A Chloroplast Retrograde Signal Regulates Nuclear Alternative Splicing

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complex than shifting FEF signals between different locations in the visual field.

IFJ may include areas that function as general executive modules (*22*, *23*). Also, IFJ is close to areas Ba45 and Ba46, homologs of which have been described in nonhuman primate recordings to encode information about object-categories in delayed match-to-sample tasks (*23*, *24*). Indeed, the "attentional template" that specifies the relevant location or object in spatial or feature attention is hardly distinguishable from working memory for these qualities (*9*), which is known to involve prefrontal cortex (*24*). Coupled interactions between prefrontal areas and visual areas (*25*–*31*) could underlie many cognitive phenomena in vision, with shared neural mechanisms but variations in the site of origin and the site of termination.

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A Chloroplast Retrograde Signal Regulates Nuclear Alternative Splicing

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Light is a source of energy and also a regulator of plant physiological adaptations. We show here that light/dark conditions affect alternative splicing of a subset of *Arabidopsis* genes preferentially encoding proteins involved in RNA processing. The effect requires functional chloroplasts and is also observed in roots when the communication with the photosynthetic tissues is not interrupted, suggesting that a signaling molecule travels through the plant. Using photosynthetic electron transfer inhibitors with different mechanisms of action, we deduce that the reduced pool of plastoquinones initiates a chloroplast retrograde signaling that regulates nuclear alternative splicing and is necessary for proper plant responses to varying light conditions.

Light regulates about 20% of the transcriptome in *Arabidopsis thaliana* and rice (*1*, *2*). Alternative splicing has been shown to modulate gene expression during plant development and in response to environmental cues (*3*). We observed that the alternative splicing of *At-RS31* (Fig. 1A), encoding a Ser-Arg–rich splicing factor (*4*), changed in different light regimes, which led us to investigate how light regulates alternative splicing in plants.

Seedlings were grown for a week in constant white light to minimize interference from the circadian clock and then transferred to light or dark conditions for different times (see the supplementary materials). We observed a two- and fourfold increase in the splicing index (SI)—defined as the abundance of the longest splicing isoform relative to the levels of all possible isoforms—of *At-RS31* [mRNA3/(mRNA1 + mRNA2 + mRNA3)] after 24 and 48 hours in the dark, respectively (Fig. 1B). This effect was rapidly reversed when seedlings were placed back in light, with total recovery of the original SI in about 3 hours (Fig. 1C), indicating that the kinetics of the splicing response is slower from light to dark than from dark to light.

The light effect is gene specific (fig. S1) and is also observed in diurnal cycles under short-day conditions (Fig. 1D and fig. S2). Furthermore, three circadian clock mutants behaved like the wild type (WT) in the response of *At-RS31* alternative splicing to light/dark (fig. S3). Changes in *At-RS31* splicing are proportional to light intensity both under constant light and in short-day–grown seedlings (fig. S4).

Both red (660 nm) and blue (470 nm) lights produced similar results as white light (Fig. 1E). Moreover, *At-RS31* alternative splicing responses to light/dark are not affected in phytochrome and cryptochrome signaling mutants (*5*, *6*), ruling out photosensory pathways in this light regulation (Fig. 1F and figs. S5 and S6).

Light-triggered changes in *At-RS31* mRNA patterns are not due to differential mRNA degradation. First, the light effect is not observed in the presence of the transcription inhibitor actinomycin D (Fig. 1G). Second, the effects are still observed in *upf* mutants, defective in the nonsense-mediated mRNA decay (NMD) pathway (*7*) (Fig. 1H and fig. S7). Third, overexpression of the constitutive splicing factor $U2AF^{65}$ (*8*) in *Arabidopsis* protoplasts mimics the effects of light on *At-RS31* alternative splicing (Fig. 1I).

mRNA1 is the only isoform encoding a full-length At-RS31 protein (*9*). *mRNA3* and *mRNA2* are almost fully retained in the nucleus (fig. S8). *mRNA1* levels decrease considerably in dark without significant changes in the total amount of *At-RS31* transcripts (Fig. 2A and fig. S9), which suggests that alternative splicing is instrumental

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in the control of *mRNA1* cellular levels and, consequently, At-RS31 protein abundance. To assess how interference with *At-RS31* alternative splicing regulation could affect *Arabidopsis* phenotype, we obtained transgenic plants overexpressing either a cDNA (mRNA1ox) or a genomic version (31 genOX) of *At-RS31* and a knock-out (31 mut) line (fig. S10). *At-RS31* total mRNA is more abundant in the mRNA1ox and 31 genOX lines compared with the WT (fig. S10). Whereas the mRNA1ox line expresses almost exclusively the *mRNA1* isoform and at higher levels than the other lines, the 31 genOX line is mostly enriched in *mRNA3* and *mRNA2* isoforms, and *mRNA1* levels are not much higher than those in WT seedlings (Fig. 2, B and C, and fig. S10). Under photoperiodic conditions (16 and 8 hours of light and dark, respectively) no major phenotypes are observed for these lines (Fig. 2D, "L" in Fig. 2E, and fig. S11). However, when plants are either exposed to long darkness (3 days) (Fig. 2E), or grown under constant low light intensity (50 μmol m^{-2} s^{-1}) (Fig. 2F), the mRNA1ox line shows a dramatic phenotype, characterized by small and yellowish seedlings concomitantly with faster degradation of chlorophylls a and b (fig. S12). This suggests that down-regulation of *mRNA1* in the dark through alternative splicing is important for proper growth of the plant in response to changing environmental light conditions.

As sensory photoreceptors do not participate, we investigated whether retrograde signals from the chloroplast were involved (*1*). Increasing concentrations of DCMU [3-(3,4-dichlophenyl)1,1-dimethylurea] (*10*), a drug that blocks the electron transfer from photosystem II (PSII) to the plastoquinone (PQ) pool, inhibited the effect of light on *At-RS31* alternative splicing (Fig. 3A), confirming chloroplast involvement. Roots have no chloroplasts, so the light effect should only be observed in the green tissue in dissected seedlings (Fig. 3B). However, the effect was observed both in dissected leaves and roots, with roots showing a time delay in the response (Fig. 3, D and F). When shoots were separated from roots before the light/dark treatment (Fig. 3C), dissected shoots retained the response (Fig. 3E), but light had no effect on *At-RS31* splicing in detached roots (Fig. 3G). Similar results were obtained by covering the different parts of the seedlings (fig. S13). No chlorophyll was detected in the roots of pre- or post-dissected plants (fig. S14), strengthening the hypothesis that a mobile signal generated in the leaves triggers root alternative splicing responses to light.

About 39% of the 93 alternative splicing events (table S1) tested using a high-resolution reverse transcriptase polymerase chain reaction (RT-PCR) panel (*11*) changed significantly in response to light (tables S2 to S4 and fig. S15). The effects of light were not due to sugar starvation during darkness (fig. S15), indicating that the chloroplast role in this light signaling pathway is not primarily

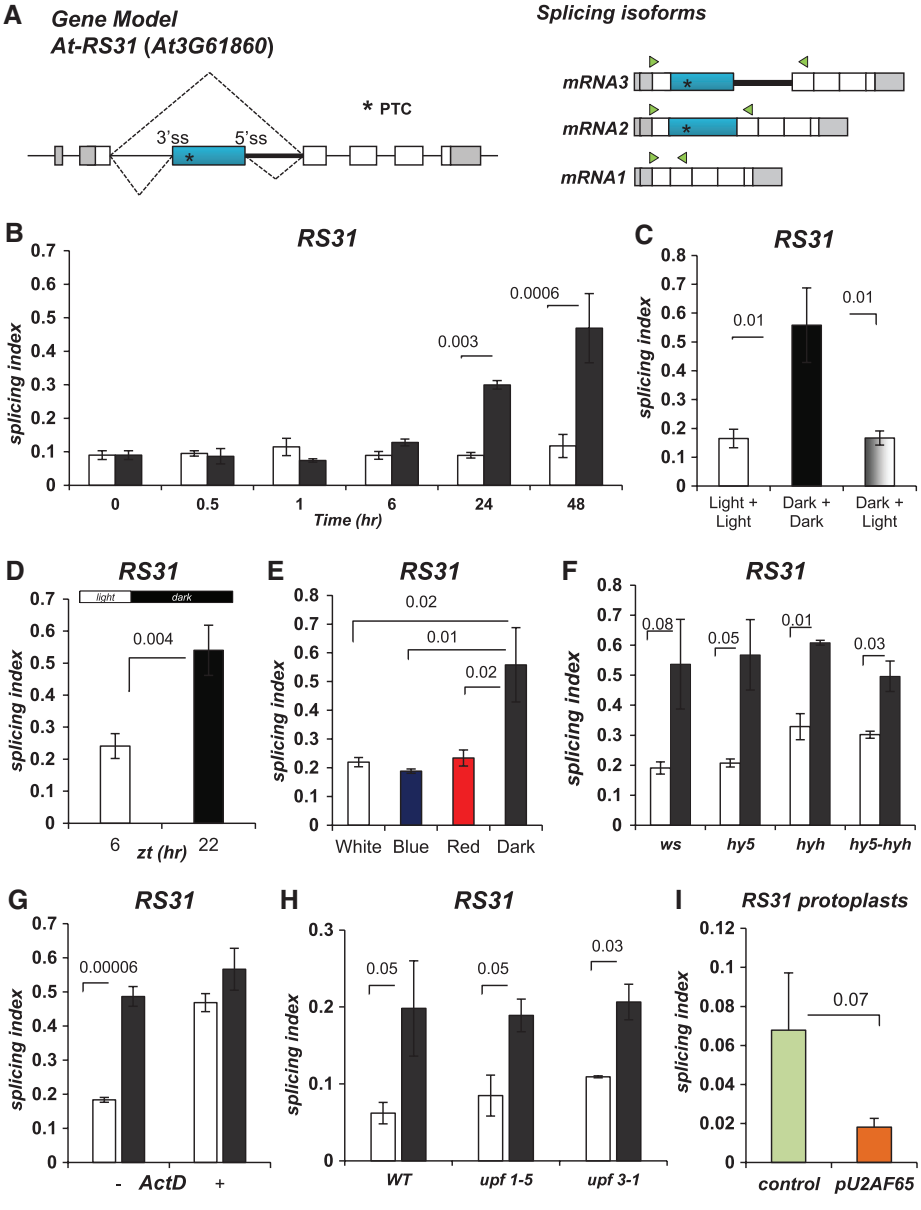
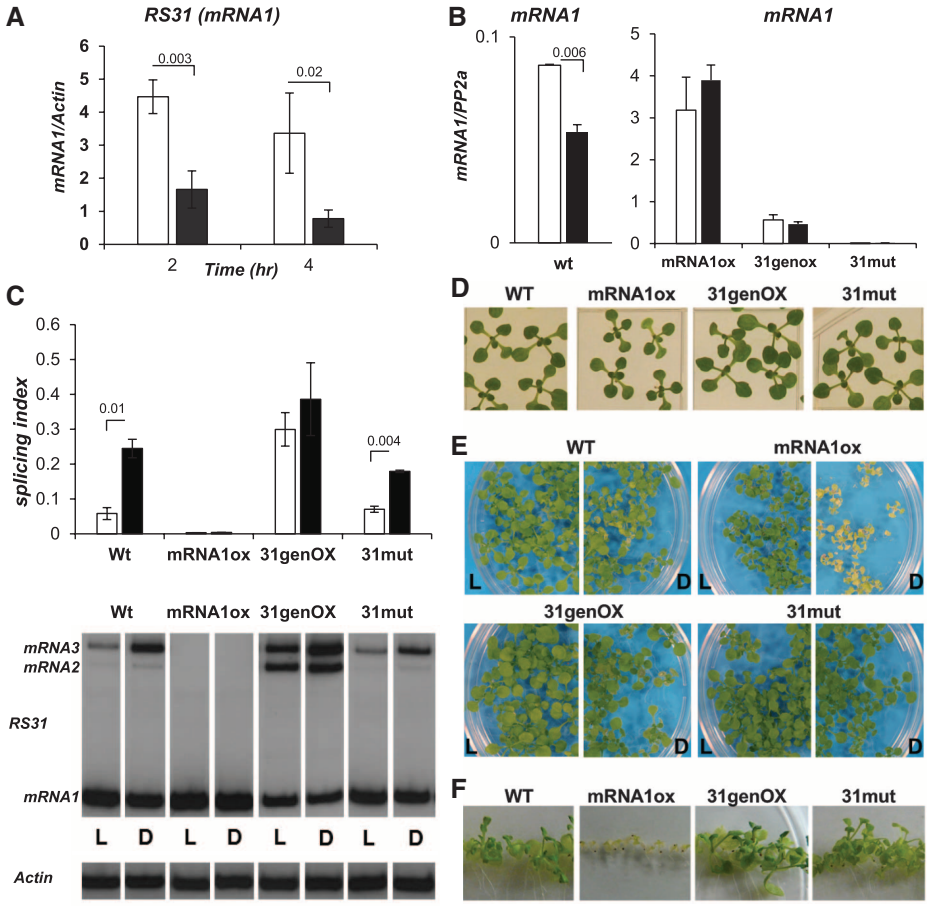


Fig. 1. Effects of light/dark transitions on *At-RS31* alternative splicing. (**A**) *At-RS31* splicing event. *PTC, premature termination codon; triangles, primers for splicing evaluation (see also fig. S10a). (**B**) *Arabidopsis thaliana* seedlings were incubated in light/dark for different times (see the supplementary materials). (**C**) After 48 hours of darkness, transferring the seedlings to light causes a rapid change in the SI of *At-RS31* to "light values" (see the supplementary materials). Light+Light, 48 hours + 3 hours light; Dark+Dark, 48 hours + 3 hours dark; Dark+Light, 48 hours dark + 3 hours light. (**D**) Seedlings were grown for 2 weeks under short-day conditions (~100 μmol m^{-2} s^{-1}). Samples were collected 2 hours before lights off [6 hours Zeitgeber time (zt)] and 2 hours before lights on (22 hours zt). (**E**) Plants were grown in constant light, transferred to dark for 48 hours, and then treated in light/dark for 6 hours with light-emitting diodes to provide specific wavelengths (see the supplementary materials). (**F**) *hyh* and *hy5* mutants for integrators of photoreceptor-signaling pathways show the same *At-RS31* alternative splicing regulation by light as WT plants. WS, *Wassilewskija* ecotype. (**G**) Actinomycin D (ActD) causes the loss of the light/dark transition effect. ActD (+) was added 2 hours before light/dark treatments. Dimethyl sulfoxide, control (–). (**H**) SI change induced by the light/dark transition is preserved in the NMD-impaired mutants *upf1-5* and *upf3-1*. (**I**) Effect of $U2AF^{65}$ overexpression in *A. thaliana* protoplasts. In all experiments: white bars, light; black bars, darkness. Data represent means ± SD ($n \geq 3$); significant *P* values (Student's *t* test) are shown.

Fig. 2. *At-RS31* alternative splicing regulation is important for proper adjustment to light changes. (**A**) Quantitative RT-PCR analysis of *At-RS31 mRNA1* levels relative to *Actin*. The graph shows the relative expression of *mRNA1* in two different time points of light/dark treatment (2 and 4 hours). (**B**) Quantitative RT-PCR analysis of *At-RS31 mRNA1* isoform expression in the different genotypes and treatments. (**C**) (Top) *At-RS31* SI in the different genotypes in response to light/dark (see the supplementary materials). (Bottom) Representative gel images for the alternative splicing pattern of *At-RS31* in the different genotypes in response to light/dark transitions. *Actin*, as control. L, light; D, dark. (**D**) All lines were grown on Murashige and Skoog medium (MS) agar plates with 1% sucrose for 1 week in light/dark cycles (16/8 hours), 120 μmol $m^{-2}s^{-1}$ white light. Similar size sections of plates are shown. (**E**) Seedlings of the different genotypes were grown under a 16/8 hours light/dark regime for 2 weeks and then transferred to dark for 3 days (D) or kept in photoperiod as controls (L). (**F**) Seedlings for each genotype were grown on MS plates in constant light conditions (~50 μmol $m^{-2}s^{-1}$). Similar size sections of plates are shown. Bar color code and statistics as in Fig. 1.



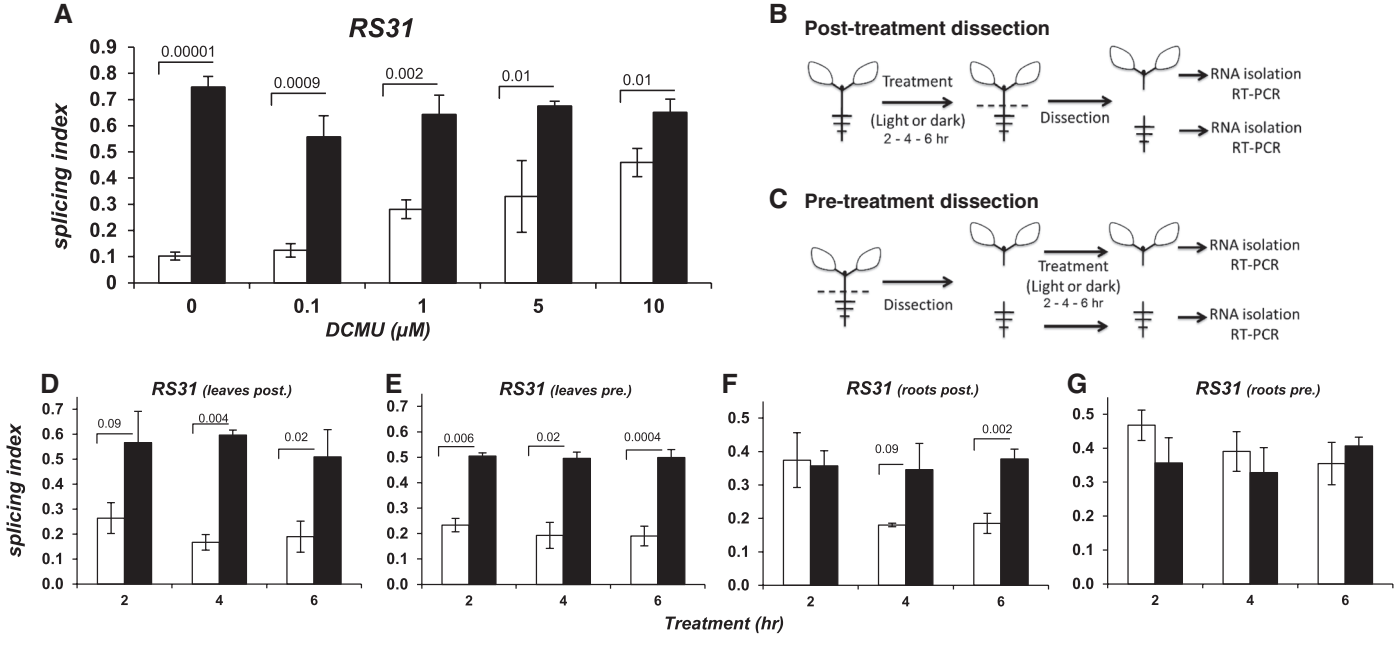


Fig. 3. The light signal is generated in the photosynthetic tissue and travels through the plant. (**A**) Seedlings were grown in constant light, transferred to darkness for 48 hours, and then treated with DCMU during a 6-hour light/dark further incubation. (**B** to **G**) Light regulation of *At-RS31* alternative splicing in green tissues and roots after a 2-, 4-, or 6-hour light/dark exposure of whole seedlings [(B), (D), and (F)] or in the isolated parts exposed separately to light or dark [(C), (E), and (G)]. [(B) and (C)] Schemes for post- and pre-exposure dissections (see the supplementary materials). [(D) and (F)] *At-RS31* alternative splicing assessment in green tissue (D) or in the roots (F) of light/dark treatments performed using intact seedlings (dissection performed after light/dark treatment). [(E) and (G)] *At-RS31* alternative splicing assessment in response to light/dark treatments using predissected (E) green tissue or (G) roots (dissection performed before light/dark treatment). Bar color code and statistics as in Fig. 1.

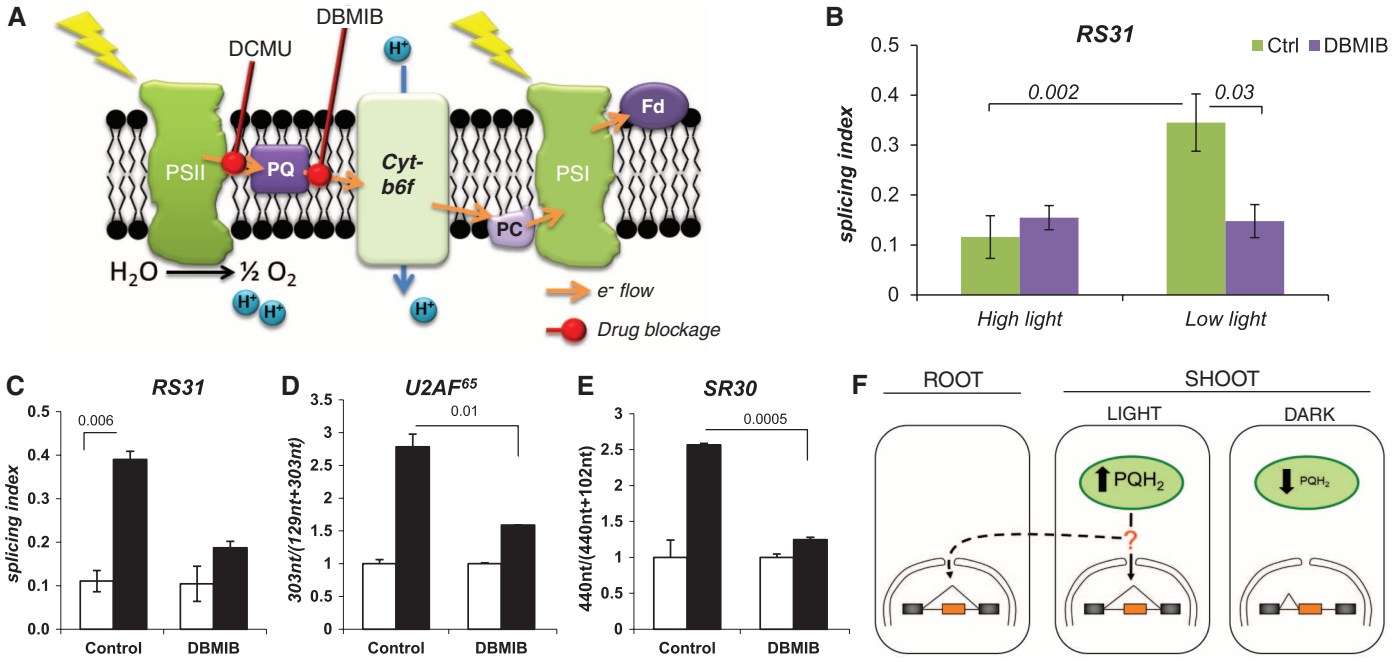


Fig. 4. The plastoquinone redox state mediates alternative splicing regulation by light. (**A**) Diagram showing the action of DBMIB and DCMU in the photosynthetic electron transport chain. (**B**) Effects of the addition of 30 μM DBMIB to seedlings under medium (80 μmol m^{-2} s^{-1}) and low (15 μmol m^{-2} s^{-1}) light on *At-RS31* alternative splicing. (**C** to **E**) Addition of 30 μM DBMIB reduces the effects of light/dark transitions on *At-RS31* (C), *At-U2AF*^{65} (D), and *At-SR30* (E) alternative splicing patterns. (**F**) Model for the light regulation of alternative splicing. Light-induced reduction of plastoquinone to plastoquinol (PQH_2) generates a signal that modulates alternative splicing in the nucleus. This signal, or a derived one, travels to the roots and provokes similar effects. Bar color code and statistics as in Fig. 1.

associated with the energy state of the cells (fig. S16a). In addition, we ruled out the participation of sugar-sensing pathways (fig. S16, b and c) reported to control nuclear gene expression (*12*, *13*).

Analysis of light-responsive splicing events revealed an enrichment in those of RNA-binding and processing protein coding genes (fig. S17 and table S3). The SR protein genes *At-SR30* and *At-U2AF*^{65} showed the biggest changes (fig. S18 and table S2), and DCMU treatment also confirmed a role for chloroplast involvement in their responses (fig. S19).

Experiments shown in figs. S20 and S21 revealed that plastid gene expression, the tetrapyrrole pathway, and reactive oxygen species (*14*) are not involved in *At-RS31* alternative splicing regulation. Because methyl viologen takes electrons from PSI (*15*) with no effect on *At-RS31* alternative splicing (fig. S21a), we inferred that the signal must be generated between PSII and PSI. To prove this, we used DBMIB (2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone). Both DCMU and DBMIB inhibit the overall electron transport chain, but whereas DCMU increases the oxidized PQ pool by blocking the electron transfer from the PSII to PQ (*10*), DBMIB keeps the PQ pool reduced by preventing the electron transfer to cytochrome b6/f (*15*) (Fig. 4A). Addition of DBMIB potentiates the decrease in *At-RS31* SI when seedlings are exposed to low light in comparison to the lack of effect under high light (Fig. 4B). It was shown that when externally added in the dark, DBMIB can act as a reduced quinone analog (*16*, *17*). Consistently, DBMIB decreases *At-RS31* SI in the dark, mimicking the effects of light (Fig. 4C). Similar results were obtained for *At-U2AF*^{65} and *At-SR30* (Figs. 4, D and E).

Our results reveal a retrograde pathway linking the photosynthetic redox state to the regulation of nuclear alternative splicing, mediated by the PQ pool, together with a signaling molecule, of yet unknown nature, that is able to travel through the plant to affect alternative splicing (Fig. 4F).

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Supplementary Materials

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