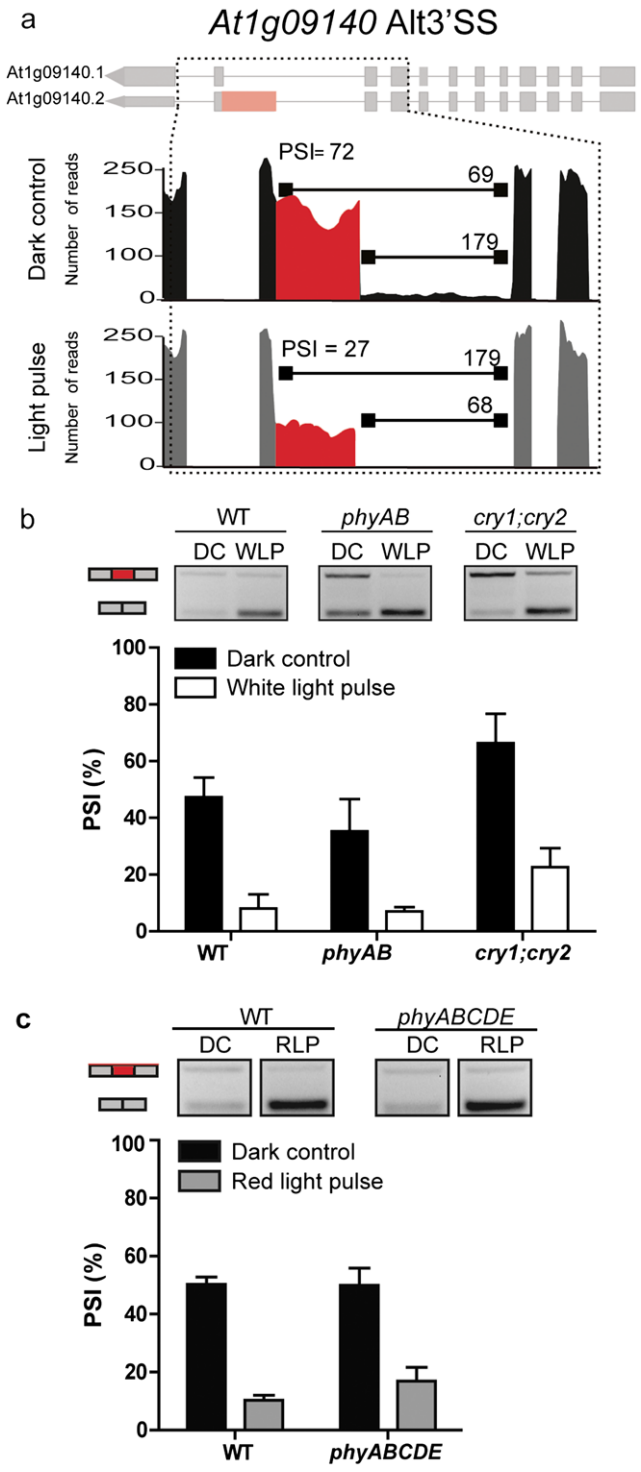

Figure 7. Experimental validation of light regulation of AS of the *SR30* gene. (a) Coverage plot area and associated gene model. (b) Gel electrophoresis of the RT-PCR splicing analysis of Col-0, *phyAphyB* and *cry1cry2* plants subjected to a 2-h white light pulse (WLP) or kept in the dark as controls (DC). (c) Gel electrophoresis of the RT-PCR splicing analysis of Col-0 and *phyABCDE* mutant plants subjected to a 2-h red-light pulse (RLP) or kept in the dark as controls (DC). The graphs represent the average of three independent biological replicates and error bars represent the standard error. Alternative regions are highlighted in red.

different *cis*-acting sequences by promoting or inhibiting the recruitment of spliceosomal particles to different donor and/or acceptor splice sites (26). Thus, regulation of AS by light may be triggered by light induced changes in the levels and/or activities of auxiliary splicing factors. Indeed, a recent report showed that the *Arabidopsis thaliana* splicing factor *REGULATOR OF CHROMOSOME CONDENSATION 1* (*RCC1*), an SR-like protein, is required for normal photomorphogenic development under red light conditions (28). Furthermore, red light and phytochrome dependent effects on a subset of AS events, including some affecting splicing factors, are reduced in the *rcc1* mutant (13,28). However, how light triggers the initial changes resulting in alterations in AS of splicing factors remains to be determined. In addition to the role of auxiliary splicing factors controlling AS, there is evidence indicating that AS is also modulated by epigenetic modifications that change the rate of transcriptional elongation, giving more time to spliceosomal particles to be recruited to weak splice sites (26,29). Histone modifications can also modulate AS by enhancing the recruitment of specific splicing factors (26). Finally, changes in the activity and/or concentration of core splicing factors can also influence AS regulation, most likely by altering the kinetics of one or more steps in spliceosome assembly (30). Which of all these potential mechanisms are affected by light remains to be elucidated.

In addition to studying the role of *trans*-acting factors, we looked for *cis* sequences that might play a role in light-induced AS. In order to do so, we analyzed the regions flanking exon-intron boundaries looking for potential *cis*-regulatory sequences involved in the mediation of light effects on AS, but could not identify any reliable *cis*-acting elements enriched in the subset of events evaluated. In particular, we focused our analysis on the 232 introns that showed differential retention in response to the light pulse. We first evaluated donor and acceptor sites and then the regions surrounding 50 bp exon-intron boundaries in that dataset compared to different control datasets. Unfortunately, we could not identify any relevant motif enriched near the 5′ss and 3′ss using software such as MEME, or by conducting a k-mer analysis looking for enriched 3–7 k-mers in those regions.

Finally, whereas it is clear that light has strong effects on AS in higher plants throughout their development, our knowledge of the biological consequences of those effects are less understood. In etiolated seedlings, light has been shown to promote the accumulation of an alternatively spliced variant of the *SUPPRESSOR OF PHYA* (*SPA*) gene that encodes a truncated protein, which acts in a dominant negative fashion promoting de-etiolation and photomorphogenic development (13). In light-grown plants, low light conditions promote a reduction in the levels of the full length isoform of *SR31* through AS, and this regulation is important for proper growth and development of plants exposed to these environmental conditions (12). Here, we studied the effect of a brief light pulse given in the middle of the night, with the aim of identifying potential posttranscriptional regulatory mechanisms controlling clock function and/or the floral transition. A recent microarray analysis of transcriptomic changes induced by a similar light treatment allowed us to identify a novel gene family, the *LNKs*, whose expression is acutely induced by light and regulate clock function and the floral transition (31). In this study, we found that a light pulse given in the middle of the night affected the AS patterns of several clock associated genes, such as *JMJD5*, *RVE8/LCL5*, *LHY*, *CKB3* and *TIC*. Interestingly, some of these are exclusively regulated by light at the posttranscriptional level and do not show changes in steady state mRNA levels. Whether any of these light-regulated AS events associated with clock genes modulate clock function or the floral transition remains to be evaluated in detail.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. (a) Summary of the sequencing data, (b) Correlation metrics.

Table S2. DE Genes database (see accompanying Excel file).

Table S3. Differentially Spliced (DS) genes database (see accompanying Excel file).

Table S4. Oligonucleotides used for RT-PCR.

Table S5. See accompanying Excel file.

Table S6. (a) Summary of differential expression and alternative splicing results, (b) Relative frequencies of the different AS identified as light regulated.

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