Alternative Splicing Diversifies the Transcriptome during Early Photomorphogenesis and Responds to Metabolic Signals in Arabidopsis

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Contents

Zusammenfassung

Pflanzen nutzen Licht als Energiequelle und Informationsträger um den Tag/Nacht Zyklus, die Jahreszeiten sowie konkurrierende Pflanzen in ihrer unmittelbaren Umgebung zu detektieren. Das Erkennen veränderter Lichtbedingungen ist für Pflanzen essentiell um ihren Metabolismus den äußeren Bedingungen anzupassen und ihre Entwicklungsprozesse zu steuern. In dieser Arbeit haben wir transkriptomweite Änderungen der Genexpression und des Alternativen Spleißens (AS) in dunkel gezogenen (etiolierten) Keimlingen untersucht, die sich unter blauem, rotem oder weißem Licht morphologisch den veränderten Lichtbedingungen anpassen, d. h. den Prozess der Photomorphogenese durchlaufen. Unsere Analyse zeigt, dass sich hierbei die Expressionslevel nahezu 20 % aller Gene, sowie die Spleißmuster hunderter Vorläufer-mRNAs (prä-mRNAs) ändern. Licht führt in mehr als 60 % der lichtregulierten Fälle mit AS zu einer vermutlich nicht-proteinbildenden Spleißariante zur verstärkten Bildung der wahrscheinlich proteinkodierenden Spleißform. Die Varianten, von denen hierbei im Ausgleich weniger gebildet wird, tragen Merkmale, die voraussichtlich zum Abbau dieser mRNAs durch den RNA-Qualitätskontrollmechanismus Nonsense-mediated decay (NMD) führen. Entsprechend zeigen wir für den mutmaßlichen Spleißfaktor *REDUCED RED-LIGHT RESPONSES IN CRY1CRY2 BACKGROUND 1 (RRC1)*, dessen Rolle in der Lichtsignalgebung in einer früheren Arbeit beschrieben wurde, dass in Licht das AS der prä-mRNA zugunsten der funktionellen Variante verschoben wird. Außerdem untersuchen wir die biologische Funktion des lichtabhängigen AS von dem Spleißfaktor *Ser/Arg-rich protein (SR) 30*, dessen alternative Variante trotz vorhandener NMD Merkmale nicht durch NMD abgebaut zu werden scheint. Um die für lichtabhängiges AS notwendige Signalgebung besser zu verstehen haben wir Bedeutung von Photorezeptoren in diesem Zusammenhang untersucht. Die Analysen zeigen, dass die Reaktion auf Weißlicht in der Rotlichtrezeptormutante *phyA phyB* unverändert ist. Dies deutet auf eine untergeordnete Rolle von Photorezeptoren für die Regulation von lichtabhängigem AS hin. Zudem führen interessanterweise die Gabe von Saccharose zu sehr ähnlichen Änderungen im AS wie Licht, was auf eine Kopplung des AS mit dem Energiestatus der Pflanze hinweist.

Abstract

Light is an important source of both information and energy for plants. Diurnal rhythms and seasonal changes, as well as surrounding competition are detected by the available light quality and quantity. Sensing changing light conditions to adjust metabolism and control development is cruicial for survival. Here, we analyse transcriptome-wide changes in gene expression and alternative splicing (AS) in dark-grown (etiolated) seedlings as they transition to growth in white, blue, or red light, undergoing photomorphogenesis. We find changes in expression levels for about 20 % of all genes and changes in splicing patterns in hundreds of transcripts. In more than 60 % of the light-regulated splicing events involving an assumed non-coding variant, production of a presumably protein-coding variant is increased in light, while levels of the other variant carrying Nonsense-mediated decay (NMD)-triggering features decline. Following this pattern, AS of the red light signalling component and putative splicing regulator *REDUCED RED-LIGHT RESPONSES IN CRY1CRY2 BACKGROUND 1 (RRC1)* shifts in favour of the functional variant upon light exposure. While AS of splicing regulator *Ser/Arg-rich protein (SR) 30* also favours the protein-coding variant in light, the alternative variant is not degraded by NMD, and we explore potential other biological functions of this AS event. Furthermore, aiming to elucidate upstream signalling components, we find light-dependent AS to be unaffected in the photoreceptor mutant *phyA phyB* exposed to white light, indicating that photoreceptor signalling only plays a minor role upstream of AS in white light. Interestingly, sucrose supply and light alter the AS output similarly, suggesting that the changes in AS correlate with the plant energy status.

1 Underlying Publications

Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, nov 2016.

Hartmann, L., Wießner, T., and Wachter, A. Subcellular Compartmentation of Alternatively Spliced Transcripts Defines SERINE/ARGININE-RICH PROTEIN30 Expression. *Plant Physiology*, 176(4):2886–903, apr 2018.

2 Personal Contributions

The results presented here have largely been published (see Section 1). Exceptions are Figure 8A, E, I-L, N, O, Table 3, and Figure 13, as well as the corresponding data in the Supplemental Figure 1 and Supplemental Table 1.

The research was designed by Philipp Drewe-Boß, Gunnar Rätsch, Andreas Wachter, and myself. Read alignment, calling of new splice events and differential AS events with FDR calculation were performed by Philipp Drewe-Boß. Subsequent filtering strategies were developed by Andreas Wachter and myself, and filtering was done by me with technical assistance from Fabian Sinz (Supplemental Data Set 1 and 2). SI values were calculated by André Kahles, SI analysis was developed by Andreas Wachter (Supplemental Data Set 3). Extraction of NMD features was done by Philipp Drewe-Boß, analysis of the resulting data was done by Andreas Wachter, and comparison to the Drechsel et al. [2013] data set was done by André Kahles and Andreas Wachter (Supplemental Data Set 4). For the Gene Ontology term analysis I used an Excel macro that was kindly provided by Gabriele Drechsel (Supplemental Data Set 5). Transcribed intergenic regions were identified by Jonas Behr, the resulting data was analysed by me (Supplemental Data Set 6). The experiments presented here, with few exceptions (see below), were performed by myself with technical assistance from Gabriele Wagner and Natalie Faiss. These include photomorphogenesis experiments, preparation of RNA-seq libraries, hypocotyl assays, cloning of constructs, generation of transgenic *Arabidopsis thaliana* lines, *Nicotiana benthamiana* infiltration, co-amplification and RT-qPCRs, generation and purification of a polyclonal antibody (except immunisations), protein extractions, immunoprecipitations, and immunoblot analyses. Theresa Wießner provided the hypocotyl and RT-qPCR data in Figure 16 using lines generated by me, one biological replicate each for Figures 17 and 18C, as well as the samples and data in Figure 18A and the RT-qPCR data from my samples in Figure 21. Illumina sequencing was performed by Christa Lanz and Jens Riexinger at the Genome Center of the Max Planck Institute for Developmental Biology. Vipin T. Sreedharan provided the visualisation of the RNA-seq data in GBrowse. Protoplast transformations were provided by the ZMBP central facilities. Eva Stauffer and Gabriele Drechsel kindly assisted me during confocal microscopy for Figure 11. All figures and accompanying statistical analyses were done by me, also in cases where data was generated by someone else. In particular the result and discussion sections are partially based on the published manuscripts [Hartmann et al. 2016; 2018]. Hartmann et al. [2016] was written by Andreas Wachter and myself, and Hartmann et al. [2018] was written by Andreas Wachter with contributions from me and Theresa Wießner. The methods from Hartmann et al. [2016] and Hartmann et al. [2018] were combined to give a coherent section. The published methods are cited directly with small modifications like updated figure and primer references, plant lines, and spelling. Data created by co-authors on the publications that is not mentioned here has not been included in

this thesis but results are discussed where appropriate.

3 Introduction

3.1 Alternative Splicing

A precursor messenger RNA (pre-mRNA) undergoes capping and polyadenylation, but often also splicing and editing, during its maturation process. These co- and post-transcriptional modifications determine ultimately every property of the mRNA by changing its sequence and structure. The arguably largest change results from splicing, where some parts, the introns, are removed and the remaining parts, the exons, are joined. Components of a ribonucleoprotein complex called spliceosome recognise specific sequences in the primary transcript as splice sites. How closely a splice site resembles the consensus sequence will determine splicing factor affinity, with strong sites containing the consensus and weak sites diverging from it. Generally, using strong splice sites results in so-called constitutive splicing. If competing weak sites exist, alternative splicing (AS) can occur. Whether and how often a particular site is used depends on a complex interplay of numerous components. *Trans*-acting splicing factors and RNA-binding proteins bind *cis*-regulatory sequences within the transcript, and the combination and positions of the different binding partners will influence splicing outcome [Kornblihtt et al. 2013; Lee and Rio 2015; Baralle and Giudice 2017]. The *trans* factors themselves are, of course, regulated in their expression levels, but also regarding their post-translational modifications [Tacke et al. 1997; Xiao and Manley 1997; Tillemans et al. 2006; Chaudhury et al. 2010; Xiang et al. 2013], and AS [Staiger et al. 2003; Schöning et al. 2008; Stauffer et al. 2010]. Accessibility of *cis* sequences and splice sites can be affected by both the pre-mRNA's secondary structure [Wachter 2010; McManus and Graveley 2011; Wachter 2014; Liu et al. 2015] and the transcription elongation rate [de la Mata et al. 2003; Ip et al. 2011; Braunschweig et al. 2013; Kornblihtt et al. 2013; Dolata et al. 2015]. In addition to this kinetic coupling of transcription and splicing, there is also evidence for recruitment coupling by association of splicing factors with the RNA polymerase [Misteli and Spector 1999; de la Mata and Kornblihtt 2006; Muñoz et al. 2010; Huang et al. 2012; Braunschweig et al. 2013; Kornblihtt et al. 2013]. Finally, chromatin structure also affects splicing by recruiting adaptor proteins via histone modifications, or through the effect nucleosome positions have on the polymerase's elongation progress [Tilgner et al. 2009; Schwartz et al. 2009; Luco et al. 2011; Luco and Misteli 2011; Braunschweig et al. 2013; Kornblihtt et al. 2013].

As a consequence of alternative pre-mRNA processing, one gene can give rise to several different mRNAs. These can differ in their regulatory potential only, or give rise to different proteins. AS events are categorised into four groups according to the differences in the isoforms: intron retention, cassette exon (regulated exon), and alternative 3' or 5' splice site (Figure 1). AS is widespread in complex organisms, affecting about 95 % of intron-containing genes in humans [Pan et al. 2008] and about

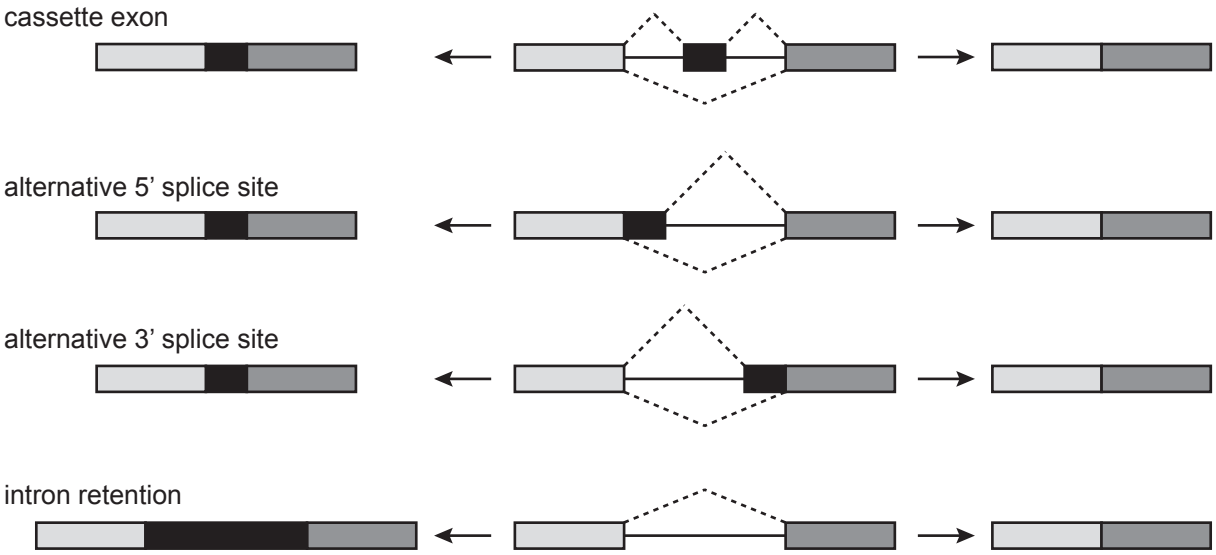


Figure 1: **Types of Alternative Splicing.** Shown are hypothetical pre-mRNAs with exons as boxes and introns as lines (middle). Dashed lines indicate which parts are removed to form the respective two variants. Alternative splicing outcomes are shown to the right and left of the pre-mRNAs.

61 % in *Arabidopsis thaliana* (Arabidopsis) [Filichkin et al. 2010; Marquez et al. 2012] according to current estimates. Biological function and regulation of the vast majority of AS events is presently unclear. However, the impact of AS on human diseases [Karni et al. 2007; Cooper et al. 2009; David and Manley 2010; Kedzierska and Piekielko-Witkowska 2017], animal and plant development [Salz 2011; Mockenhaupt and Makeyev 2015; Zhang et al. 2016; Baralle and Giudice 2017], as well as plant adaptative processes [Sanchez et al. 2010; Staiger and Green 2011; James et al. 2012; Wang et al. 2012; Yang et al. 2014; Zhang et al. 2014a; Dong et al. 2017] highlight its overall importance.

3.1.1 Alternative splicing in quantitative gene expression control

Functionally distinct proteins are one possible outcome of AS. Examples that have been described recently in plants include functional differences for protein variants of PHYTOCHROME INTERACTING FACTOR (PIF) 6, with the alternative variant affecting seed germination, but not the full-length protein [Penfield et al. 2010; Rühl et al. 2012]. Differential localisation of proteins derived from splicing variants has been reported for the flavin-dependent mono-oxygenase YUCCA4 [Kriechbaumer et al. 2012] and the ZINC-INDUCED FACILITATOR-LIKE 1 (ZIFL1) transporter [Remy et al. 2013], with roles in biosynthesis of the phytohormone auxin in flowers, and auxin polar transport or drought tolerance, respectively.

By regulating the balance between splicing variants with differences in their regulatory potential, AS fine tunes gene expression. The resulting mRNAs may differ, for example, in their stability, ultimately

affecting protein levels [Sureau and Perbal 1994; Sureau et al. 2001], or in their localisation, or translation efficiency [Kalsotra and Cooper 2011; Parton et al. 2014]. A number of cases have been described in which unstable splicing variants are degraded by the Nonsense-mediated decay (NMD) pathway [Lewis et al. 2003; Soergel et al. 2006; Lareau et al. 2007; Stauffer et al. 2010; Kalyna et al. 2012; Drechsel et al. 2013].

NMD had initially been described as a eukaryotic RNA quality surveillance system identifying and degrading aberrant transcripts [Chang et al. 2007; Isken and Maquat 2008; Nicholson et al. 2010]. These transcripts can arise from mutations, transcription errors, and incorrect RNA processing. More recently, NMD has been recognised to control physiological transcripts as well and thus to have gene expression regulatory function [Soergel et al. 2006; Kalyna et al. 2012; Drechsel et al. 2013; Karam et al. 2013; Karousis et al. 2016; Lykke-Andersen and Jensen 2015]. To be recognised as an NMD target, the mRNA usually carries a premature termination codon (PTC), PTC-independent long 3' untranslated regions (UTRs), 3' UTR-positioned introns, and/or upstream open reading frames [Kerényi et al. 2008; Karousis et al. 2016; Kertész et al. 2006; Wu et al. 2007; Hori and Watanabe 2007; Kalyna et al. 2012; Nyikó et al. 2013]. Factors bound to these features are recognised during translation, and the mRNA subsequently undergoes either endonucleolytic cleavage or deadenylation and decapping [Nicholson and Mühlemann 2010; Lykke-Andersen and Jensen 2015].

NMD regulates its own components [Yepiskoposyan et al. 2011; Huang et al. 2011], has a role in axon guidance [Colak et al. 2013], and is involved in plant and mammalian immunity [Jeong and Choi 2013; Balistreri et al. 2014; Garcia et al. 2014; Gloggnitzer et al. 2014]. In physiological target transcripts, use of alternative splice sites can give rise to the NMD-eliciting features. This coupled AS-NMD has been shown to regulate expression of Arabidopsis heterogeneous nuclear ribonucleoprotein (hnRNP) proteins GLYCINE-RICH PROTEIN (GRP) 7 and GRP8 [Staiger et al. 2003; Schöning et al. 2008], POLYPYRIMIDINE TRACT BINDING PROTEIN (PTB) 1 and PTB2 [Stauffer et al. 2010], RZ-1B/RZ-1C [Wu et al. 2016], as well as members from the Ser/Arg-rich (SR) protein family [Palusa and Reddy 2010; Kalyna et al. 2012], and is widespread in animals as well [Lewis et al. 2003; Kalsotra and Cooper 2011; Lareau and Brenner 2015; Baralle and Giudice 2017].

3.1.2 Splicing regulators

SR proteins and hnRNP proteins form two major classes of splicing regulators in animals and plants [Chen and Manley 2009; Wachter et al. 2012; Reddy et al. 2013; Staiger 2015]. They were initially shown to act antagonistically with SR proteins enhancing splicing and hnRNPs repressing it [Cáceres et al. 1994; Eperon et al. 2000; Zhu et al. 2001; Olson et al. 2007], but, at least in some cases, they activate and inhibit splicing in a sequence- and binding position-dependent manner [Erkelenz et al. 2013;

Zhou et al. 2014; Bradley et al. 2015; Lee and Rio 2015]. The hnRNP protein family is very diverse and members do not all share one particular functional or structural feature [Han et al. 2010]. They are involved in all aspects of nucleic acid metabolism, including trafficking [Shan et al. 2003], translation [Kosturko et al. 2006; Chaudhury et al. 2010; Torvund-Jensen et al. 2014], and decay [Reznik et al. 2014; Geissler et al. 2016] in addition to splicing. SR proteins are also involved in several aspects of mRNA biology, including nuclear export [Huang and Steitz 2001; Huang et al. 2003; Lai and Tarn 2004; Twyffels et al. 2011], translation [Sanford et al. 2004; Michlewski et al. 2008; Kim et al. 2014], and degradation [Zhang and Krainer 2004]. Furthermore, they have been found to play a role in miRNA biogenesis [Sanford et al. 2008; Änkö et al. 2012; Twyffels et al. 2011; Howard and Sanford 2015], RNA granule formation [Yoon et al. 2013], and genome stability [Li and Manley 2005].

Plant SR proteins contain one or two RNA recognition motifs (RRMs) at their N-terminus and an RS domain downstream of the RRMs. By definition, the RS domain contains at least 20 % RS or SR dipeptides and is longer than 49 amino acids [Barta et al. 2010]. This RS domain interacts with other proteins and can be heavily phosphorylated [de la Fuente van Bentem et al. 2006; Reddy 2007; Barta et al. 2008], affecting protein activity and localisation [Tillemans et al. 2006; Zhang et al. 2014b], and possibly binding of both protein and RNA, which has been demonstrated for human SR proteins [Tacke et al. 1997; Xiao and Manley 1997]. SR proteins and related regulators can have splice form specific phosphorylation sites [de la Fuente van Bentem et al. 2006]. It is assumed that the RS domain has a role in spliceosome assembly [Reddy et al. 2013]. Generally, plant SR proteins localise to the nucleus, where they shuttle between the nucleoplasm and speckles [Fang et al. 2004; Lorković et al. 2004; Tillemans et al. 2005; Ali and Reddy 2006; Tillemans et al. 2006; Lorković et al. 2008; Mori et al. 2012].

In Arabidopsis, SR proteins are a family of 18 genes [Reddy and Ali 2011]. These undergo extensive AS, with 14 genes giving rise to about 90 splicing variants in seedlings [Palusa et al. 2007]. AS of SR proteins is regulated under high light, temperature and salt stress, as well as in response to hormone treatment [Palusa et al. 2007; Tanabe et al. 2007; Filichkin et al. 2010; Cruz et al. 2014], and in development [Lopato et al. 1999; Palusa et al. 2007]. Abundance of splice variants can differ between tissues [Lopato et al. 1999; Palusa et al. 2007], and both splicing auto- and cross-regulation for members of the family have been demonstrated [Lopato et al. 1999; Kalyna et al. 2003; 2006; Palusa et al. 2007; Palusa and Reddy 2010; Thomas et al. 2012].

There are a number of other genes acting in splicing or AS that are very similar to SR proteins but do not meet the criteria for inclusion in the family [Barta et al. 2010; Shikata et al. 2012b]. For one of these SR-like proteins, SR45, splice form specific functions have been described. Expression of *SR45.1* and *SR45.2* was shown to complement a mutant in a strictly tissue-specific manner, the former rescuing a petal and the latter a root phenotype [Zhang and Mount 2009]. SR45 has also been described as a negative regulator of sugar signalling [Carvalho et al. 2010], and as an important

factor in DNA methylation [Ausin et al. 2012]. A binding motif for SR45 has also been uncovered [Day et al. 2012], and more than 4000 RNA targets of SR45 have been identified in a transcriptome-wide assay [Xing et al. 2015]. Such detailed knowledge is not yet available for any other SR- or SR-like protein, especially regarding splice form-specific functions, but some of their properties and roles in diverse processes have come to light. RSZ33 is involved in pollen wall formation by acting in the splicing of *CALLOSE SYNTHASE 5* transcripts [Huang et al. 2013b]. SC35 and SCL proteins have a role in splicing of *FLOWERING LOCUS C* transcripts [Yan et al. 2017], and an intronic *cis* element necessary for SCL33 autoregulatory splicing has been identified [Thomas et al. 2012]. RS40 and RS41 have been linked to miRNA biogenesis [Chen et al. 2015]. Furthermore, SR34b regulates cadmium tolerance by controlling expression of IRON-REGULATED TRANSPORTER 1 [Zhang et al. 2014a], and *SR30* splicing variants, among those from other SR-protein genes, have been reported to differ in their recruitment to polysomes during development and upon stress [Palusa and Reddy 2015]. The SR-like protein REDUCED RED-LIGHT RESPONSES IN CRY1CRY2 BACKGROUND 1 (RRC1) affects AS of SR proteins *SR34*, *SR34b*, *RS31*, *RS31a*, and *RS40*, as well as *PIF6*. *SR34b* and *RS31* were alternatively spliced in response to red light, linking splicing regulation by RRC1 to photoreceptor PHYTOCHROME B-mediated red light signalling [Shikata et al. 2012b;a].

3.1.3 Alternative splicing and light signalling

AS has been shown to also affect other light signalling components. Proteins with distinct subcellular localisation patterns are made from alternatively spliced transcripts of the *PHY-SPECIFIC TYPE 5 PHOSPHATASE* [de la Fuente van Bentem et al. 2003], and a transcription factor involved in light signalling, HY5-HOMOLOG (HYH), varies in stability depending on the splicing variant it is translated from [Sibout et al. 2006].

In addition to the aforementioned effect of light on the splicing patterns of splicing regulators, light has also been described to affect the splicing outcome of other genes. AS of a chloroplastic ascorbate peroxidase gene resulted in proteins with different suborganellar localisation during pumpkin (*Cucurbita sp.*) development and was regulated in a light dependent manner [Mano et al. 1997]. Similar results were reported in case of a peroxisomal and a cytosolic variant of pumpkin hydroxypyruvate reductase [Mano et al. 1999].

3.1.4 Alternative splicing programmes in adaptation and development

Effects on single genes aside, AS could be an important mechanism to coordinately adjust the expression of gene sets [Jangi and Sharp 2014], and recent studies have indeed found such a role for AS. This is becoming particularly evident in animal nervous systems, where synapse specification, embryonic

development, and even evolutionary differences in vertebrate nervous systems were all recently shown to involve underlying AS programmes [Li et al. 2014; Gueroussov et al. 2015; Traunmüller et al. 2016]. It was also recently discovered that mitotic cell cycle progression of human cells is accompanied by changes in splicing patterns of a large number of genes [Dominguez et al. 2016].

In plants, early indications for a larger role for AS in shaping transcriptomes came from a study using a high-resolution reverse transcription-polymerase chain reaction (RT-PCR) panel for *A. thaliana*. Differences in AS depended on the growth conditions, including differences between dark- and light-grown seedlings [Simpson et al. 2008]. Opposite splicing patterns of several transcripts were found in rice seedlings grown either in light or darkness when comparing their transcriptomes using microarrays [Jung et al. 2009]. AS of the core clock component *PSEUDO RESPONSE REGULATOR 9 (PRR9)* in Arabidopsis is affected in mutants of PROTEIN ARGININE METHYL TRANSFERASE 5 (PRMT5), as are many other genes, based on Affymetrix and tiling array data [Sanchez et al. 2010]. Interestingly, PRMT5 expression is regulated in a circadian fashion, and seems to act on spliceosomal proteins, possibly linking circadian rhythms to gene expression control by AS. Furthermore, several clock genes are affected by mutations in the splicing factor SNW/SKI-INTERACTING PROTEIN [Wang et al. 2012], and clock splicing patterns are modulated by high light, temperature and drought stress, as well as pathogen infection [James et al. 2012; Filichkin et al. 2010; 2015]. A single light pulse in the middle of the night also affected splicing of clock genes [Mancini et al. 2015], indicating that AS could be important for entraining circadian rhythms in general, and not just under stress.

More recently, high-throughput analyses using RNA-seq have found widespread red light-mediated AS in the moss *Physcomitrella patens* [Wu et al. 2014] and etiolated *A. thaliana* seedlings [Shikata et al. 2014]. Light-dependent changes in AS have also been reported for older green Arabidopsis plants using a high resolution RT-PCR panel [Petrillo et al. 2014]. Very recently, coordinated AS in cell differentiation of the Arabidopsis root has been described [Li et al. 2016]. The study looked into root development by generating expression data from different cell types in three developmental zones and, interestingly, found the splicing programme underlying cell maturation to be independent of cell type. As this coordinated change in AS co-occurs with differential SR-protein isoform expression across developmental zones, the results strongly suggest that the SR-protein family is responsible for the change in splicing programme. Overall, AS has emerged as an essential mechanism in gene expression control, also in plant light-driven adaptive and developmental processes.

3.2 Photomorphogenesis

3.2.1 Plant photosensory receptors

Plants use light to drive photosynthesis and gather information about their surroundings. Light quality and quantity, indicative of, among other things, the diurnal rhythm and turn of the seasons, have an enormous impact on plant development and physiology. Absorption of light by photosensory receptors (photoreceptors), but also by pigments in the chloroplast results in complex signalling affecting circadian rhythms as well as processes like chloroplast movement, stomatal opening, shade avoidance, phototropism, flowering, seed germination, and photomorphogenesis [McCree 1972; Inada 1976; Evans 1987; Jiao et al. 2007; Woodson and Chory 2008; Franklin and Quail 2010; Kami et al. 2010; Galvão and Fankhauser 2015; Exposito-Rodriguez et al. 2017].

Plant photoreceptors are currently grouped into the five different classes, phytochromes, cryptochromes, phototropins, Zeitlupes, and ultra violet (UV) B receptor UV RESISTANCE LOCUS 8 (UVR8) [Jiao et al. 2007]. In addition to these there may be a yet unidentified class responsible for green light effects [Folta and Maruhnich 2007].

Phytochromes Phytochromes are red (R) and far-red (FR) light receptors. They exist in two interconvertible conformers with distinct absorption spectra, the Pr form absorbing R and the biologically active Pfr form absorbing FR maximally [Kami et al. 2010; Li et al. 2011; Wang and Wang 2015]. However, both forms also absorb blue (B) light weakly [Furuya and Song 1994] so that the entire visible light spectrum can be monitored by phytochromes [Shinomura et al. 1996; Li et al. 2011]. Irradiation with light of a suitable wavelength changes the conformation between Pr and Pfr [Mancinelli 1994; Chen et al. 2005], making phytochromes R/FR switches, however, Pfr converts back to the Pr form in a slow dark reversion reaction as well [Rockwell et al. 2006]. The active Pfr form can translocate from the cytoplasm to the nucleus where it accumulates in speckles [Kircher et al. 1999; Gil et al. 2000; Kim et al. 2000; Hisada et al. 2000; Kircher et al. 2002; Kevei et al. 2007] and alters gene expression, in part through interaction with transcription factors [Martinez-Garcia et al. 2000; Chen et al. 2012; 2014]. In the cytoplasm, Pfr has so far been described to regulate translation, but likely has other cytosolic effects as well [Paik et al. 2012]. Expanding from their classical function as light receptors, it has been suggested that phytochromes also function as temperature sensors [Jung et al. 2016; Legris et al. 2016].

Of the five phytochromes (PHYA-PHYE) found in Arabidopsis [Clack et al. 1994], PHYA and PHYB are the most influential in photomorphogenesis under R or FR [Tepperman et al. 2006]. PHYA is most abundant in etiolated seedlings due to its light labile nature [Li et al. 2011]. When the seedling emerges from the soil PHYA enables it to detect very low levels of any light between 300 and 780 nm to initiate

photomorphogenesis. PHYA mediates responses to prolonged irradiation with FR, as it is found under a dense canopy, and may have a role in B light signalling based on the *phyA* mutant's phenotype under B light [Whitelam et al. 1993; Neff and Chory 1998]. In continuous R light, mainly PHYA is responsible for early and rapid changes in gene expression [Tepperman et al. 2006]. PHYB is considered to be the dominant phytochrome in de-etiolation under prolonged R and white (W) light, as it is the most abundant phytochrome in green plants and seems solely responsible for long-term R light effects. In early responses, PHYB appears to act additively or synergistically with PHYA [Tepperman et al. 2006]. Under B light phytochromes modulate signalling from B/UV-A receptors during photomorphogenesis [Ahmad and Cashmore 1997; Casal and Mazzella 1998; Neff and Chory 1998; Casal 2007; Li et al. 2011; Yang et al. 2017].

Phytochromes C-E are relatively light stable phytochromes like PHYB [Li et al. 2011]. PHYC regulates de-etiolation under R light, but depends on PHYB for its function [Franklin et al. 2003; Monte et al. 2003; Clack et al. 2009]. PHYD's contributions to R light-mediated photomorphogenesis are minor [Aukerman et al. 1997], while PHYE appears to have essentially no role in this process [Devlin et al. 1998; Li et al. 2011].

Cryptochromes Two of the three Arabidopsis cryptochromes mediate signalling in response to B/UV-A light [Kami et al. 2010; Christie et al. 2015; Liu et al. 2016; Yang et al. 2017]. Irradiation does not affect their subcellular localisation, but initiates interaction with, for example, transcription factors. One aspect of cryptochrome signalling requires phosphorylation which is proportional to the intensity and duration of the light signal [Shalitin et al. 2002; 2003] and is accompanied by a conformational change [Kondoh et al. 2011; Yang et al. 2017]. However, many of the exact mechanisms underlying cryptochrome signalling are still under investigation, including its photoactivation [Christie et al. 2015; Liu et al. 2016; Yang et al. 2017].

CRY2 is a nuclear protein which is degraded in blue light and mainly associated with the photoperiodic control of flowering [Guo et al. 1998; Yang et al. 2017], but has some influence on hypocotyl elongation in low light as well [Lin et al. 1998]. CRY1 is found in both the nucleus and the cytosol, and is the receptor mainly responsible for de-etiolation in response to B/UV-A [Ahmad and Cashmore 1993; Yang et al. 2017]. The cytoplasmic pool of CRY1 is responsible for cotyledon expansion and root elongation, all other effects are mediated by the nuclear pool [Wu and Spalding 2007]. CRY3 has a role in mitochondria and chloroplast DNA repair [Kleine et al. 2003; Pokorny et al. 2008; Liu et al. 2016].

Phototropins Phototropins are also B/UV-A responsive receptors [Briggs and Christie 2002; Christie et al. 2015]. They autophosphorylate upon irradiation [Christie et al. 2015]. Both Arabidopsis phototropins are associated with the plasma membrane, but PHOT1 is partially released into the cyto-

plasm upon light exposure [Sakamoto and Briggs 2002], and PHOT2 is targeted to the Golgi [Aggarwal et al. 2014]. PHOT1 and 2 mainly mediate other responses like phototropism but are also responsible for rapid inhibition of hypocotyl elongation upon a B light signal [Folta and Spalding 2001]. In addition, they modulate signalling by phytochromes and cryptochromes [Wang et al. 2013; Wang and Folta 2014], and are in turn modulated by them [Casal 2007].

Zeitlupe The zeitlupe class of B/UV-A photoreceptors has three members, ZEITLUPE, FLAVIN-BINDING KELCH REPEAT F-BOX 1, and LOV KELCH PROTEIN 2 which are mostly involved in regulating components of the circadian clock and photoperodic control of flowering [Nelson et al. 2000; Somers et al. 2000; Schultz et al. 2001; Ito et al. 2012; Christie et al. 2015]. They are cytosolic or nuclear proteins, and have partially overlapping functions [Fornara et al. 2009; Baudry et al. 2010; Takase et al. 2011].

UVR8 UV-B radiation elicits stress responses in plants if the intensity is high and the wavelength short. Longer wavelengths and low fluence rates, however, result in regulatory signalling, leading to photomorphogenic development in seedlings [Kim et al. 1998; Boccalandro et al. 2001; Ulm et al. 2004; Jenkins 2017]. This regulatory signalling begins with detection of low-level UV-B light by the receptor UVR8 [Rizzini et al. 2011] and ultimately alters gene expression patterns [Ulm et al. 2004]. UVR8 localises mainly to the cytosol, and to a lesser extent to the nucleus [Kaiserli and Jenkins 2007]. In absence of UV-B light the receptor forms homodimers which are separated upon absorption of UV-B [Rizzini et al. 2011] and the protein accumulates in the nucleus [Kaiserli and Jenkins 2007] where the monomers initiate signalling.

3.2.2 Signalling underlying photomorphogenesis

Photomorphogenesis is a seedling's morphological development in light. In the absence of light, an emerging seedling will undergo skotomorphogenesis, or etiolation: the hypocotyl elongates in search of light, with the apical hook protecting the cotyledons. Once there is a light signal, the seedling switches from skoto- to photomorphogenesis where hypocotyl elongation is inhibited and the apical hook opens letting the greening cotyledons expand. This altered morphology is accompanied by a substantial change in gene expression, with more than 20 % of the Arabidopsis and rice genomes being differentially expressed between photo- and skotomorphic seedlings [Ma et al. 2001; Tepperman et al. 2001; Jiao et al. 2005; 2007]. The reported differences in gene expression between the two developmental programmes account for transcriptional changes, and both transcription factors [Oyama et al. 1997; Duek and Fankhauser 2005; Wu 2014] and chromatin remodelling [Charron et al. 2009; Bourbousse

et al. 2012] are involved in adapting transcription, with a role for non-coding RNAs [Wang et al. 2014]. Furthermore, a recent report described phytochrome-induced alternative promoter selection during early photomorphogenesis, and this has been shown to affect subcellular localisation of several corresponding proteins [Ushijima et al. 2017]. Contributions of gene expression control on a post-transcriptional level have been uncovered in the past decade (see above, [Simpson et al. 2008; Jung et al. 2009; Wu et al. 2014; Shikata et al. 2014]). In addition to the transcriptional and post-transcriptional layers of regulation, translational and post-translational mechanisms also shape the proteome in response to a light signal [Zhang et al. 2011; Liu et al. 2012b; Paik et al. 2012; Aguilar-Hernández et al. 2017].

A central switch from skoto- to photomorphogenesis downstream of all major receptors is halting the degradation of photomorphogenesis-promoting transcription factors [Arsovski et al. 2012]. In darkness, these transcription factors like ELONGATED HYPOCOTYL 5 (HY5) or HYH are marked for proteasomal degradation by the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) [Osterlund et al. 2000]. COP1 acts in oligomeric complexes with SUPPRESSOR OF phyA-105 (SPA) proteins [Seo et al. 2003; Yang and Wang 2006; Zhu et al. 2008]. SPAs may contribute to substrate recognition in these complexes [Saijo et al. 2008]. The activated photoreceptors interact with COP1 or the SPA proteins, inactivate the complexes and thereby enable accumulation of HY5 and other transcription factors driving photomorphogenesis [Favory et al. 2009; Lian et al. 2011; Liu et al. 2011a; Zuo et al. 2011; Huang et al. 2013a; Sheerin et al. 2015]. In addition, COP1 is exported from the nucleus upon light exposure [von Arnim and Deng 1994], which may contribute to early stabilisation of its targets and help maintain the commitment to photomorphogenic development [von Arnim et al. 1997].

Inhibition of PIFs, transcription factors that promote etiolation, is another central switch underlying the change to photomorphogenesis [Leivar and Quail 2011; Leivar and Monte 2014]. Of the 15 family members, PIF1, PIF3, PIF4, PIF5, and PIF7 repress phytochrome-mediated de-etiolation [Leivar and Monte 2014]. PIF3 has been shown to at least partially do this by recruiting the histone deacetylase HDA15 to target genes involved in chlorophyll synthesis and photosynthesis [Liu et al. 2013]. In light, activated phytochromes inhibit PIFs' binding of target promoters and facilitate their phosphorylation and polyubiquitylation, leading to their degradation [Leivar et al. 2008; Shin et al. 2009]. So far, a role of cryptochromes in PIF-mediated transcriptional control has only been demonstrated for shade avoidance [Keller et al. 2011; Pedmale et al. 2016]. PIFs are an important signalling hub integrating both internal and environmental pathways. The former include the circadian clock, signalling from the sugar metabolism, and hormonal signals; the latter temperature and defense responses in addition to light signalling [Leivar and Monte 2014; Paik et al. 2017]. Hypocotyl elongation, for example, is additively regulated by PIF1, PIF3, PIF4, and PIF5, both in darkness and upon sucrose treatment [Leivar et al. 2008; Shin et al. 2009; Liu et al. 2011b]. Interestingly, PIF6 function in seed dormancy and germination was shown to involve regulation by AS (see above, [Penfield et al. 2010; Rühl et al.

2012]), however, no such a role for AS has been described for PIFs involved in photomorphogenesis.

3.2.3 Integration of chloroplast signals with photoreceptor signalling

An important part of photomorphogenesis is the development of photosynthesis-competent chloroplasts, prerequisite for switching from hetero- to autotrophic growth. To coordinate seedling and organelle development, growth, and maintenance, as well as adequate responses to stresses, bidirectional signalling between chloroplasts and the nucleus is necessary [Pogson and Albrecht 2011; Chan et al. 2016; de Souza et al. 2017]. Anterograde control by the nucleus over chloroplast function is exerted, for example, via the many plastidial proteins that are encoded in the nuclear genome, like transcription factors and components of large multi-protein complexes [Bräutigam et al. 2007; Oh and Montgomery 2013; 2014]. Retrograde signalling from the chloroplast relays information on the state of the chloroplast and detected stresses to the nucleus, affecting gene expression. Among the elicitors of the seven currently discussed signalling pathways are reactive oxygen species (ROS), like hydrogen peroxide or singlett oxygen, the redox state of the plastoquinone pool, and metabolites like dihydroxyacetone phosphate [Häusler et al. 2014; Chan et al. 2016; Dietz et al. 2016; de Souza et al. 2017]. While ROS may be able to directly elicit a signalling cascade [Kovtun et al. 2000; Apel and Hirt 2004], it is assumed that different ROS react with a variety of other molecules to form oxidation products which serve as the actual signals, because ROS are highly reactive and therefore short-lived [Dietz et al. 2016; de Souza et al. 2017]. These secondary signalling molecules could provide specificity to a signal that otherwise could often be ambiguous, as the production of ROS occurs in several compartments under many different conditions, and also elicits retrograde signalling from other organelles [de Souza et al. 2017]. Exactly how the different retrograde signals are transduced to the nucleus remains unknown.

There is extensive crosstalk between chloroplast and photoreceptor signalling. About half of the light-regulated genes in seedlings shifting from low to higher intensity light were found to be regulated by the plastid [Ruckle et al. 2012]. If chloroplast biogenesis is inhibited, retrograde signals can turn normally transcription-promoting HY5 into a negative regulator of *CHLOROPHYLL A/B BINDING PROTEIN 1 (CAB1,* also called *LHCB1)* expression [Ruckle et al. 2007; Ruckle and Larkin 2009; Ruckle et al. 2012]. Perception of excess light in the chloroplast was recently shown to suppress phytochrome- and PIF-mediated growth in a PIF-independent manner [Martín et al. 2016]. Thus, light signals in favour of photomorphogenesis are modulated by retrograde signals to ensure the integrity and functionality of the developing chloroplast. Furthermore, core photoreceptor signalling components PIF1, PIF3, HY5, and HYH directly regulate ROS responsive genes [Chen et al. 2013].

The effects of retrograde signals on nuclear gene expression have mostly been examined on the transcriptional level [Strand et al. 2003; Koussevitzky et al. 2007; Aluru et al. 2009; Ruckle et al. 2012],

however, changes in AS have recently been reported as well [Petrillo et al. 2014]. In green leaves splicing patterns of SR-protein *RS31* changed in response to darkness and renewed light exposure. No involvement of the major red or blue light photoreceptors was apparent when tested. Application of two drugs inhibiting the electron transfer in photosynthesis up- and downstream of the plastoquinone pool, however, inhibited and mimicked the effect of light, respectively. The authors thus concluded that a retrograde signal reflecting the photosynthetic redox state in the plastoquinone pool affects nuclear splicing. Interestingly, it appears that this signal travels from leaves to roots to also influence AS in tissues not exposed to light/dark transitions. Furthermore, the small zinc finger protein METHYLENE BLUE SENSITIVITY, which is required for a full response to singlett oxygen stress, localises to stress granules and processing bodies upon oxidative stress, where it may control translation or affect mRNA stability [Shao et al. 2013], indicating that retrograde signals could possibly also affect gene expression outside of the nucleus.

4 Objectives

Photomorphogenesis is accompanied by substantial changes to the transcriptome, and alternative splicing (AS) is increasingly viewed as an important mechanism in plant transcriptome adaptation. The central goal of this work was to establish whether AS plays a role in seedling adaptation from growth in darkness to growth in light and what the contributions of blue and red light signalling are. To this end, etiolated seedlings were kept in darkness, or exposed to white, blue, or red light and their transcriptomes sequenced. Pair-wise comparisons of samples with and without light exposure showed statistically significant light-dependent AS changes in hundreds of cases.

Using the computational analysis as a starting point, the next goal was to independently validate the results, and to choose interesting candidates to determine the biological role of their light-dependent AS. Splicing regulators SR30 and RRC1 were chosen for their potential to affect splicing of many other transcripts, and in case of RRC1 also because of its published connection to red light signalling. Based on the properties of the respective AS variant, we initially expected in both cases expression regulation by NMD. For examining RRC1, a known mutant was complemented splice form-specifically or using a genomic construct, under control of either a constitutive or the putative endogenous promoter. These lines, as well as overexpression lines that were generated, were used in hypocotyl assays to prove that only the representative variant is capable of complementing a previously described phenotype.

While trying to confirm coupled AS-NMD for *SR30*, we discovered that, unexpectedly, the alternative variant does not accumulate when NMD is impaired. This led us to examine potential other functions of this variant. To enable detection and enrichment of endogenous protein in immunoblots and immunoprecipitations, an antibody was raised against SR30. The subcellular localisation of the protein possibly arising from each splicing variant was analysed, as was the variants' ability to autoregulate their own pre-mRNA splicing. Splice form-specific and general artificial microRNA lines as well as overexpression lines were generated, and a T-DNA knock-out line was identified. Some of the misexpression lines were screened for a phenotype in hypocotyl assays in order to test a functional connection between SR30 and light-dependent development.

To further analyse the contributions of red light photoreceptors to light-dependent AS, light-induced splicing patterns in the *phyA phyB* mutant were examined in different light settings. Finding that sucrose supplementation in darkness can mimic light-dependent AS, we tried to identify potential upstream signalling components that link AS to metabolic signalling.

5 Results

5.1 Light Exposure Changes the Alternative Splicing Patterns in Etiolated Seedlings

5.1.1 Analysis of transcriptome-wide splicing patterns reveals light-dependent alternative splicing for hundreds of genes during early photomorphogenesis

The switch from skoto- to photomorphogenesis is accompanied by massive transcriptome reprogramming [Jiao et al. 2007]. We were interested in the contribution of alternative splicing (AS) to changes in the transcriptome and therefore analysed etiolated *Arabidopsis thaliana* (Arabidopsis) seedlings exposed to blue, red, or white light for 1 or 6 h using RNA-seq. We also generated dark controls and sequenced duplicates of each sample. The 100-bp reads were mapped to the TAIR10 annotation, resulting in 37.08 x 10^6 to 145.22 x 10^6 reads per sample for two biological replicates (Table 1). AS events annotated in TAIR10 were complemented by unannotated events found in our data resulting in 56,270 AS events. We used a previously established and validated computational pipeline [Rühl et al. 2012; Drechsel et al. 2013; Drewe et al. 2013] (Supplemental Data Sets 1 and 2) to analyse quantitative changes in both AS and gene expression.

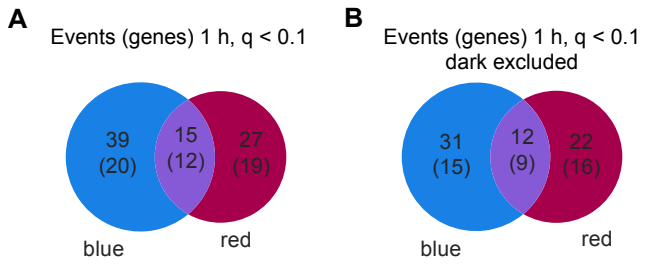


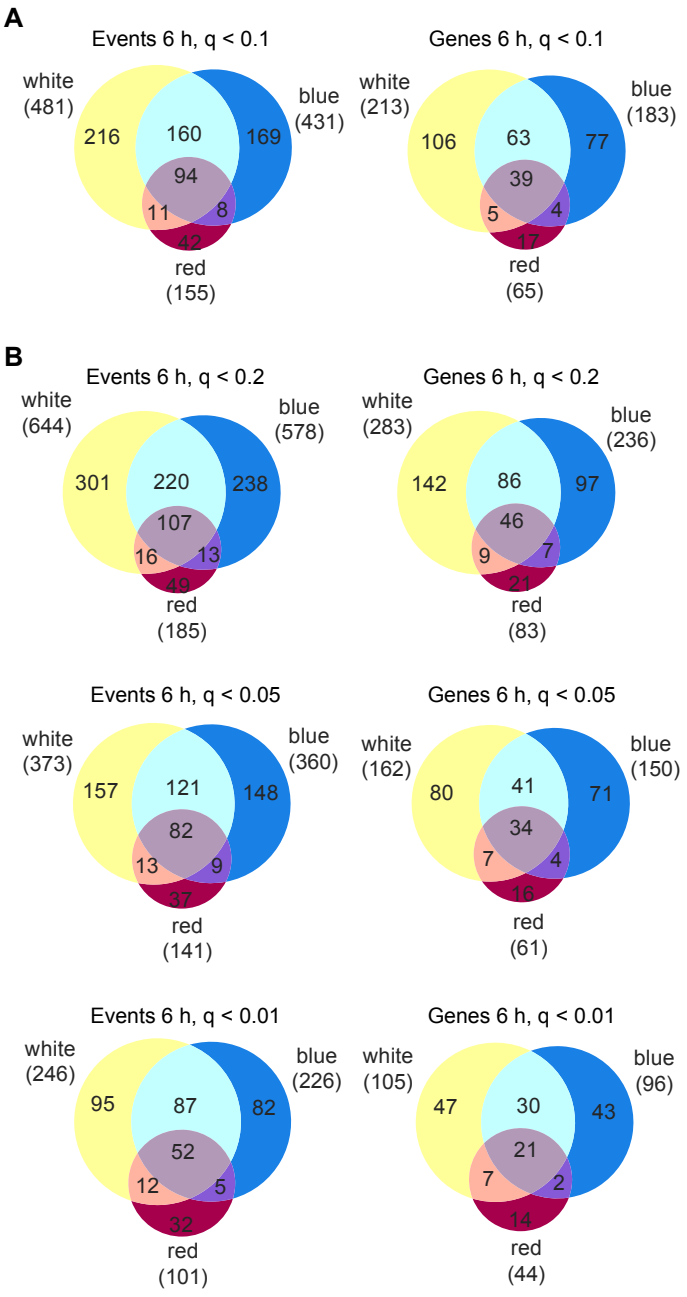
Figure 2: **Light-triggered Alternative Splicing after 1 h Blue or Red Light Exposure.** AS events and associated genes (number in parentheses) with significant (FDR < 0.1) changes in AS comparing 1 h exposure to blue (~6 µmol m^{-2} s^{-1}) or red (~14 µmol m^{-2} s^{-1}) light and respective 0 h samples. Events changing in opposite directions in blue and red were excluded. In B, events changing in the same direction between 0 h und 6 h dark samples as between 0 h and 1 h sampes were additionally excluded.

After 1 h exposure to blue or red light we found significant changes to 81 AS events in 51 genes (false discovery rate FDR < 0.1, Figure 2). Early changes in AS are expected to be detectable for highly unstable transcripts only since steady state levels need to adjust for the change to be detectable. As therefore expected, 6 h light exposure to blue or red light gave rise to many more changes (Figure 3A). After 6 h in white light, additional AS events were significantly altered. Overall, 700 AS events in 311 genes showed significant changes after 6 h light exposure (Figure 3A).

Comparing the events after 1 h and after 6 h light exposure to blue or red light, we found only very few consistent changes. A combination of weak changes in AS after 1 h and many activated downstream signalling cascades after 6 h likely causes the small overlap between the time points. Further examination of AS in light-regulated processes focused on the 6 h time point to include these

Table 1: **Read Statistics of RNA-seq data.** B = blue light, R = red light, W = white light, R1, 2 = biological replicate, D = dark, Lane = lane on flow cell, Adapter = multiplexing adapter used. Raw sequencing data was processed and analysed by Philipp Drewe-Boß. *From:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

Library	All reads ($x10^6$)	Unmapped reads ($x10^6$)	Mapped reads ($x10^6$)	Spliced reads ($x10^6$)	Unspliced reads ($x10^6$)	Lane	Adapter
B 0h R1	47.84	4.40	43.44	12.36	31.08	2	012
B 0h R2	46.47	3.87	42.60	12.48	30.11	2	006
B 6hD R1	48.09	3.74	44.35	12.71	31.64	2	019
B 6hD R2	65.36	5.05	60.31	17.74	42.58	2	005
R 0h R1	48.47	4.69	43.78	13.02	30.77	4	012
R 0h R2	79.89	7.83	72.06	21.20	50.86	4	006
R 6hD R1	50.06	5.15	44.91	12.99	31.92	4	019
R 6hD R2	50.05	4.80	45.25	13.09	32.15	4	005
W 0h R1	42.05	4.28	37.77	11.36	26.41	6	012
W 0h R2	85.66	9.02	76.64	22.64	54.00	6	006
W 6hD R1	41.49	4.41	37.08	10.91	26.16	6	019
W 6hD R2	74.55	7.20	67.35	19.83	47.52	6	005
B 1h R1	76.62	6.62	70.00	19.87	50.13	1	012
B 1h R2	148.10	13.73	134.37	38.84	95.53	1	006
B 6h R1	68.61	6.27	62.34	18.55	43.78	3	012
B 6h R2	161.13	15.91	145.22	43.43	101.79	3	006
R 1h R1	92.95	6.56	86.39	25.25	61.13	8	012
R 1h R2	90.51	5.94	84.57	24.79	59.78	8	006
R 6h R1	90.63	7.40	83.23	24.49	58.74	5	012
R 6h R2	90.95	7.15	83.80	24.40	59.40	5	006
W 6h R1	89.97	6.40	83.57	24.87	58.70	7	012
W 6h R2	103.10	6.91	96.19	29.30	66.90	7	006



E

Events

q <	white	blue	red	blue & red	sum
0.2	100	34.2	2.5	16.6	53.3
0.1	100	33.3	2.3	19.5	55.1
0.05	100	32.4	3.5	22.0	57.9
0.01	100	35.4	4.9	21.1	61.4

Genes

q <	white	blue	red	blue & red	sum
0.2	100	30.4	3.2	16.3	49.8
0.1	100	29.6	2.3	18.3	50.2
0.05	100	25.3	4.3	21.0	50.6
0.01	100	28.6	6.7	20.0	55.2

Figure 3: **Light-triggered Alternative Splicing after 6 h Light Exposure.** Significant changes in AS between 6 h light and 6 h dark samples using different FDR cutoffs (A, B) and additional filter criteria (C, D). **(A, B)** The FDR cutoff q was set as indicated. Separate diagrammes are shown for events and genes. Numbers in parentheses give total events or genes under the respective light colour. **(C)** The events shown in (A) were additionally filtered by their SI, excluding cases with coverage < 10 reads in one replicate and a maximum SI difference between replicates > 0.25. Changes in average SI between dark and light samples had to be > 0.05. FDR and SI criteria had to be met in the same light colour. Events with opposite changes in two colours were excluded. **(D)** Events from (A) were grouped according to their SI (as defined for (C)) only. **(E)** Percentages of events and genes overlapping between white and the other light colours for different FDR cutoffs (q) as shown in (A) and (B). White light ~130 µmol m^{-2} s^{-1}, red and blue light see Figure 2.

downstream effects.

Surprisingly, the significantly changing AS events overlapped only partially between the three light colours. While a difference is expected for red and blue, as these light conditions elicit primarily phytochrome or cryptochrome responses, respectively, all these signalling pathways should become active in white light. Changing the FDR cutoff did not influence the overlap (Figure 3B, E). We implemented an additional filter for effect size (change in splicing index, SI > 0.05; Figure 3C, Supplemental Data Set 3) to remove events with minor quantitative changes, high variance between replicates, or low read coverage. This resulted in fewer events, but did not increase the overlap between light qualities. Upon relaxing the FDR criterion, however, to require a value < 0.1 in one light condition, and subsequently applying only the SI filter to group the events, 87.5 % - 98.0 % of the events changing in one light colour were also affected by another light colour (Figure 3D). Most of the events were detected in all conditions, suggesting the effect on AS is largely independent of the light colour.

Red light appeared to have a weaker effect on AS than blue or white light, as the FDR filter gave fewer significant events and the SI filter a lower median SI change of 0.079 versus 0.137 and 0.136 in blue and white light, respectively (Supplemental Data Set 3). Indeed, validation experiments confirmed both the mostly common effect on AS under different light conditions and a weaker quantitative effect of red light in two cases (see Figure 8).

We detected 56,270 AS events, of which 46.9 %, 22.4 %, 21.2 %, and 9.5 % correspond to alternative 3' splice sites, regulated introns (varying rate of intron retention/splicing), alternative 5' splice sites, and regulated exons (cassette exons), respectively (Figure 4A). In contrast, among the 700 light-regulated events cassette exons (18.0 %) and regulated introns (37.1 %) were enriched, with alternative 3' and 5' splice sites accounting for only 27.3 % and 17.6 %, respectively. In light the shorter splicing variant was dominant for 74 % of the significantly changing intron retention events and 67 % of the cassette exon events. For alternative up- and downstream 5' and 3' splice site usage, respectively, an enrichment in light was detected but weaker than for cassette exon and intron retention (Figure 4B).

Looking into the potential effect of light-dependent AS on gene expression we compared the positions of light-dependently changing events to those of all events (Figure 4C and Supplemental Data Set 4A-D). We found events located within the coding sequence to be reduced and those associated with the 3' untranslated region (UTR) enriched among light-dependently spliced events. The largest number of events aligned with the coding sequence in all subsets. Thus, both the coding and the regulation potential of mRNAs are expected to be influenced by light-dependent AS.

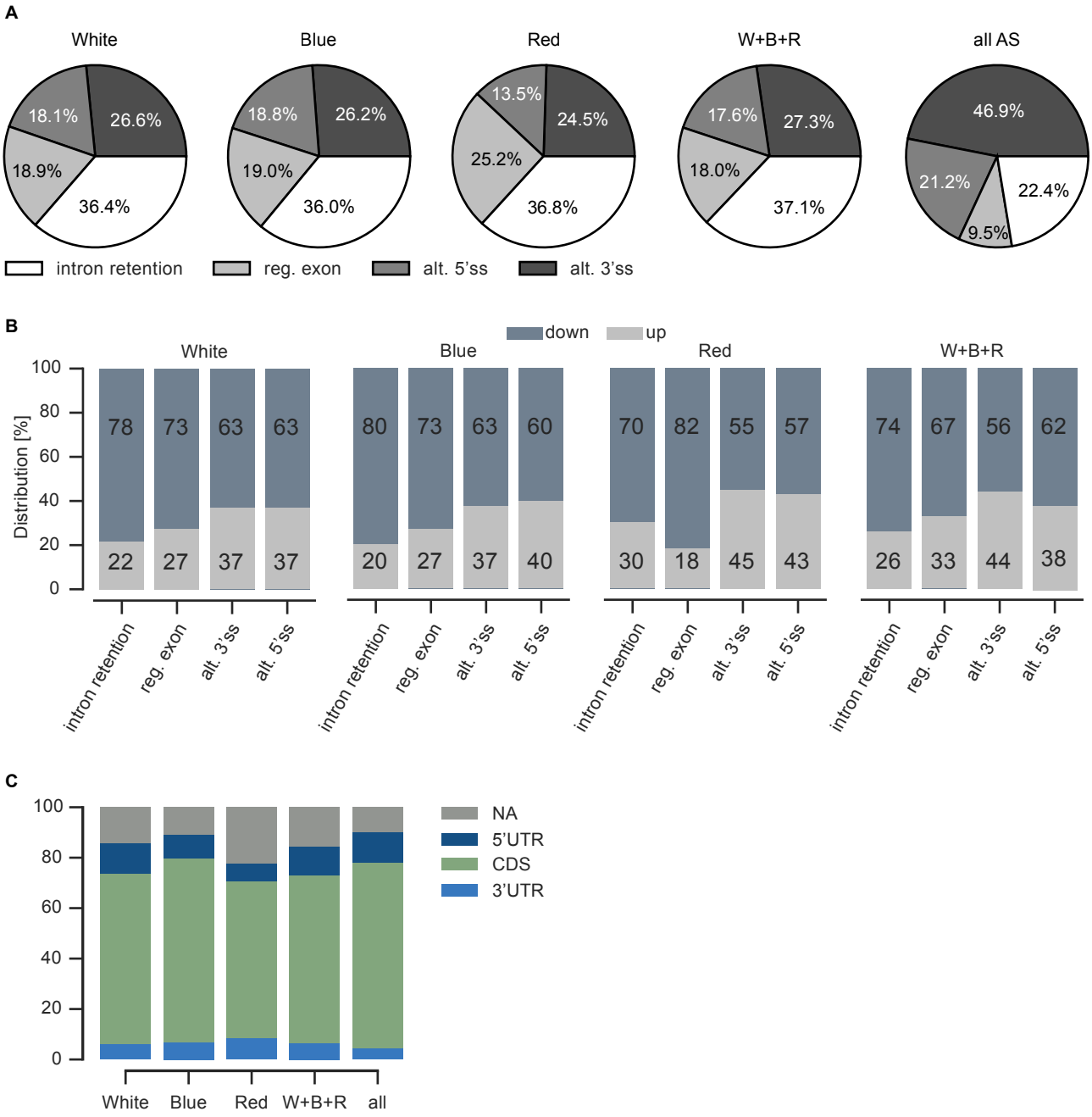


Figure 4: **Properties of Alternative Splicing Events. (A)** AS type distribution of AS events significantly affected by white, blue and red light, and of all detected events. **(B)** Direction of changes in AS. "up" and "down" refer to a relative increase and decrease, respectively, of the longer splicing variant in significantly changing AS events under the indicated condition. **(C)** Location of light-dependent AS events within the transcript. All detected AS events were mapped to either the 5' UTR, coding sequence (CDS), or 3' UTR. Events with ambiguous mappings were assigned to NA. Distributions are shown for events changing significantly between 6 h darkness and 6 h light with FDR < 0.1 and all detected events. reg. = regulated, alt. = alternative, ss = splice site. Analyses were done by Philipp Drewe-Boß and Andreas Wachter. *Slightly modified from:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

5.1.2 Light-regulated alternative splicing may often be coupled to NMD

Earlier studies in *A. thaliana* have demonstrated coupling of AS and nonsense-mediated decay (NMD) to occur [Kalyna et al. 2012; Drechsel et al. 2013]. To analyze the extent of coupled AS-NMD during the initiation of photomorphogenesis we looked for NMD-triggering features within the splicing variants. To do so we integrated the AS events into the representative isoform according to TAIR10, and identified upstream open reading frames (uORFs), premature termination codons, and long 3' UTRs (Supplemental Data Set 4A-D). We found 77.2 % of all light-regulated AS events to contain NMD-triggering features within the isoform enriched in darkness and 61.1 % of these to relatively switch from a putative NMD target to a non-NMD regulated transcript variant upon light exposure. When we considered only AS events in the coding sequence the fractions were even larger. $\sim$ 10 % of all light-regulated events from this study were shown to be controlled by NMD in a previous analysis of NMD-impaired mutants [Drechsel et al. 2013] (Supplemental Data Set 4E-G), further corroborating coupled light-regulated AS and NMD. The seedlings used in the NMD study were older and light-grown while the data from this study indicates a downregulation of the putative NMD isoform in light. Thus, we expect the overlap we report to be an underestimation. Overall, it appears that light-dependent AS often leads to a switch from a putative NMD-regulated isoform to a protein-coding variant leading to the activation of gene expression at the onset of photomorphogenesis.

5.1.3 Light-dependent transcriptional regulation affects thousands of genes

In addition to analysing AS, we also looked at differential gene expression in our data [Anders and Huber 2010] (FDR $\leq$ 0.1, Supplemental Data Set 2). As previously reported [Jiao et al. 2007] we found many genes to be up- or downregulated in light (Figure 5A). 23,432 genes were expressed in our data set of the 33,602 genes annotated in TAIR10. (FDR $\leq$ 0.1; method based on Gan et al. 2011). For 10,271 (43.8 %) of the expressed genes 6 h light exposure resulted in altered transcript levels under at least one condition. 9,336, 4381, and 4,251 genes showed a change in expression level under white, blue, or red light, respectively. Requiring an at least two-fold change in expression reduced the numbers to 3,439, 2,406, and 2,020 respectively for the three light colours (Figure 5B). Taken together, 4,310 genes, or 18.4 % of all expressed genes showed at least two-fold light-dependent changes in expression level.

Genes with changes in expression level overlapped greatly between blue and red light, with most also being detected under white light. Unlike for the analysis of AS, blue and red light affected the expression of a similar number of genes. Interestingly, a large number of transcriptional changes were detected under white light only, pointing towards an adaptive program that does not become active in response to weak monochromatic light.

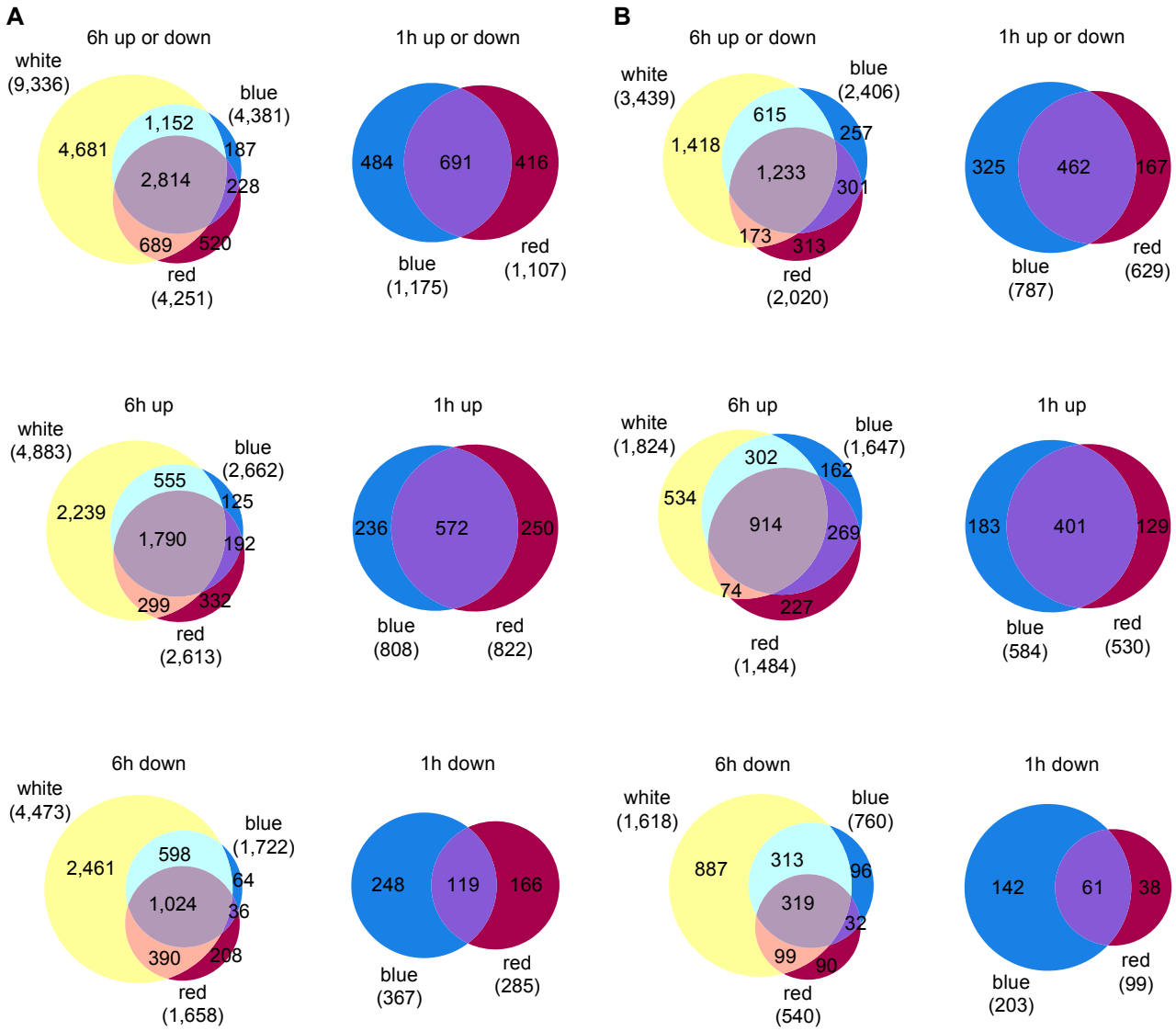


Figure 5: **Changes in Total Transcript Levels after Light Exposure.** Differential gene expression after 6 h or 1 h light exposure with FDR ≤ 0.1 **(A)** and with an additional 2-fold threshold implemented **(B)**. Changes at the 6 h time point are compared to 6 h darkness samples, changes after 1 h to 0 h samples. The total number of genes affected in each light colour is given in parentheses. For Venn diagrammes showing combined up and down changes, genes changing in opposing direction under two conditions were excluded. *From:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

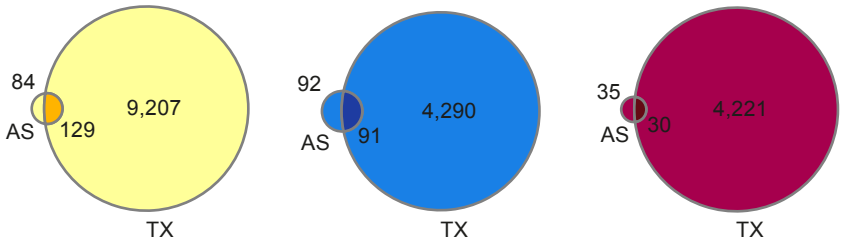


Figure 6: **Overlap between Light-Dependent Changes in Alternative Splicing and Transcript Levels.** Genes changing significantly in total expression (TX), AS, or both after 6 h illumination with white (left), blue (middle), or red (right) light.

Separating up- from down-regulated genes, we find more induced genes than repressed ones after 6 h (Figure 5A). After 1 h light exposure, fewer changes were detectable, and about twice as many genes were up- as down-regulated. Again, transcript stability has to be considered a contributing factor, since only highly unstable transcripts will have detectable changes to their steady-state levels in response to reduced transcription after 1 h.

We compared the gene lists from the AS and expression level analysis separately for the three light colours, and discovered that many of the genes with changes in AS did not change in total transcript levels (Figure 6). Given that quantitative gene expression can also be altered by AS, it is likely we overestimate the number of genes exhibiting AS and differential gene expression independent of AS.

In summary, illumination of etiolated seedlings results in complex changes to the transcriptome on the level of AS for a few hundred genes, and on the level of gene expression for thousands of genes with only a partial overlap between the two.

5.1.4 Light-dependent alternative splicing affects genes involved in RNA metabolism

The functional categories "RNA" and "metabolism" for blue light and "RNA" for white light were overrepresented for genes with light-dependent AS (Figure 7, Supplemental Data Set 5). The term "RNA" had also been overrepresented in previous studies of AS [Filichkin et al. 2010; Rühl et al. 2012; Drechsel et al. 2013], demonstrating that regulation of genes involved in RNA metabolism by AS is widespread.

A previous study found that many intergenic regions are expressed in an NMD-regulated manner [Drechsel et al. 2013]. Therefore we looked into read accumulation in intergenic regions and compared dark- and light-exposed samples (Supplemental Data 6). In some cases there was a difference between the samples, but mostly the expression levels were quite low and more work will be needed to examine whether these transcripts have functional relevance. A few of the transcribed intergenic regions had been previously identified to encode long intergenic RNAs [Liu et al. 2012a].

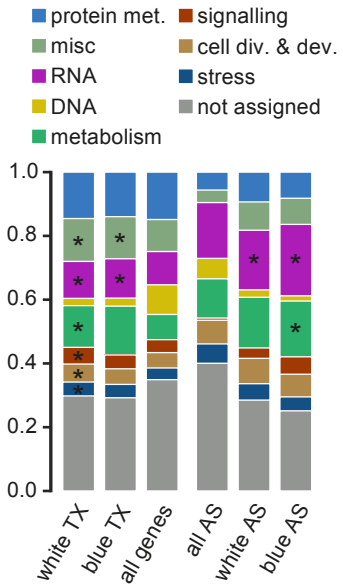


Figure 7: **Gene Ontology Term Analysis.** Terms found for genes undergoing AS or TX changes after 6 h white or blue light. Asterisks indicate terms overrepresented compared to all AS events and all genes in MapMan, respectively, with Bonferroni-corrected $p < 0.05$. met. = metabolism; misc. = miscellaneous; cell div. & dev. = cell division & development.

5.1.5 Expression of circadian genes is unchanged in darkness

To find out if a rhythmic expression pattern is already established in the dark-grown samples, we looked at the transcript levels of several of known circadian genes (Table 2). There was no significant change between the 0 h and 6 h dark samples for any of the genes. In contrast, light exposure changed the expression for a number of the circadian genes, in line with the established effect of light on rhythmic expression patterns [Jiao et al. 2007].

Table 2: **Circadianly regulated genes are not differentially expressed in darkness.** Given are the smallest up- and down-FDR values in indicated comparisons for eight known circadianly regulated genes. Background colors indicate light conditions blue (left), red (middle), and white (right). Darker shades represent darkness controls. FDRs < 0.1 are bold. *From:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

Gene	Gene ID	0 vs 6D	0 vs 6	6D vs 6	0 vs 6D	0 vs 6	6D vs 6	0 vs 6D	0 vs 6	6D vs 6
CCA1	AT2G46830	1.00	0.21	**0.00**	1.00	**0.00**	**0.00**	1.00	0.91	0.85
LHY	AT1G01060	1.00	**0.00**	**0.00**	1.00	**0.00**	**0.00**	1.00	0.41	0.48
PRR7	AT5G02810	1.00	**0.01**	0.48	1.00	0.32	**0.05**	1.00	0.18	0.26
PRR9	AT2G46790	1.00	**0.01**	0.48	1.00	**0.09**	**0.06**	1.00	0.29	0.25
GI	AT1G22770	1.00	**0.00**	**0.00**	1.00	**0.00**	**0.00**	1.00	**0.00**	**0.00**
TOC1	AT5G61380	1.00	0.49	0.62	1.00	0.76	0.94	1.00	**0.09**	0.78
LUX	AT3G46640	1.00	0.25	**0.10**	1.00	0.62	**0.09**	1.00	**0.00**	**0.00**
ZTL	AT5G57360	1.00	0.12	0.37	1.00	0.98	0.99	1.00	**0.00**	**0.00**

5.1.6 Experimental validation of computational results

To validate our findings on light-dependent AS we selected candidates from the list of significant events for independent analysis (Figure 8). Some of the events were selected based on the downstream regulatory potential of the corresponding genes, which encode splicing factors (Figures 8A-E, 9A-E, and 14A, B), transcription factors (Figure 8F-H), and a regulator of chromosome condensation (RCC1) family protein (Figure 8L), and therefore might play an important role in early photomorphogenesis. Moreover, nuclear genes encoding organellar proteins including photosynthesis components were considered in this analysis (Figure 8M-P). Select light signalling components (Figure 8I-K) for which we could detect alternatively spliced regions with an FDR < 0.5 were also included. We were able to confirm the presence of the respective splicing variants for all candidates selected. In some cases we detected additional isoforms and limited our analysis to the major ones which were also confirmed by sequencing (Supplemental Figure 1). All events showed a change in AS ratio in white light, or, when tested, in all light colours. However, there was a difference in the extent of splicing change between colours for some candidates. In these cases white light was most effective in causing the change in splice ratio.

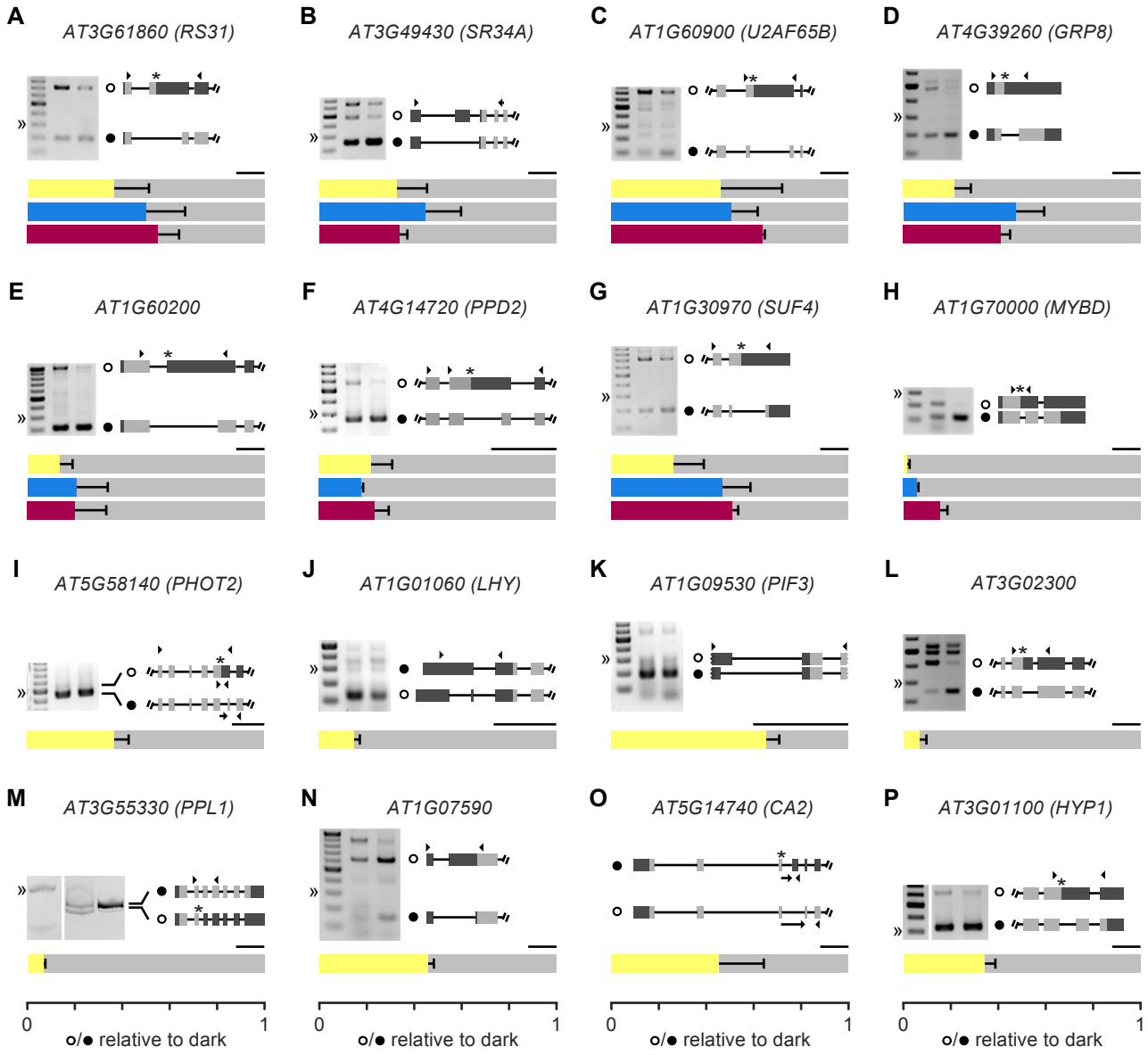


Figure 8: **Independent Experimental Validation of Light-Dependent Alternative Splicing.** See also Figures 9A-E and 14A, B. Splicing variants of genes encoding splicing factors (A-E), (putative) transcription factors (F-H), light signalling components (I-K), and other proteins (L-P) were co-amplified and quantified using a Bioanalyzer (A-H, J-N, P), or analysed by RT-qPCR (I, O) from samples grown in darkness and collected at 0 h or after 6 h exposure to white (~130 µmol m^{-2} s^{-1}, A-P), blue (~6 µmol m^{-2} s^{-1}), or red (~18 µmol m^{-2} s^{-1}, A-H) light (top, middle, bottom bars in A-H). Shown are representative agarose gels with double arrowheads pointing at 300 bp (A-H, J-L, N, P) or 500 bp (I) of a DNA size ladder with 100 bp increments, or at 200 bp of a ladder with 50 bp increments (M), and PCR products from 0 h (left) and 6 h (right) samples. The variants quantified are marked with circles and partial (A-C, E-G, I-L, N-P) or full (D, H, M) gene models are shown with introns represented by lines and exons by boxes. Regions colored in dark grey are UTRs, and asterisks mark the introduction of a premature termination codon. Solid arrowheads show the positions of the primers used, and arrows (B, I, O) indicate splice junction-spanning primers. Primer pairs shown below a gene model are specific to that variant (I, O). Bars give average relative splice form ratios with the ratio in darkness set to 1, as indicated by the light grey background bar for each color. Error bars are +SD, n = 3. Scale bars represent 500 bp in each gene model. (E) Splicing factor PWI domain-containing protein. (F) The outer forward primer was used in the gel picture, the inner for quantification. (H) Myb-like transcription factor family protein. (L) regulator of chromosome condensation (RCC1) family protein. (O) The topmost primers were used for co-amplification. (N) Tetratricopeptide repeat (TPR)-like superfamily protein.

5.2 Light Mediates a Rapid and Transient Shift in *SR30* AS

The set of genes displaying light-dependent AS was enriched in factors involved in RNA metabolism, including several splicing regulators. Looking specifically into the SR protein family showed that 7 out of the 18 SR protein genes from *A. thaliana* contained at least one AS event that was significantly changed upon exposure to 6 h white light (Table 3). SR protein genes from *A. thaliana* are known to display extensive and regulated AS of their own pre-mRNAs [Kalyna et al. 2003; Palusa et al. 2007; Palusa and Reddy 2010], which might critically affect their function in orchestrating downstream AS programs.

Table 3: **Members of the SR-Protein Family Undergoing Light-Dependent Changes in Alternative Splicing and Gene Expression.** Significant changes according to the FDR < 0.1 (AS) and FDR ≤ 0.1 (TX) thresholds as applied before. Column "2-fold" indicates an at least 2-fold change in TX. Column "up/down" indicates direction of change in TX.

Gene	AS	TX	2-fold	up/down
SR30	x	-	-	-
SR34	-	x	x	down
SR34A	x	-	-	-
SR34B	-	x	-	down
SCL28	-	x	x	up
SCL30	-	x	-	down
SCL30A	x	-	-	-
SCL33	x	-	-	-
RS2Z32	-	x	-	down
RS2Z33	x	x	-	down
RS31	x	x	-	down
RS40	-	x	-	down
RS41	x	-	-	-

To investigate the consequences of light-triggered AS, we functionally characterised an event within *SR30* that was significantly altered under all light conditions tested in this study (Figure 9A). Co-amplification RT-PCR of the corresponding region confirmed that *SR30.1* and *SR30.2* constitute the two major splicing variants (Figure 9B). First, the AS shift was resolved at higher temporal resolution (Figure 9C). Opposite changes in the levels of the two major splicing variants were detectable already 0.5 h after onset of white light exposure, and became more pronounced over time, showing a maximum at the 6 h time point. Similar but less pronounced AS pattern changes were observed in response to blue and red light (Figure 9D, E). After one day of growth in light, relative amounts of the two splicing variants returned closer to the starting levels detected in etiolated seedlings. As the monochromatic light exposures were done at comparatively low intensities (R: ~ 18 µmol m^{-2} s^{-1}; B: ~ 6 µmol m^{-2} s^{-1}), we assume that the effect on AS we observed is independent of previously reported light stress responses. Furthermore, no AS change was seen for seedlings kept in darkness (see also Supplemental Data Set 1), excluding a light-independent effect on *SR30* expression. In general, the observed light-triggered changes in AS of *SR30* are in accordance with previous reports [Petrillo et al. 2014; Shikata et al. 2014].

The rapid and pronounced change in AS of the *SR30* pre-mRNA suggested its potential involvement in the early steps of light-triggered AS. The two major splicing variants *SR30.1* and *SR30.2* differ in the position of the stop codon. As the 3' UTR of *SR30.2* is considered to be long and contains an intron, both hallmarks of an NMD target, we tested whether NMD is involved in the regulation of this gene.

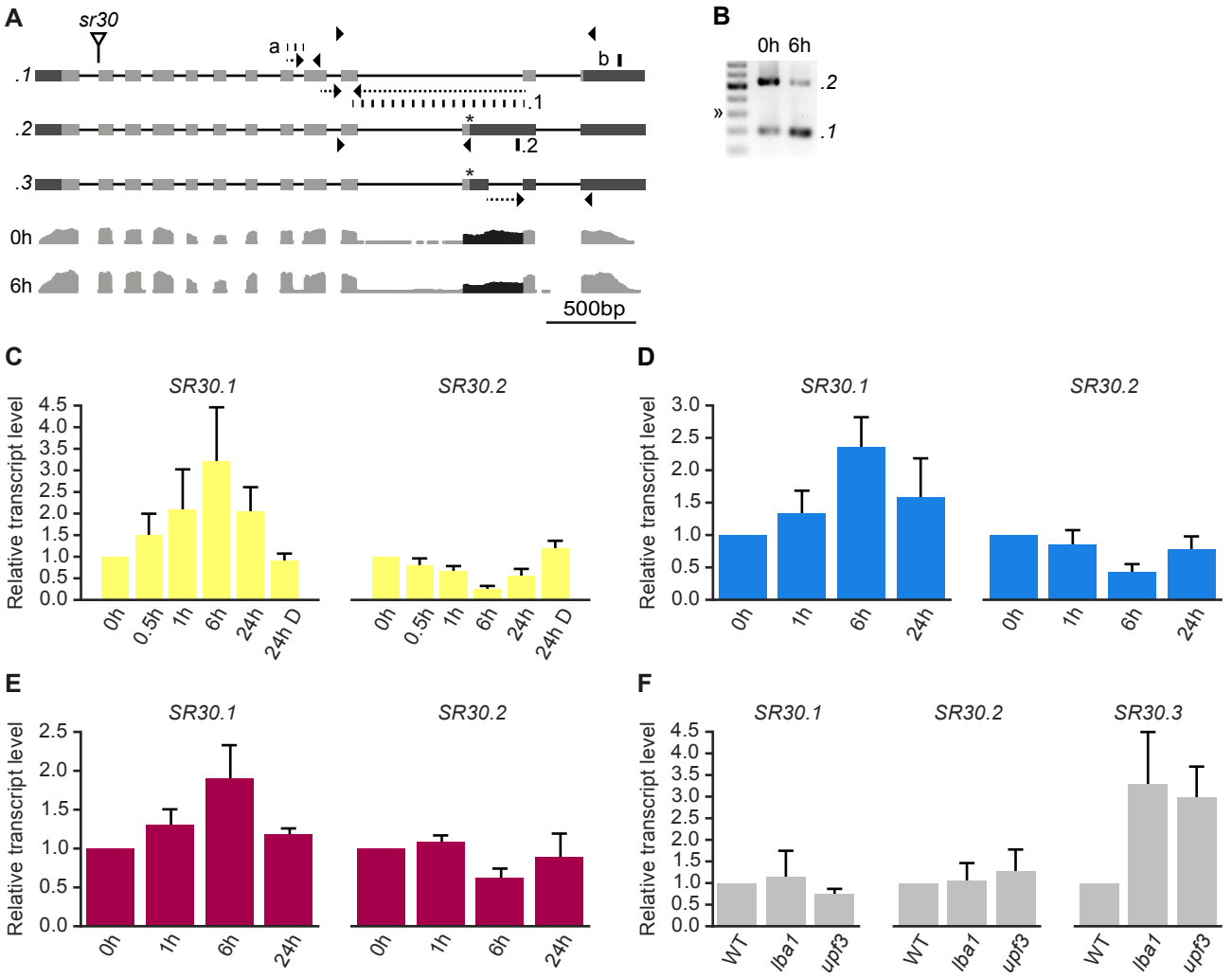


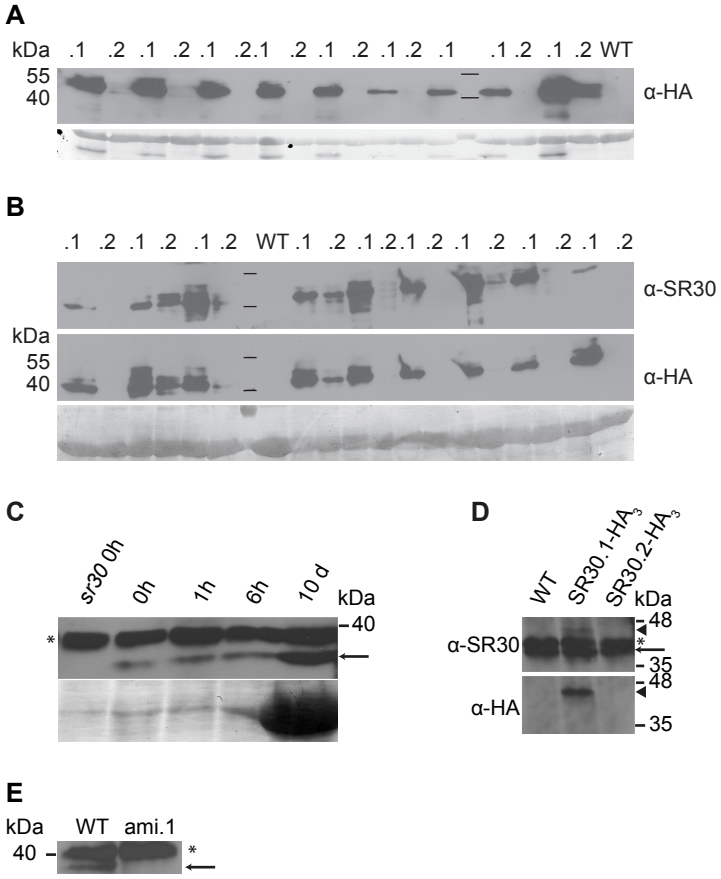
Figure 9: **Light-Regulated Alternative Splicing of *SR30* affects major variants in opposing directions. (A)** Gene models of relevant *SR30* splicing variants depicting exons as boxes and introns as lines. UTRs are dark grey. Below each splicing variant, the binding site of primers used for quantification in RT-qPCR is indicated by an arrowhead or, when exon border-spanning, arrow. The primer pair directly above the first variant was used to measure total transcript from the gene. The topmost pair are co-amplification primers used in downstream analyses. Arrowheads are not drawn to scale, thus cover a larger area than the actual binding site in the model. The asterisk marks the introduction of a PTC by the AS event. Small black boxes indicate amiRNA binding sites with strong dashed lines indicating splice junction-spanning binding sites (a, b, .1, .2). The triangle points at the T-DNA insertion site of *sr30*. Underneath the gene models, coverage plots are shown for RNA-seq results at 0 h and 6 h white light. **(B)** *SR30* splice variants were co-amplified in etiolated seedlings sampled at 0 h and 6 h light exposure and separated on an agarose gel. Primer positions are indicated in (A). The double arrowhead points at 300 bp of a DNA size ladder with 100 bp increments. **(C)** Splicing variants were quantified using RT-qPCR with the primers shown in A in samples exposed to white light as indicated. Levels are relative to total transcript of the gene and were normalised to the 0 h sample. D = dark, n = 3-7. **(D)**, **(E)** Splicing variants were quantified and normalised as in (C) in samples exposed to blue (D) or red (E) light for indicated periods. n = 3. **(F)** *SR30* variant transcript levels were quantified as in C in 10-d-old green seedlings of WT, *lba1*, or *upf3*. Levels are relative to WT and were normalised to total *SR30*. n = 3. All barplots are mean values + SD. For all light intensities see Figure 8.

Levels of the individual splicing variants were measured in the NMD-impaired mutants *lba1* and *upf3* (Figure 9F) and compared to wild type (WT). Neither of the two splicing variants had altered levels in the NMD factor mutants. To confirm NMD targets do accumulate in the mutants used, the additional minor splicing variant *SR30.3*, containing two introns in the 3' UTR, was analysed and showed elevated levels in both mutant lines. This finding suggested that both *SR30.1* and *SR30.2* could be translated into protein species, which are distinct at their C-termini and might have varying functions.

To further address the coding potential of the two splicing variants, they were transiently expressed in *Nicotiana benthamiana*. A protein corresponding to *SR30.1* was readily detectable in all samples, while for *SR30.2* no or a much weaker signal was observed (Figure 10A, B). This was consistently seen for constructs with and without the 3' UTR, suggesting varying stability or extractability of the two proteins account for the difference in signals rather than NMD. Generation of a polyclonal antibody allowed us to additionally analyse endogenous SR30 protein levels in *A. thaliana*. We were able to detect one specific signal for SR30, which was absent in both a T-DNA knockout and an artificial microRNA line (Figure 10C, E, G). SR30 levels were higher in light- than in dark-grown samples, however, no evidence for the presence of alternative proteins was found. Furthermore, upon constitutive expression of the two splicing variants in *A. thaliana*, again only SR30.1 but not SR30.2 was detectable (Figure 10D), despite high and comparable transcript levels for the two constructs (Figure 10G). All immunoblots using the SR30 antibody resulted in an additional signal for a protein slightly larger than SR30, which might correspond to the other members of the SR subfamily (SR34, SR34a, SR34b; Barta et al. 2010).

To address whether a potential alternative protein would differ in subcellular localisation or in *in vivo* accumulation we transiently co-expressed fluorescent fusion proteins of the splicing variants in Arabidopsis protoplasts without their endogenous UTRs to make translation more likely. Colocalisation of each SR construct with NLS-DsRed confirmed that both SR30.1 and SR30.2 are present in the nucleus (Figure 11A, B). Colocalisation experiments of the variants showed identical fluorescence patterns, regarding both compartmentation and fluorescence intensity (Figure 11C, D). Moreover, nuclei displaying speckle localisation for the reporter fusions had identical numbers and sizes of foci for the two SR30 variants (Figure 11E).

In a functional analysis of the SR30 variants, we tested each splice form's ability to affect the splicing pattern of a reporter construct based on the genomic sequence of *SR30* (Figure 12A). In a previous study, ectopic expression of *SR30* was found to alter the splicing outcome for the endogenous *SR30* gene [Lopato et al. 1999]. Accordingly, the AS output of the splicing reporter was changed upon co-expression of the CDS constructs in comparison to a neutral control protein (Figure 12B). *SR30* CDS co-expression caused a relative shift to the reporter variant equivalent to *SR30.2*, with the outcome being almost identical for the two CDS constructs.



G

Line	Total	.1	.2	Segr.
UTR-SR30.1-HA3-UTR #8	130.4	225.3	1.5	~1.3%
UTR-SR30.2-HA3-UTR #7	112.2	0.6	262.7	~2.5%
SR30.1-HA3 #8	5.2	84.3	0.6	0%
SR30.2-HA3 #15	18.9	0.8	81.2	~2.5%
ami.1 #2	0.34	0.12	0.92	NA

Figure 10: **Immunoblot Analyses Show Robust Detection of SR30.1 but not SR30.2.** (**A, B**) Transient overexpression of SR30 in *N. benthamiana* leaves. Leaf halves were infiltrated with Agrobacteria carrying constructs with SR30.1 or SR30.2, respectively, under the CaMV 35S promoter. Corresponding leaf halves infiltrated with .1 or .2 were loaded next to each other. WT is an uninflitrated leaf. As a loading control, the RUBISCO band stained by amido black ist shown. In (A) the constructs carried the splice form-specific CDS followed by an HA_3-tag without the endogenous UTRs and 15 µg of total protein was loaded in each lane. In (B) the constructs contained both endogenous UTRs in addition, and fresh weight equivalents were loaded. **(C)** Detection of endogenous SR30 protein in seedlings grown in darkness and exposed to light for the indicated time, or grown in white light for 10 d. Identical fresh-weight equivalents were loaded. The asterisk and arrow mark a cross-detection band and SR30, respectively. The lower panel shows the RUBISCO band from the stained membrane as loading control. Samples are WT unless indicated otherwise. **(D)** HA_3-tagged CDS constructs of the two major splice variants were overexpressed in WT, followed by immunoprecipitation from protein extracts of light-grown seedlings (10 d) using α-SR30. For detection, α-SR30 (upper panel) and α-HA (lower panel) antibodies were used. Identical fresh weight equivalents were loaded. The asterisk marks a cross-detection band, the arrow endogenous SR30, and the arrowhead tagged SR30. **(E)** Protein was extracted from 10-d-old WT seedlings or seedlings stably expressing an amiRNA directed specifically against *SR30.1*. SR30 was immunoprecipitated using the α-SR30 antibody. Fresh-weight equivalences were loaded in each lane. The arrow points at the SR30 band, the asterisk marks a cross-detection band. **(F)** The genomic sequence spanning the insertion site was amplified from *sr30* and sequenced from both ends. T-DNA sequence is shown in italics, sequence aligning to *SR30* is shown in bold. The sequence in green normal font cannot be assigned to either. Primer sequences are underlined. **(G)** *SR30* expression levels and segregation of lines used in (D), Figure 13, and (E). Levels of total transcripts and specific splice variants were measured using RT-qPCR with the primers indicated in Figure 9A. Measurements were normalised to *PP2A* levels. Segr. gives the percentage of dead seedlings when growing the lines on selective medium.

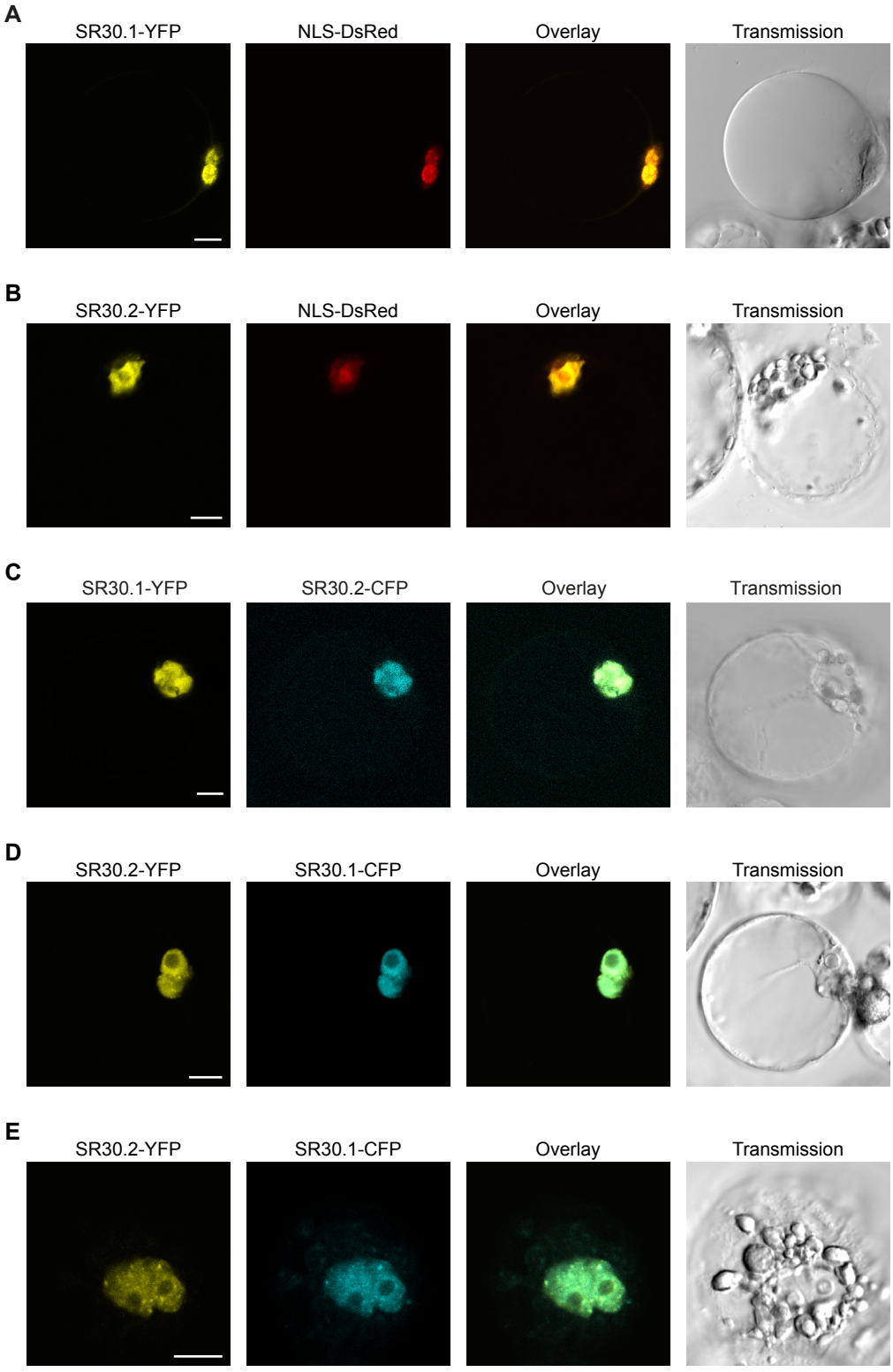

A
SR30.1-YFP
NLS-DsRed
Overlay
Transmission
B
SR30.2-YFP
NLS-DsRed
Overlay
Transmission
C
SR30.1-YFP
SR30.2-CFP
Overlay
Transmission
D
SR30.2-YFP
SR30.1-CFP
Overlay
Transmission
E
SR30.2-YFP
SR30.1-CFP
Overlay
Transmission

Figure 11: **SR30.1 and SR30.2 Fusion Proteins Show Identical Subcellular Localisation.** Confocal microscopy of *A. thaliana* protoplasts expressing NLS-DsRed and SR30.1-YFP **(A)**, NLS-DsRed and SR30.2-YFP **(B)**, SR30.1-YFP and SR30.2-CFP **(C)**, or SR30.2-YFP and SR30.1-CFP **(D, E)**. All bars represent 10 µm.

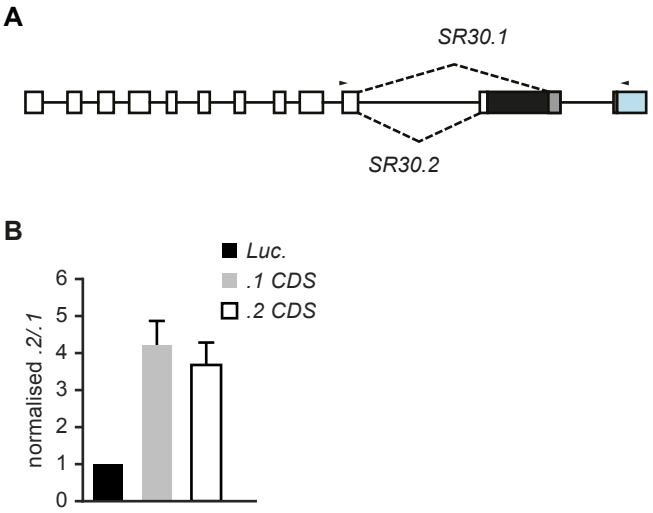


Figure 12: **SR30.1 and SR30.2 both affect splicing of an *SR30* reporter. (A)** Gene model of the reporter. Exons are shown as boxes, introns as lines. White: CDS, black: 3' UTR in SR30.2, grey: CDS in SR30.1 and 3' UTR in SR30.2, blue: HA-tag, arrowheads indicate primer positions for co-amplification of resulting splicing variants. Model drawn to scale except for the HA-tag. **(B)** The genomic *SR30* construct (A) was co-infiltrated either with splice-form-specific CDS constructs (*.1*/*.2* CDS) or control (*Luc.*) in *N. benthamiana* leaves. Splicing variants specifically from the genomic reporter were co-amplified and quantified using a Bioanalyzer. Splice form ratios were normalised to the levels measured for co-infiltrated control protein (Luciferase, Luc.). Mean values are displayed + SEM, n = 14-15.

Finally, to test for a possible role of SR30 in light signalling, we determined hypocotyl lengths of mutant seedlings either carrying a T-DNA knockout allele or constitutively expressing *SR30* under control of the CaMV 35S promoter (Figure 13). WT and mutant seedlings showed identical hypocotyl lengths at all light intensities tested. Again, there was no difference between seedlings overexpressing either SR30.1 or SR30.2. The absence of a phenotype under these conditions might be caused by a number of reasons: restriction of SR30 action in photomorphogenesis to the early signalling phase, functional redundancy between SR30 and related splicing factors, or an involvement of this AS event in a process other than light signalling, given its complex regulation under diverse conditions that has been described before [Lopato et al. 1999; Palusa et al. 2007; Tanabe et al. 2007].

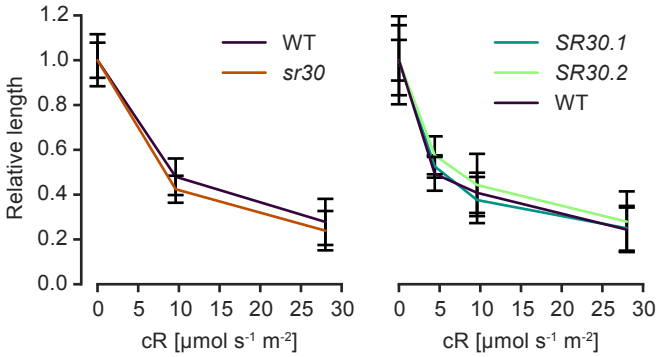


Figure 13: ***SR30* mis-expression does not alter hypocotyl length.** Etiolated seedlings were exposed to different intensities of red light for 6 d. Each experiment was done once with one line. *SR30.1* and *SR30.2* are overexpression lines, the constructs do not contain endogenous UTRs. For expression levels see Figure 10G. Lengths were normalised to the average length measured in darkness. n = 24-51 for the plot on the right, and 23-56 for the one on the left. Error bars are SD.

5.3 AS of *RRC1* is Light-Regulated and Promotes Expression of the Protein Coding Variant

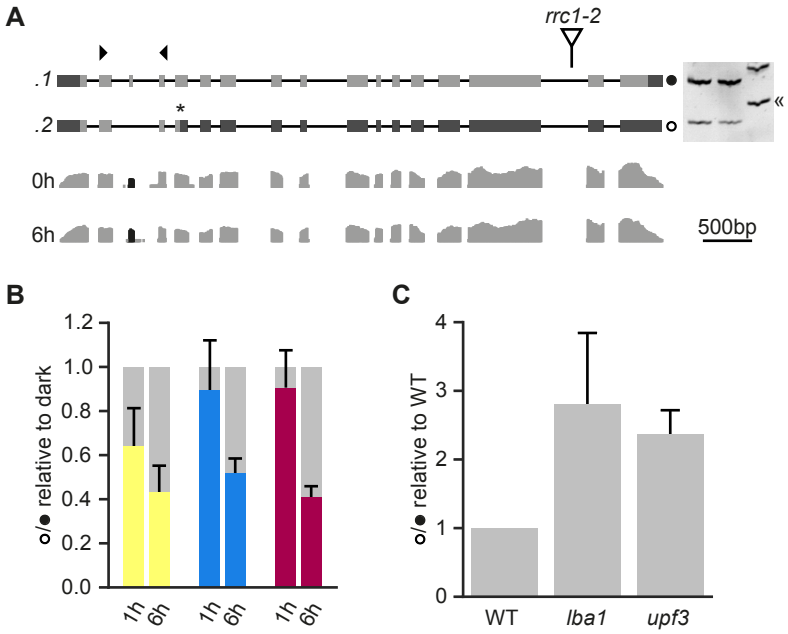


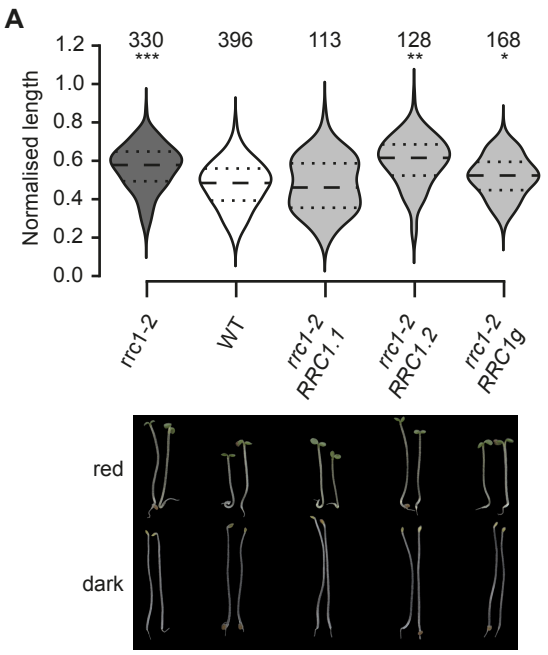
Figure 14: **Light Promotes Splicing of *RRC1* to the Protein-Coding Variant. (A)** Gene model of *RRC1* major splicing variants showing exons as boxes and introns as lines. UTRs are dark grey. The position of the co-amplification primers are given by arrowheads, and the position of the T-DNA in the *rrc1-2* mutant is indicated. The asterisk marks the introduction of a PTC by the AS event. The co-amplified PCR products in 0 h (left) and 6 h (right) samples separated on a gel are shown with the double arrowhead pointing at 100 bp of a 50 bp ladder. Below the gene model, coverage plots show representative RNA-seq results for a 0 h and 6 h sample. The alternatively spliced region is colored in black. **(B)** Confirmation of light-dependent AS under white (left), blue (middle), and red (right) light. Splicing variants were co-amplified from samples grown in darkness and collected at 0 h or after 1 h or 6 h exposure to light, and quantified using a Bioanalyzer. Bars give average splice form ratios calculated as shown by circles with the ratio in darkness set to 1. Error bars are + SD, n = 3. Light intensities: see Figure 8. **(C)** Splicing variants were co-amplified from etiolated WT, or indicated NMD-deficient mutants, and quantified as in (B). Ratio in WT is set to 1; error bars are + SD, n = 3.

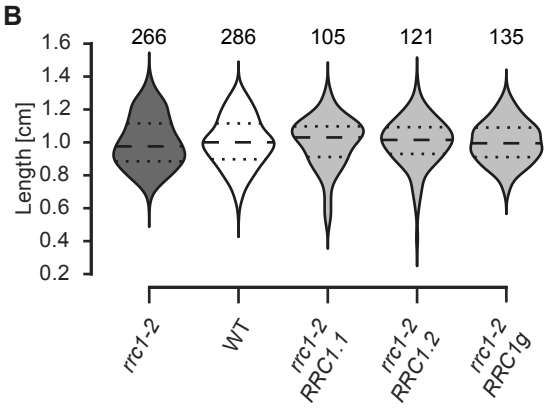
RRC1 is a putative splicing factor that has been described as a new component of PHYB-dependent signalling [Shikata et al. 2012b]. PHYB-dependent light signalling was impaired in *rrc1* mutants, and AS patterns of SR genes were altered [Shikata et al. 2012a]. The C-terminal RS domain was crucial to RRC1 function in light signalling [Shikata et al. 2012b], suggesting that regulating AS through its protein-protein interactions could be the central role of RRC1 in this context.

Based on our RNA-seq data, the third exon of RRC1 is alternatively spliced light-dependently (Figure 14A). We could validate a shift in splice ratios using RT-PCR upon exposure to red, blue and white light in favour of the inclusion variant (Figure 14B). The exclusion of the third exon in *RRC1.2* results in a frame shift leading to a PTC further downstream in the transcript. This hallmark of an NMD target and the accumulation of the isoform in two NMD-impaired mutants (Figure 14C) point to a regulation

of this variant by NMD.

To learn whether this light-dependent AS event is functionally relevant we complemented the *rrc1-2* knock-down mutant [Shikata et al. 2012b] with either splice variant or the gene. We measured hypocotyl lengths in T2 generation seedlings after growth in red light or darkness (Figure 15A-C). Hypocotyl lengths of seedlings grown in red light were normalised to the average hypocotyl length in darkness for each line to remove the impact of potential light-independent growth phenotypes. As previously reported [Shikata et al. 2012b], *rrc1-2* seedlings were elongated in red light compared to the WT. Complementation under the 35S promoter with the *RRC1.1* isoform, but not with the *RRC1.2* isoform, rescued the phenotype. Introducing the genomic construct also shortened the hypocotyls but did not fully complement the phenotype. The difference in lengths of complementing with *RRC1.1* or the genomic contruct may result from different levels of overexpression (Figure 15D). *RRC1* isoforms were specifically expressed in all transgenic lines, but the introduction of the genomic construct resulted in a massive overexpression compared to the moderate expression in the cDNA lines. To test if the strong constitutive promoter had an effect on hypocotyl lengths we also introduced the cDNA constructs into the WT background. All of these lines had hypocotyl lengths comparable to the WT (Figure 15E, F). Immunoblot analysis detected a protein corresponding to *RRC1.1* but none that could have been *RRC1.2*, further corroborating that the alternative variant is degraded via NMD (Figure 15G). As for the transcript levels, the amount of RRC1 protein detectable in the lines expressing the genomic constructs was much higher than in the lines expressing RRC1.1. This higher expression level could interfere with downstream signalling which in turn affects hypocotyl lengths. To remove this effect we complemented the *rrc1-2* mutant with constructs under the control of the putative endogenous promoter. The genomic construct fully rescued the phenotype (Figure 16A). Expression of the two splice variants, however, gave transcript levels that were substantially lower than in the WT; possibly because the introns were missing. Only in the line with the highest expression level of *RRC1.1* the phenotype was fully rescued (Figure 16C). Expression of *RRC1.2* again did not rescue the phenotype. In summary, light-dependent AS reinforces light signalling by increasing the level of functional RRC1.

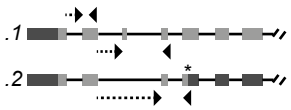




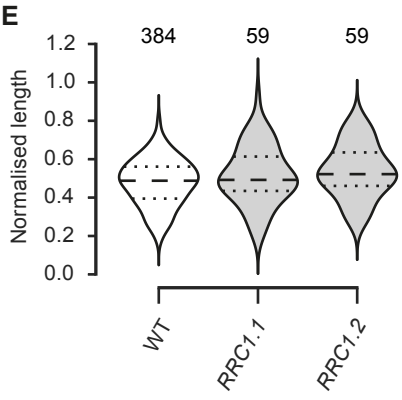
C

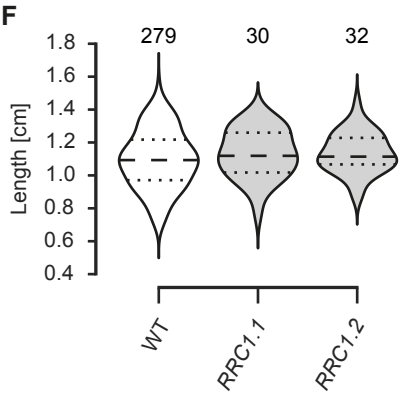
Line 1	Line 2	t-test	median test
rrc1-2	WT	1.58×10^{-22}	6.91×10^{-18}
rrc1-2	*rrc1-2 RRC1.1*	2.87×10^{-10}	5.36×10^{-06}
rrc1-2	*rrc1-2 RRC1.2*	8.60×10^{-03}	1.66×10^{-02}
rrc1-2	*rrc1-2 RRC1g*	3.10×10^{-05}	2.00×10^{-05}
WT	*rrc1-2 RRC1.1*	9.06×10^{-01}	5.25×10^{-01}
WT	*rrc1-2 RRC1.2*	4.19×10^{-21}	2.95×10^{-12}
WT	*rrc1-2 RRC1g*	5.80×10^{-05}	6.79×10^{-04}
rrc1-2 RRC1.1	*rrc1-2 RRC1.2*	3.17×10^{-11}	8.28×10^{-08}
rrc1-2 RRC1.1	*rrc1-2 RRC1g*	4.94×10^{-03}	1.58×10^{-01}
rrc1-2 RRC1.2	*rrc1-2 RRC1g*	1.92×10^{-08}	1.29×10^{-07}
WT	*RRC1.1*	3.61×10^{-02}	9.85×10^{-01}
WT	*RRC1.2*	9.23×10^{-04}	4.81×10^{-02}
RRC1.1	*RRC1.2*	4.28×10^{-01}	4.61×10^{-01}

D



Genotype	Line	.1	.2	Total	Segr.
rrc1-2 RRC1g	#4	66.2	31.2	44.1	15%
	#21	23.1	16.2	27.0	23%
	#25	123.2	92.9	167.9	18%
rrc1-2 RRC1.1	#1	12,2	0,7	10,7	14%
	#5	6.9	0.5	5.2	16%
	#34	14.9	1.4	16.5	20%
rrc1-2 RRC1.2	#7	0.6	3.8	1.3	2%
	#10	1.3	8.3	3.4	25%
	#23	1.3	14.8	4.9	17%
RRC1.1	#10	138.5	4.0	99.8	30%
RRC1.2	#17	0.4	194.8	47.4	8%





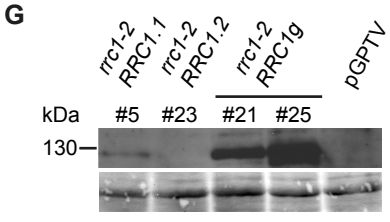
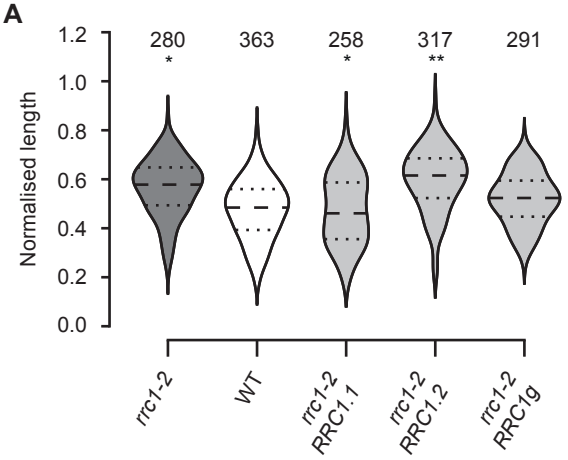


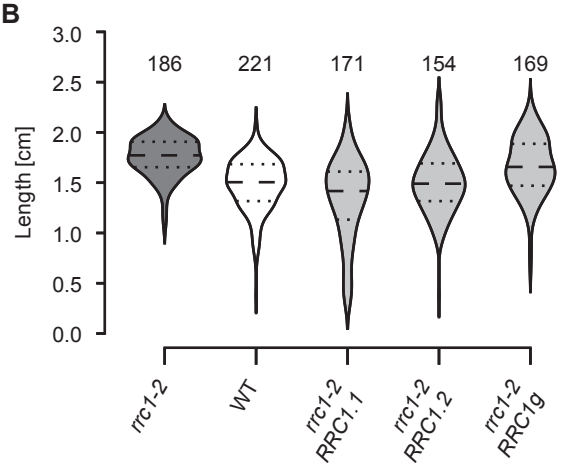
Figure 15: **Complementing *rrc1-2* with *RRC1.1* rescues the elongated hypocotyl phenotype. (A)** Violinplots showing the distribution of the relative hypocotyl lengths measured after growth in red light ($\sim$ 10 µmol m^{-2} s^{-1}) for *rrc1-2*, WT, and complementation lines (top). The dashed line represents the median, dotted lines the quartiles. All hypocotyls were normalised to the average length in darkness of each line. Complementation constructs express tagged splicing variants (*.1*, *.2*) or the genomic sequence (*g*) under the CaMV 35S promoter. Asterisks indicate p-values from Mood's median test compared to WT: * $p < 10^{-2}$, ** $p < 10^{-11}$, *** $p < 10^{-17}$. Exact p-values for all comparisons are provided in (C); n is indicated above each genotype. For each complementation construct, 3 independent F1/T2 lines were used. Bottom panel shows representative seedlings from hypocotyl assays in darkness and under red light. **(B)** Hypocotyl lengths of lines used in (A) grown in darkness. **(C)** p-values of pairwise comparisons between lines used in (A) and (E). **(D)** Partial gene model of *RRC1* showing the position of RT-qPCR primers. The pair above *.1* was used to amplify total *RRC1* transcript. The pairs shown below each variant were specific to the respective variant. Transcript levels were measured using RT-qPCR and normalised to the levels of a control line (pGPTV). Segregation (Segr.) shows percentage of dead seedlings for each line grown on selective medium. **(E)** Relative hypocotyl length of *RRC1* overexpression lines in WT background grown in red light. The length of each hypocotyl was normalised by the mean hypocotyl length in darkness. **(F)** Hypocotyl lengths of dark-grown seedlings corresponding to (E). **(G)** Western Blot of *RRC1* complementation lines. 20 µg total protein extract from dark-grown seedlings were loaded in each lane. The transgene was detected using α-Flag. The stained membrane is shown as loading control.

A



Line 1	Line 2	Median test
rrc1-2	WT	1.33×10^{-08}
rrc1-2	*rrc1-2 RRC1.1*	8.50×10^{-02}
rrc1-2	*rrc1-2 RRC1.2*	8.87×10^{-04}
rrc1-2	*rrc1-2 RRC1g*	9.93×10^{-09}
WT	*rrc1-2 RRC1.1*	1.00×10^{-07}
WT	*rrc1-2 RRC1.2*	2.77×10^{-17}
WT	*rrc1-2 RRC1g*	8.75×10^{-01}

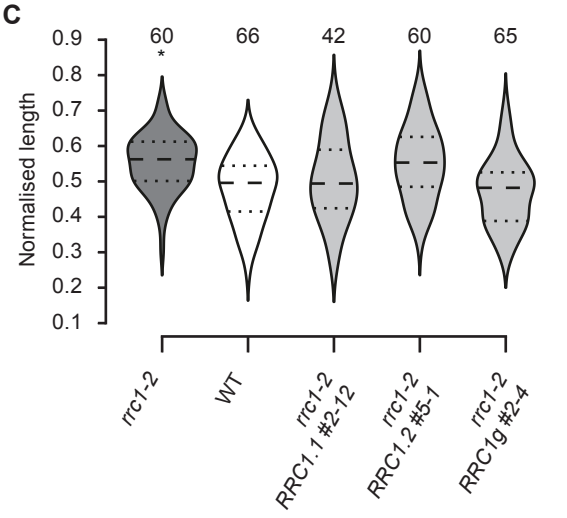
B



D

Genotype	Line	.1	.2	Total	WT	Segr.
	#1-4	2.3	4.4	2.8	2.1	22%
rrc1-2 RRC1g	#2-2	1.8	2.3	2.1	3.1	8%
	#2-4	4.8	5.2	3.9	3.5	12%
	#1-3	0.8	0.3	0.8	0.06	21%
	#2-6	1.0	0.5	1.1	0.08	17%
rrc1-2 RRC1.1	#2-8	0.8	0.3	0.8	0.05	22%
	#2-10	4.0	3.0	3.5	NA	0%
	#2-12	0.8	0.4	0.8	0.17	15%
	#3-6	0.7	1.5	0.9	0.18	26%
rrc1-2 RRC1.2	#4-4	0.5	3.5	1.0	0.58	9%
	#5-1	0.5	2.3	0.9	0.20	25%

C



Line 1	Line 2	Median test
rrc1-2	WT	2.43×10^{-03}
rrc1-2 RRC1.1	WT	1.00
rrc1-2 RRC1.2	WT	4.97×10^{-02}
rrc1-2 RRC1g	WT	3.36×10^{-01}

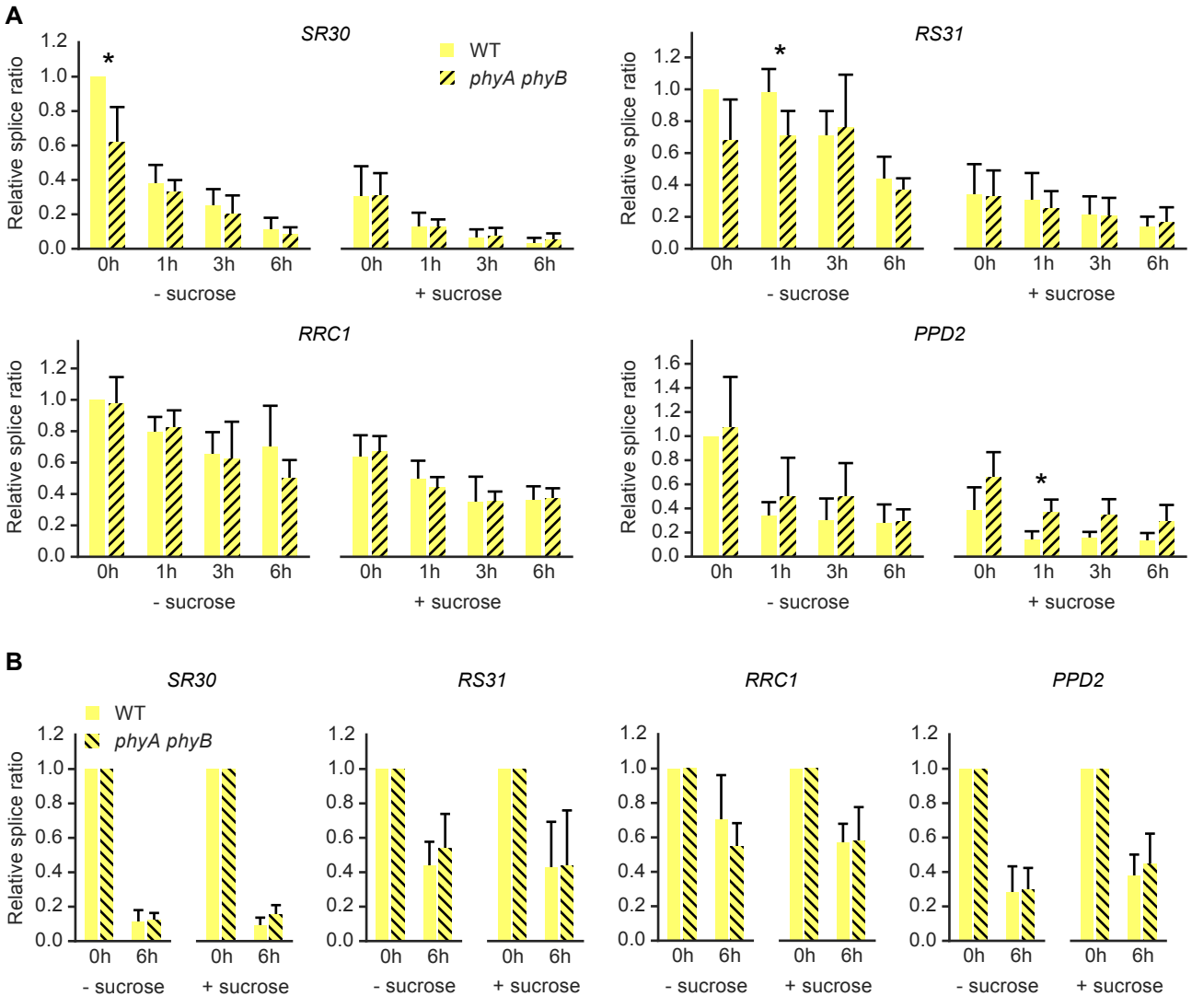
Figure 16: **Complementation of the *rrc1-2* Mutant Using Constructs under Control of the Endogenous Promoter.** **(A)** Violin plots depicting relative hypocotyl lengths of lines grown in red light (~ 10 µmol m^{-2} s^{-1}). The length of each hypocotyl was normalised by the mean hypocotyl length in darkness. Replicate numbers are provided above the plot. p-values for pair-wise comparisons of hypocotyl lengths using Mood's median test are shown on the right. Data from 3 independent F1 lines per construct, analysed in 4-5 independent experiments. **(B)** Violin plots showing hypocotyl length distribution of dark-grown seedlings of the different genotypes in (A). **(C)** Violin plots depicting relative hypocotyl length of individual lines grown in red light. This plot is a subset of the data shown in (A), including the *rrc1-2 RRC1.1#2-12* and other lines that were grown in parallel. Other details as described in legend to (A). **(D)** Transcript levels of *RRC1.1* (*.1*), *RRC1.2* (*.2*), total *RRC1* (total), and total *RRC1* from the non-mutated allele (WT, specific for WT allele and complementation constructs; does not detect T-DNA allele) in the complementation lines used for the hypocotyl assays. Transcript levels were measured using RT-qPCR and normalised to the levels of a control (mean of WT and empty vector control, pGPTV). Segregation (Segr.) shows percentage of dead seedlings grown on selective medium. The dashed lines represent the medians, dotted lines the quartiles. Asterisks indicate p-values from Mood's median test compared to WT: * $p < 10^{-2}$, ** $p < 10^{-11}$. Hypocotyl assays and RT-qPCRs performed by Theresa Wießner. *Partially from:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

5.4 Photoreceptor Mutant *phyA phyB* is Hardly Affected in Light-Dependent AS

Recent publications on light-dependent AS have come to different conclusions concerning the involvement of photoreceptors in eliciting changes to splicing patterns. A study on *A. thaliana* leaves incubated in light or darkness found changes in AS to be independent of photoreceptors, and, instead, a result of retrograde signalling [Petrillo et al. 2014]. Similarly, splicing of *SR30* changed in green plants upon a light signal during the night without detectable contributions of the photoreceptors [Mancini et al. 2015]. In contrast, a study in *A. thaliana* seedlings [Shikata et al. 2014] and another in *Physcomitrella patens* [Wu et al. 2014] using red light found evidence for a major role of phytochrome photoreceptors in light-triggered AS. It is possible that these seemingly antithetic results stem from different experimental settings. To see how the results from our experimental setup fit into this controversy, we illuminated etiolated *phyA phyB* double mutants with white light and compared changes in AS of four candidates to those in WT (Figure 17A, Table 4). In all cases, the patterns were very similar between WT and the mutant. For single events and time points we found statistically significant differences between WT and *phyA phyB*, but these did not correlate with the overall response to light. Intriguingly, growing seedlings on sucrose-containing medium affected the AS patterns similarly as light exposure did. The relative change in AS ratio, however, was the same under either growth condition and for either line (Figure 17B, C).

White light appeared to affect AS independently of the major red light photoreceptors PHYA and PHYB. Since white light can induce light signalling through red and blue light receptors, we next illuminated etiolated WT and mutant seedlings with red light. The splicing pattern of *SR30* changed red light-dependently in both WT and *phyA phyB* seedlings (Figure 18A, B), with and without external sugar supply. This effect was slightly more pronounced in the WT compared to *phyA phyB*, but not statistically significantly so in most samples (Table 4). As observed in the white light experiments, sugar supply resulted in a general AS ratio shift, yet did not alter the final relative AS change in response to light. Overall the results suggest there is only a minor contribution from phytochromes to white and red light-dependent AS and point towards the existence of another signalling pathway. As suggested by Petrillo et al. [2014], such a signal could be associated with photosynthesis. In this case, irradiation with far-red light should result in more pronounced differences between the two lines, as photosynthesis and related signalling are inactive under this condition.

Exposing etiolated WT seedlings to far-red light for 6 h resulted in a much weaker AS ratio change for *SR30* compared to the effects seen with red or white light (Figure 18C). Almost no response was visible in the *phyA phyB* double mutant, showing that AS changes are PHYA/B dependent under this light regime. Overall, our data indicate that PHYA and PHYB play only a minor role in the AS ratio



C

Sucrose	SR30 WT	SR30 *phyA phyB*	RS31 WT	RS31 *phyA phyB*	RRC1 WT	RRC1 *phyA phyB*	PPD2 WT	PPD2 *phyA phyB*
-	0.11	0.12	0.44	0.54	0.70	0.55	0.28	0.30
+	0.09	0.16	0.43	0.44	0.57	0.58	0.38	0.45

Figure 17: **White Light Triggers Similar AS Changes in WT and *phyA phyB* Seedlings. (A)** Etiolated seedlings grown on plates with or without 2 % sucrose for 6 d were exposed to white light for the indicated periods. Splice variants were co-amplified and quantified using a Bioanalyzer. Ratios *.2/.1* were calculated and normalised to the one measured for WT 0 h on plates without sucrose. Displayed are mean values + SD. n = 5-7 (- sucrose) and 4-7 (+ sucrose). p-values * < 0.01, comparing WT and *phyA phyB* in an independent t-test, or, if WT is set to 1, in a 1-sample t-test. A list of all p-values is provided in Table 4. **(B)** Based on the same data as (A), light-dependent AS in WT and *phyA phyB* were normalised to their respective 0 h sample. Splice form ratios (*.2/.1*) were normalised to WT 0 h and *phyA phyB* 0 h, respectively, for growth on sucrose and without sucrose separately. **(C)** For direct comparison, all mean values at 6 h normalised to the respective 0 h value are given. One biological replicate provided by Theresa Wießner.

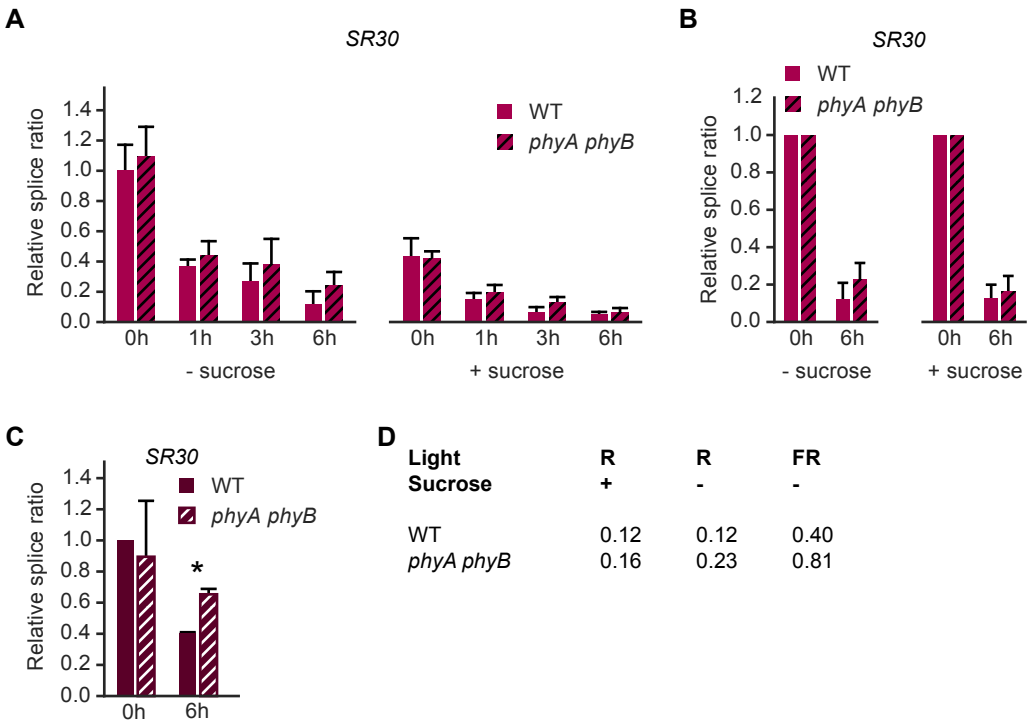


Figure 18: **Red Light also Triggers Similar AS Changes in WT and *phyA phyB* Seedlings.** Etiolated seedlings grown on plates with (A, B) or without (A, B, C) 2 % sucrose for 6 d were exposed to red **(A)**, or far-red **(C)** light for the indicated periods. Splice variants were co-amplified and quantified using a Bioanalyzer. Ratios .2/.1 were calculated and normalised to the one measured for WT 0 h on plates without sucrose. In (A), ratios were normalised to the mean value of the WT replicates at 0 h (- sucrose). Displayed are mean values + SD (A: n = 4; C: n = 3). p-values * < 0.01, comparing WT and phyA phyB in an independent t-test, or, if WT is set to 1, in a 1-sample t-test. A list of all p-values is provided in Table 4. **(B)** Based on the same data as (A), light-dependent AS in WT and *phyA phyB* were normalised to their respective 0 h sample. Splice form ratios (.2/.1) were normalised to WT 0 h and phyA phyB 0 h, respectively, for growth on sucrose and without sucrose separately. **(D)** For direct comparison, all mean values at 6 h normalised to the respective 0 h value are given. Data in (A) provided by Theresa Wießner.

shifts observed in white and red light.

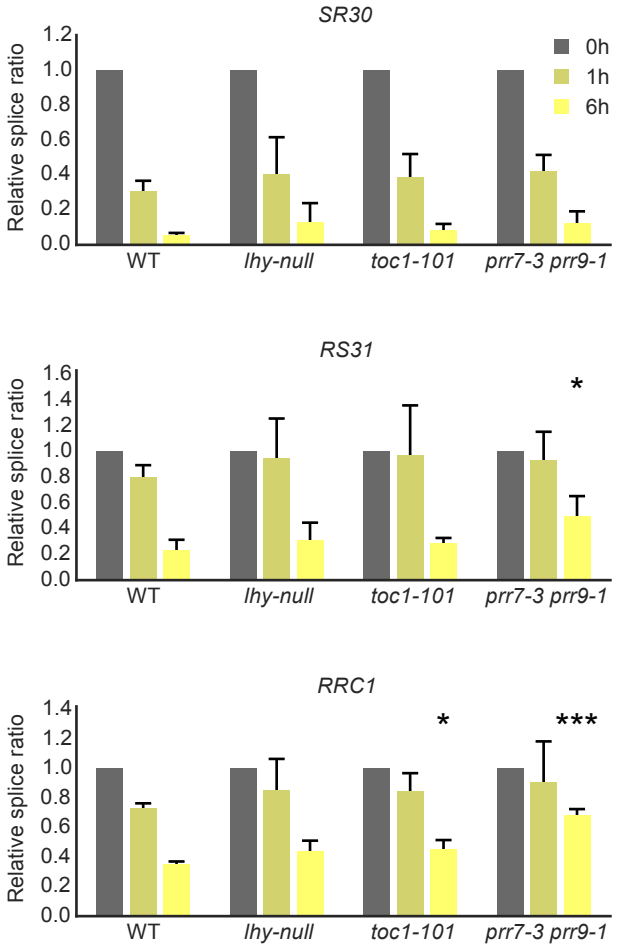
Table 4: **p-values for all comparisons WT versus *phyA phyB* shown in Figures 17 and 18.** Suc. = sucrose, Exp. = exposure, 1sam. = 1-sample t-test, indep = independent t-test.

Gene	Light	Suc.	Exp.	p-value	Test
SR30	FR	-	0h	0.6628	1sam.
SR30	FR	-	6h	0.0024	indep
SR30	R	+	0h	0.8180	indep
SR30	R	+	1h	0.2450	indep
SR30	R	+	3h	0.0369	indep
SR30	R	+	6h	0.3164	indep
SR30	R	-	0h	0.5134	indep
SR30	R	-	1h	0.2088	indep
SR30	R	-	3h	0.3125	indep
SR30	R	-	6h	0.0853	indep
SR30	W	+	0h	0.9271	indep
SR30	W	+	1h	0.9793	indep
SR30	W	+	3h	0.7276	indep
SR30	W	+	6h	0.2943	indep
SR30	W	-	0h	0.0026	1sam.
SR30	W	-	1h	0.3327	indep
SR30	W	-	3h	0.4110	indep
SR30	W	-	6h	0.3935	indep
RS31	W	+	0h	0.8797	indep
RS31	W	+	1h	0.5760	indep
RS31	W	+	3h	0.9603	indep
RS31	W	+	6h	0.5863	indep
RS31	W	-	0h	0.0163	1sam.
RS31	W	-	1h	0.0061	indep
RS31	W	-	3h	0.7492	indep
RS31	W	-	6h	0.2720	indep
RRC1	W	+	0h	0.6147	indep
RRC1	W	+	1h	0.3752	indep
RRC1	W	+	3h	0.9842	indep
RRC1	W	+	6h	0.7409	indep
RRC1	W	-	0h	0.7312	1sam.
RRC1	W	-	1h	0.6418	indep
RRC1	W	-	3h	0.7845	indep
RRC1	W	-	6h	0.1123	indep
PPD2	W	+	0h	0,0395	indep
PPD2	W	+	1h	0,0047	indep
PPD2	W	+	3h	0,0406	indep
PPD2	W	+	6h	0,0336	indep
PPD2	W	-	0h	0,7079	1sam.
PPD2	W	-	1h	0,2758	indep
PPD2	W	-	3h	0,2343	indep
PPD2	W	-	6h	0,8751	indep

5.5 No Role for Circadian Regulators in Changing Alternative Splicing

As light exposure of etiolated seedlings will also entrain the circadian clock, and AS and the circadian clock have been linked in previous reports [Sanchez et al. 2010; James et al. 2012; Filichkin et al. 2015; Mancini et al. 2015], we tested light-dependent changes to AS in several mutants with defects in circadian components (Figure 19). In case of the *prr7-3 prr9-1* double mutant we found a slightly weaker change in AS for *RRC1*, in the other cases there was no difference in comparison to the WT. This indicates that circadian regulators do not majorly contribute to these AS changes early in photomorphogenesis.

A



B

Gene	Line	Time	p-value
SR30	*lhy-null*	1h	0.4056
SR30	*prr7-3 prr9-1*	1h	0.0883
SR30	*toc1-101*	1h	0.3170
SR30	*lhy-null*	6h	0.2629
SR30	*prr7-3 prr9-1*	6h	0.0927
SR30	*toc1-101*	6h	0.2277
RS31	*lhy-null*	1h	0.4204
RS31	*prr7-3 prr9-1*	1h	0.3367
RS31	*toc1-101*	1h	0.4360
RS31	*lhy-null*	6h	0.4014
RS31	*prr7-3 prr9-1*	6h	0.0254
RS31	*toc1-101*	6h	0.3312
RRC1	*lhy-null*	1h	0.2749
RRC1	*prr7-3 prr9-1*	1h	0.2354
RRC1	*toc1-101*	1h	0.1005
RRC1	*lhy-null*	6h	0.0606
RRC1	*prr7-3 prr9-1*	6h	5.91×10^{-6}
RRC1	*toc1-101*	6h	0.0192

Figure 19: **Circadian Regulators Do Not Majorly Influence Light-Dependent Alternative Splicing of Select Candidates. (A)** 6-d-old etiolated seedlings were exposed to white light for the indicated periods. Co-amplified splicing variants were quantified on a Bioanalyzer. Ratios *.2/.1* were calculated and normalised to each 0 h value. n = 4, error bars are + SD. p-values * < 0.05, *** < 0.001 from independent t-tests against the ratio in WT, values given in **(B)**. *From:* Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. (www.plantcell.org, Copyright American Society of Plant Biologists).

5.6 Light Exposure and Sugar Supply both Change Alternative Splicing Patterns

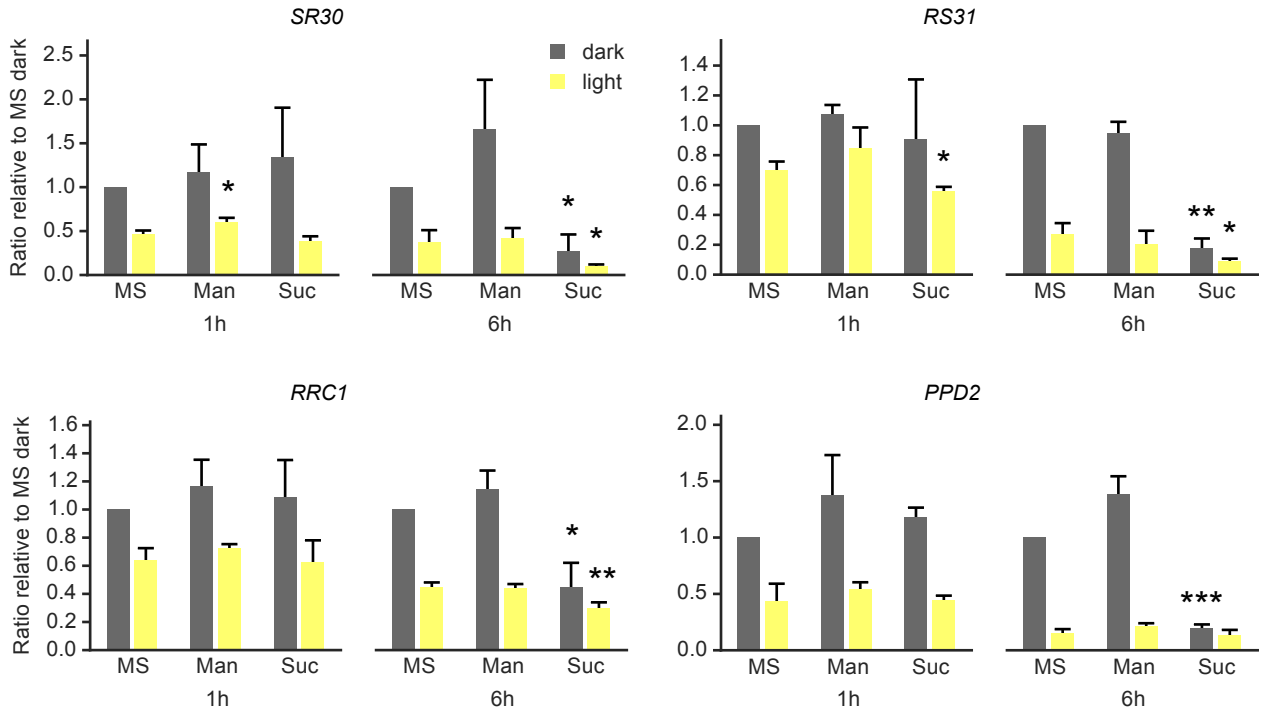
As mentioned above, not only light but also sucrose supplementation influenced splicing outcome. Growth on sucrose-containing medium in darkness shifted AS patterns into the same direction as light exposure did for seedlings grown without sugar and exposed to light for 6 h (Figures 17, 18). To investigate the interplay of light and sugar we grew etiolated seedlings on sucrose-free medium, transferred them to liquid medium with or without sugar under green light, and exposed them to white light or kept them in darkness. An additional control with an equimolar concentration of mannitol was included to control for a potential osmotic effect. After 1 h in darkness, we found no significant difference in AS between the samples from the three media (Figure 20A). In light, the splice ratio was shifted in all samples after 1 h. After 6 h, the sample kept in darkness in sucrose and the one exposed to light without sucrose showed similar AS changes. For *SR30* and *RS31* it seemed sugar and light had additive effects and caused a stronger shift in splice ratio, but the difference was not statistically significant (Figure 20B, C).

5.6.1 Expression patterns of SnRK1 targets correlate with changes in alternative splicing upon light or sucrose exposure

Activation of photosynthesis and sugar feeding both affect energy levels, thus sensors of the energy status could be integrators of the respective signals, leading to downstream changes in AS. Both HEXOKINASE 1 (HXK1) and SUCROSE-NON-FERMENTATION1-RELATED KINASE 1 (SnRK1) have been described as independently acting plant energy sensors [Rolland et al. 2006; Sheen 2014]. HXK1 senses glucose levels independent from its metabolic functions [Moore et al. 2003] and affects gene expression [Sheen 2014]. SnRK1 is activated when the supply in energy decreases [Crozet et al. 2014; Baena-González and Hanson 2017], changing gene expression [Baena-González et al. 2007] and adjusting metabolism [Sugden et al. 1999; Harthill et al. 2006] among other effects [Crozet et al. 2014; Hulsmans et al. 2016; Broeckx et al. 2016].

In order to test for a potential role of these two energy sensors in light- and sugar-dependent AS, we measured transcript levels of *HXK1*, *CAB1*, a gene known to be induced by light [Brusslan and Tobin 1992], and *DARK INDUCED (DIN) 1* and *DIN6*, both targets of SnRK1 [Baena-González et al. 2007] in seedlings incubated in control or supplemented medium with or without exposure to 6 h light (Figure 21). *HXK1* levels were unresponsive under both sugar and light exposure. *CAB1* levels were only increased by light. *DIN1* and *DIN6*, as previously reported [Thum et al. 2003; Baena-González et al. 2007], showed reduced transcript levels in the presence of either sugar or light, with the strongest

A



B

Gene	Treatment	Time	p-value	Test
SR30	MS	1h	2.77×10^{-03}	1samp
SR30	MS	6h	1.55×10^{-02}	1samp
SR30	Mannitol	1h	4.11×10^{-02}	indep
SR30	Mannitol	6h	2.03×10^{-02}	indep
SR30	Sucrose	1h	4.22×10^{-02}	indep
SR30	Sucrose	6h	2.07×10^{-01}	indep
RS31	MS	1h	1.18×10^{-02}	1samp
RS31	MS	6h	3.47×10^{-03}	1samp
RS31	Mannitol	1h	5.76×10^{-02}	indep
RS31	Mannitol	6h	4.09×10^{-04}	indep
RS31	Sucrose	1h	2.00×10^{-01}	indep
RS31	Sucrose	6h	6.69×10^{-02}	indep
RRC1	MS	1h	1.84×10^{-02}	1samp
RRC1	MS	6h	1.16×10^{-03}	1samp
RRC1	Mannitol	1h	1.65×10^{-02}	indep
RRC1	Mannitol	6h	8.69×10^{-04}	indep
RRC1	Sucrose	1h	6.22×10^{-02}	indep
RRC1	Sucrose	6h	2.04×10^{-01}	indep
PPD2	MS	1h	3.45×10^{-02}	1samp
PPD2	MS	6h	3.98×10^{-04}	1samp
PPD2	Mannitol	1h	5.50×10^{-02}	indep
PPD2	Mannitol	6h	3.69×10^{-04}	indep
PPD2	Sucrose	1h	3.61×10^{-03}	indep
PPD2	Sucrose	6h	2.51×10^{-01}	indep

C

Gene	Treatment		Time	p-value	Test
SR30	Mannitol	dark	1h	4.80×10^{-01}	1samp
SR30	Sucrose	dark	1h	4.00×10^{-01}	1samp
SR30	Mannitol	light	1h	3.14×10^{-02}	indep
SR30	Sucrose	light	1h	1.69×10^{-01}	indep
SR30	Mannitol	dark	6h	1.81×10^{-01}	1samp
SR30	Sucrose	dark	6h	2.27×10^{-02}	1samp
SR30	Mannitol	light	6h	6.84×10^{-01}	indep
SR30	Sucrose	light	6h	2.59×10^{-02}	indep
RS31	Mannitol	dark	1h	1.57×10^{-01}	1samp
RS31	Sucrose	dark	1h	7.31×10^{-01}	1samp
RS31	Mannitol	light	1h	1.61×10^{-01}	indep
RS31	Sucrose	light	1h	1.83×10^{-02}	indep
RS31	Mannitol	dark	6h	3.48×10^{-01}	1samp
RS31	Sucrose	dark	6h	1.88×10^{-03}	1samp
RS31	Mannitol	light	6h	3.89×10^{-01}	indep
RS31	Sucrose	light	6h	1.48×10^{-02}	indep
RRC1	Mannitol	dark	1h	2.72×10^{-01}	1samp
RRC1	Sucrose	dark	1h	6.40×10^{-01}	1samp
RRC1	Mannitol	light	1h	1.82×10^{-01}	indep
RRC1	Sucrose	light	1h	9.06×10^{-01}	indep
RRC1	Mannitol	dark	6h	2.02×10^{-01}	1samp
RRC1	Sucrose	dark	6h	3.09×10^{-02}	1samp
RRC1	Mannitol	light	6h	8.09×10^{-01}	indep
RRC1	Sucrose	light	6h	8.96×10^{-03}	indep
PPD2	Mannitol	dark	1h	2.22×10^{-01}	1samp
PPD2	Sucrose	dark	1h	2.07×10^{-01}	1samp
PPD2	Mannitol	light	1h	4.33×10^{-01}	indep
PPD2	Sucrose	light	1h	8.29×10^{-01}	indep
PPD2	Mannitol	dark	6h	5.65×10^{-02}	1samp
PPD2	Sucrose	dark	6h	1.21×10^{-03}	1samp
PPD2	Mannitol	light	6h	6.15×10^{-02}	indep
PPD2	Sucrose	light	6h	9.04×10^{-01}	indep

Figure 20: **Sucrose and Light Cause Comparable Alternative Splicing Shifts. (A)** Seedlings were grown in darkness and incubated in control medium (MS), mannitol (Man), or sucrose (Suc) solutions for 1 h or 6 h in darkness or light. Alternative splice forms were co-amplified and quantified using a Bioanalyzer. Ratios (*.2/.1*) were calculated as before for each candidate, and normalised to the control sample in darkness. Displayed are mean values + SD, n = 3. p-values: * < 0.05, ** < 0.01, *** < 0.001 comparing Man and Suc to the MS light and dark sample, respectively. **(B)** p-values of comparing light versus dark samples. **(C)** p-values of comparing sugar versus MS control treatment, as indicated in (A). Tests are independent t-tests (indep) when not tested against 1, and 1-sample t-test (1samp) when tested against 1.

decrease when both sugar and light were present. This pattern correlates perfectly with the observed changes in AS ratios, suggesting a coupling of SnRK1-mediated energy signalling and light-dependent AS.

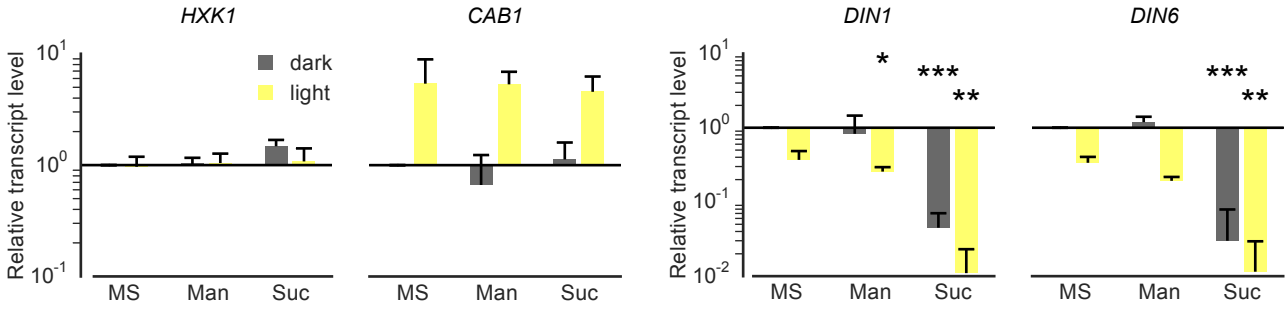


Figure 21: **Expression of Targets of SnRK1 Correlates with Changes in Alternative Splicing.** RT-qPCR analysis of transcript levels for genes involved in sensing the energy status after 6 h exposure. Sample description and data normalisation, depiction, and statistical analysis as described in Figure 20A. Data is displayed on a log scale. RT-qPCR run by Theresa Wießner on my samples.

6 Discussion

6.1 Total Transcript Levels and AS Patterns Change During Photomorphogenesis

Studies using microarrays to examine rice and *A. thaliana* transcriptomes described differences in expression for about 20 % of their genomes in seedlings undergoing skoto- or photomorphogenesis [Jiao et al. 2005]. Here, we used RNA-seq to compare the transcriptomes of skotomorphogenic seedlings with and without exposure to red, blue, or white light, and found 18.4 % of the expressed genes to change in total transcript level by at least two-fold after 6 h in one or several light conditions. Thus, the extent of transcriptomic adaptation appears to be roughly comparable between continuous growth in darkness or light and during the transition from darkness to light. However, while expression data from microarray and RNA-seq analyses are highly correlated when compared directly, the results from these two methods do differ with numerous changes in expression being only detected by one of the methods [Marioni et al. 2008; Sekhon et al.; Xu et al. 2013; Perkins et al. 2014; Zhao et al. 2014]. Furthermore, the numbers stated above are relative to the genome and expressed genes, respectively, so there may actually be larger difference than is apparent from these numbers.

Blue and red light changed the transcription of genes similarly. The numbers are comparable between the two colours, with a large overlap in affected genes. This is in accordance with previous studies reporting that expression of only a few genes is specifically controlled by monochromatic light [Ma et al. 2001; Jiao et al. 2005]. We also found many of these genes to be affected by white light. In addition, white light seemed to change expression of a large number of genes seemingly uninfluenced by blue or red light illumination alone, which is likely explained by the higher light intensity used with white light to follow standard growth conditions, but possibly also by activating both red and blue light signalling pathways which could be necessary to elicit significant changes in transcript levels for some genes.

Our RNA-seq data was also analyzed with regard to changes in AS at the onset of photomorphogenesis. After 6 h of light exposure, we found 700 AS events changing significantly in at least one light condition based on an FDR ≤ 0.1. Requiring at least 5 % change in SI (see Section 5.1.1 for definition) and thus removing events with insufficiently covered splicing variants and those with large variation between replicates resulted in 179 events. Of these, most events were affected under all three light conditions, indicating AS is regulated by light in general and not mainly by a specific light colour. However, we also found that red light can be less effective in triggering changes to AS compared to blue or white light, shown both by a lower median SI change and in independent experimental validations of selected AS events. It is possible that the differences we observed are due to the different

light intensities we applied. The conditions chosen for blue and red light should saturate effects on hypocotyl elongation [Young et al. 1992]. We cannot exclude, however, that there are additional or stronger effects on AS to be found under higher light intensities. A corresponding difference in changing total expression of genes between red and blue light was not present, excluding that the red light signal was generally too weak or the signalling too inefficient to trigger proper responses. Comparing genes affected in their total transcript levels with the ones with changing AS patterns showed that these two mechanisms often target different genes. The subset of genes underlying both AS and, independent of that, transcriptional control is likely even smaller than the overlap we report here, since changes to the generation of NMD targets as a result of AS will affect total transcript levels.

Looking into a potential role for coupled AS-NMD in early photomorphogenesis we analysed what types of splicing are prevalent and how light affects the production of potential NMD targets. Overall, the use of alternative 3' splice sites was the most frequent type of AS in our data set. This is in line with previous studies using the same analysis pipeline [Rühl et al. 2012; Drechsel et al. 2013], but also a study using a different analysis [Mancini et al. 2015], and, in addition, is the most common type found in the TAIR10 annotation [Hartmann et al. 2016]. In contrast, a 2012 survey of AS in *A. thaliana* described intron retention as the prevalent AS type [Marquez et al. 2012]. The discrepancy can likely be ascribed to differences in variant definition and to the inclusion of low abundant isoforms with possibly no biological relevance. This is underlined by intron retention becoming the dominant type of AS when considering only significantly changing events in our datatset. Furthermore, Marquez et al. [2012] examined a mixture of flowers and green seedlings whereas we used etiolated seedlings. A study in maize has demonstrated that the prevalent AS type may be tissue-dependent by finding mutually exclusive exon inclusions to be the dominant type of AS in the endosperm while intron retentions were most common in five other tissues [Wang et al. 2016]. This finding exemplifies the limited comparability of data generated from different tissues, at least regarding frequencies of AS types.

We discovered an enrichment of cassette exon and intron retention events in light-regulated AS events, both of which often lead to the introduction of NMD-targeting features [Stauffer et al. 2010; Kalyna et al. 2012; Drechsel et al. 2013]. Illumination mostly led to a relative increase of the shorter transcript variant, i.e. cassette exons were skipped and regulated introns were spliced in light. Interestingly, increased intron retention in light, not splicing, has been described in *P. patens* [Wu et al. 2014]. Aside from the obvious biological differences between moss filaments and seedlings that may begin to explain the fundamental discrepancy, light-grown and darkness-adapted, not strictly etiolated protonemata were used in this study, further limiting the comparability to our study.

Given that we found increased splicing to the relatively shorter variants in cassette exon and intron retention events, we expected the number of potential NMD targets to decline upon light exposure. Indeed, light exposure changed AS in favour of transcripts without NMD features for 61.1 % out of

the light-regulated AS events involving an isoform with NMD features, which make up 77.2 % of all significant events. It seems that expression of many genes is restricted post-transcriptionally by their splicing outcome and released, possibly in a concerted manner, by light-triggered changes in AS leading to more stable and productive mRNA variants. This potential role for AS-NMD in photomorphogenesis adds to the growing list of processes with NMD involvement beyond mere RNA surveillance, such as immune responses to viral [Balistreri et al. 2014; Garcia et al. 2014] and bacterial [Gloggnitzer et al. 2014] infection, as well as salt stress [Drechsel et al. 2013], and temperature-mediated flowering time control [Sureshkumar et al. 2016]. However, future research will have to show for each case whether NMD is truly involved, or if a different process affects translation of the respective isoform, such as nuclear retention of unproductive transcripts arising from intron retention [Göhring et al. 2014] or use of alternative 3' splice sites [Hartmann et al. 2018]. This is highlighted by the different findings for our candidates *RRC1* and *SR30*. The splicing variant predominantly produced in darkness is likely unproductive and carries NMD-eliciting features in both cases, but the AS event in *RRC1* is regulated by coupled AS-NMD, while the one in *SR30* triggers nuclear retention of the alternative variant [Hartmann et al. 2018].

6.2 Light-Dependent Alternative Splicing Affects Expression of Splicing Regulator *SR30* and Light Signalling Component *RRC1*

The functional category 'RNA' was overrepresented among the genes exhibiting light-dependent AS, including many splicing regulators. Extensive AS of splicing regulators has been reported previously and can contribute to quantitative gene expression control when being coupled to NMD [Staiger et al. 2003; Schöning et al. 2008; Palusa and Reddy 2010; Wachter et al. 2012]. This has been shown, for example, for POLYPYRIMIDINE TRACT BINDING PROTEINS (PTB) 1 and PTB2, which shift splicing of their pre-mRNAs to NMD-sensitive variants in an auto- and crossregulatory feedback loop [Stauffer et al. 2010]. However, regulated AS may also increase functional diversity of splicing regulators, as has been showcased for *SR45* [Zhang and Mount 2009].

Splicing components from the family of SR proteins would be well suited to function as master regulators of light-responsive AS programmes. We indeed found altered gene expression and AS patterns for several SR genes upon illumination of etiolated seedlings. Previous studies have shown AS of SR transcripts in green plants to respond to changing light conditions [Petrillo et al. 2014; Mancini et al. 2015], and established the occurrence of complex AS outputs for most of the SR genes from *A. thaliana* with hormones and stresses influencing their AS outcomes [Palusa et al. 2007; Tanabe et al. 2007; Filichkin et al. 2010; Cruz et al. 2014]. Studies reporting developmental phenotypes of SR and SR-like misexpression lines [Lopato et al. 1999; Kalyna et al. 2003; Ali et al. 2007; Carvalho et al. 2010; Shikata

et al. 2012b; Yan et al. 2017] additionally indicated a role for members from the SR protein family in early plant development. Our data further support this notion.

6.2.1 *SR30* AS regulates gene expression independent of NMD

We studied the functional impact of a light-controlled AS event within *SR30* in more detail. The two major splicing variants *SR30.1* and *SR30.2* showed reciprocal changes in their steady state levels upon illumination of etiolated seedlings. This effect was detectable already after 0.5 h light exposure and reached its maximum between 6 and 24 h after onset of light. Usage of an upstream 3' splice site in *SR30.2* changes the 3' end of the coding sequence and introduces NMD target features, namely an extended 3' UTR and an intron positioned more than 50 nts downstream of the stop codon. In line with the presence of NMD-triggering features, a previous study showed a relative increase in the ratio of *SR30.2* to *SR30.1* in the NMD factor mutant *upf3* compared to WT [Palusa and Reddy 2010]. However, analysing the individual transcript levels in the same *upf3* mutant and additionally in a *upf1* mutant we did not find evidence for NMD targeting of *SR30.2*, while accumulation of a third minor splicing variant with an additional intron 110 nts downstream of the stop codon proved efficient NMD impairment and accumulation of target transcripts in the mutants. Differences in seedling age and growth conditions between the earlier study and ours affecting AS may contribute to these opposing findings. Furthermore, we separately quantified the *SR30* splicing variants, whereas the other study used co-amplification assays. We found slightly decreased levels of *SR30.1* in the *upf3* mutant compared to WT, which might play a part in the ratio change described by Palusa and Reddy [2010]. Other than stabilization of *SR30.2*, altered AS could result in this observed effect, which appears quite likely given the many different conditions that have been reported to affect AS of *SR30*. Finally, the half lives of *SR30.1* and *SR30.2* were found to differ substantially, with *.2* having a much longer half life than *.1* [Hartmann et al. 2018], further indicating that *.2* turnover is not a central parameter in determining *SR30* splice form ratios.

Instead of being degraded by the NMD pathway, *SR30.2* could either be translated to give rise to a protein variant of SR30, or it could be kept from being translated altogether and thus not be accessible to the NMD machinery. Immunoblot analyses resulted in a single band for endogenous SR30. While this may indicate that only one of the splicing variants is productive, it could also be explained by very similar sizes of the two variants, differences in their extractability, or low steady state levels of one of the proteins. Transient expression and stable overexpression in *N. benthamiana* and Arabidopsis, respectively, also led to consistent detection of the SR30.1 variant, while the .2 variant was not detectable in most cases. This was true irrespective of the presence or absence of the natural UTRs in the expression constructs, which further supports that NMD does not play a role in the fate of *SR30.2*.

We also expressed CDS-fluorescent protein fusions of either splicing variant in protoplasts to analyse potentially differing subcellular localisation patterns as the two protein variants differ in a few amino acids at the end of their C-terminal RS domain which has been suggested to define nuclear localisation of SR30 [Mori et al. 2012]. In confocal microscopy both variants were detectable, which could be due to low level artificial expression of SR30.2 protein from constructs lacking the natural UTRs. We found either splicing variant to localise to the nucleus *in vivo*. In some cases the protein accumulated in speckles, as SR30.1 has been shown to do previously when expressed under its endogenous promoter [Fang et al. 2004]. Both variants were also able to alter the splicing pattern of a reporter based on the *SR30* pre-mRNA, in line with a previous report of a feedback control mechanism [Lopato et al. 1999]. Possible translation of *SR30.2* is further supported by a study suggesting its association with polysomes [Palusa and Reddy 2015], but this has later been shown to be a minor occurance [Hartmann et al. 2018]. Overall our immunoblot data suggest that *SR30.2* does not give rise to significant levels of protein, while the functional experiments indicate that even if a low level of protein were made there are no differences in function between the variants. We conclude, therefore, that the light-dependent switch in the AS of *SR30* serves quantitative gene expression control purposes.

The possibility of SR30.2 being kept from translation altogether has been examined following up on the work shown here. These experiments revealed that while some *SR30.2* can be found on ribosomes, it is mostly retained in the nucleus [Hartmann et al. 2018] and thus kept from translation and the NMD machinery. Nuclear retention of PTC-containing isoforms has been reported previously, including for SR protein family members *RS31* [Kim et al. 2009; Petrillo et al. 2014] and *RS2Z33* [Göhring et al. 2014]. Furthermore, based on the RNA-seq data, several other SR-protein family members exhibit similar AS events as *SR30* and it is possible that the production of alternative transcript variants with altered subcellular localisation extends to these [Hartmann et al. 2018]. It was also shown that *SR30.2* possibly is further processed to yield *SR30.3* in a form of consecutive splicing. This could be a mechanism employed to degrade *SR30.2*, leading to its export from the nucleus as *SR30.3* and subsequent degradation by the NMD pathway. It is unclear at this point whether the *SR30.2* accumulation in the nucleus serves a purpose, such as indirectly regulating processing of transcripts by sequestering RNA binding proteins, similar to the function of a long non-coding RNA which affects splicing outcome by interacting with speckle components [Bardou et al. 2014].

An *sr30* knockout as well as overexpressing lines for *SR30.1* and *SR30.2* showed WT-like inhibition of hypocotyl elongation in response to red light. Accordingly, SR30 function is not critical in this assay of light responses, but might be restricted to an early phase of light signalling, in line with the transient nature of the corresponding AS shift. Three SR proteins closely related to SR30 are present in *A. thaliana* and, in general, complex AS patterns for the pre-mRNAs of many SRs are assumed to originate from feedback loops and intertwined expression patterns. AS of the *SR30* pre-mRNA is regulated in a

feedback loop [Lopato et al. 1999] and connected to the SR-like protein SR45 [Ali et al. 2007; Day et al. 2012]. These mechanisms are expected to buffer SR protein production and might also compensate misexpression of *SR30* in the mutants analysed here.

A recent study reported the creation of a quadruple *sr* mutant which is defective in expressing all members of the SR subfamily, *SR30*, *SR34*, *SR34a*, and *SR34b* [Yan et al. 2017]. This mutant showed no obvious phenotype. Considering the pleiotropic phenotypes that have been described for a mutant overexpressing *SR30* [Lopato et al. 1999], this is surprising. Unfortunately, the report does not include any data proving misexpression of any of the genes in the quadruple mutant. In fact, we found the *sr30* T-DNA insertion that is present in the reported mutant to reside in the 5'UTR and the single mutant to exhibit a WT-like *SR30* expression pattern [Hartmann et al. 2018], indicating expression, at least of *SR30*, may not be impaired in the quadruple mutant. Furthermore, *SR30* AS was previously shown to be altered during development, in different tissues, upon glucose exposure, and in response to stresses such as heat and high light [Palusa et al. 2007; Tanabe et al. 2007]. To truly characterise *sr30* mutants they may have to be examined under these specific conditions to reveal a phenotype.

Interestingly, *SR30* AS was shown to also be light-regulated in green plants given a light pulse during their night [Mancini et al. 2015], and affected by the application of a photosynthesis inhibitor [Petrillo et al. 2014]. This could be indicative of a central function for *SR30* AS in physiological light responses in general.

6.2.2 RRC1 expression is restricted in darkness by coupled AS-NMD

Another interesting candidate due to its reported connection to PHYB signalling was *RRC1*, a putative SR-like splicing factor [Shikata et al. 2012b;a]. The red light-dependent hypocotyl phenotype of an *rrc1* mutant could only be complemented by the full-length protein encoding variant. Unlike for *SR30*, the alternative PTC-containing isoform is degraded by the NMD pathway in this case, while light exposure enhances production of the protein-coding variant. Thus, it appears that expression of a positive PHYB signalling component is restricted in darkness by coupled AS-NMD, and the light-dependent shift in AS increases protein production and should lead to an enhanced light response. The C-terminal RS domain of the protein is required for proper function in PHYB signalling [Shikata et al. 2012b], and, generally, is essential for the splicing regulatory activity of splicing factors [Graveley 2000; Reddy and Ali 2011]. Hypomorphic *rrc1* mutants lacking the RS domain exhibited changes in splicing patterns of several SRs [Shikata et al. 2012b], suggesting that RRC1 could be higher up than SR proteins in a splicing cascade. Direct involvement of RRC1 in regulating AS of those SR protein genes affected by RRC1 mutations remains to be shown. Whether RRC1 is a key regulator of light-dependent AS in general will have to be addressed in future studies as well as exactly how RRC1 is linked to red light

signalling on a molecular level. The recently established methods for profiling interaction sites of RNA binding proteins on a transcriptome-wide level [Zhang et al. 2015; Xing et al. 2015; Meyer et al. 2017] may be useful in addressing some of these aspects.

6.3 Signalling from Photoreceptors and the Chloroplast Affect Splicing Patterns

6.3.1 Previously published data indicate phytochrome signalling can change AS

Having established that light triggers changes in AS patterns that, at least in case of *RRC1,* can affect light signalling, we wanted to understand the upstream signalling pathways leading to the changes in AS. Previous studies have on the one hand shown a role for phytochrome in changing AS in *P. patens* [Wu et al. 2014] and etiolated *A. thaliana* seedlings [Shikata et al. 2014] under red light, and on the other hand photoreceptor-independent white or red light-triggered AS in green plants [Petrillo et al. 2014; Mancini et al. 2015].

The report by Shikata et al. [2014] is closest to our experimental setup as it also looked into changes in AS upon red light exposure of etiolated Arabidopsis seedlings. They compared AS in WT and *phyA phyB* mutants exposed to red light for 1 h or 3 h. AS events were classified as phytochrome-dependent if, when comparing WT seedlings kept in darkness to illuminated WT seedlings, the direction of the change in AS was the same as when comparing WT and *phyA phyB* seedlings illuminated for a given time. The authors reported 1,505 and 1,714 genes to give rise to phytochrome-dependent and phytochrome-independent AS events after 1 h illumination, respectively, and AS changes in 1,116 and 2,098 genes, respectively, after 3 h. Most of the genes with phytochrome-dependent AS changes were found either in the 1 h sample or in the 3 h sample, raising the question of what the function of such short-lived changes could be. A decrease in detection of phytochrome-dependently regulated genes over the time course is surprising, considering the time it takes to establish new steady state levels and the accumulation of downstream changes with time. More importantly, Shikata et al. found more genes affected in their AS red light-dependently but independent from phytochrome signalling than ones with phytochrome-dependent AS changes. The numbers exceed expected effects from red light signalling through the other phytochromes which so far have been found to play only minor roles in de-etiolation (Tepperman et al. 2006; Franklin and Quail 2010). Also, analysis of a light-grown *phy* quintuple mutant showed no involvement of any phytochrome in red light-dependent AS [Mancini et al. 2015].

One important difference between this previous study and ours is the use of different analysis pipelines which may lead to very different outcomes. The fact that Shikata et al. [2014] could validate only 10 out of 19 events they independently analysed while the validation of the pipeline used here confirmed

9 out of 10 randomly selected events below threshold [Drechsel et al. 2013] indicates that stringency may vary greatly between the analyses. To separate technical issues from real differences in the results as much as possible, the data published by Shikata et al. was analysed using our pipeline [Hartmann et al. 2016]. This resulted in far fewer significantly changing AS events and an increase in numbers with time. Both the scale at which light-dependent AS changes happen and the increase over time in this new analysis are in line with the results from our own data set. The new analysis additionally redefined the phytochrome-dependent events because the control for the mutant background was missing in the original definition. Light-dependent changes in the WT were required to be absent in both the darkness controls and the light-exposed *phyA phyB* mutant to be counted as phytochrome-dependent and resulted in 67 and 329 events after 1 h and 3 h red light illumination, respectively. With 2 and 11 events, respectively, only few phytochrome-independent changes were identified. Thus, according to this new analysis, the majority of AS changes under these particular red light conditions are dependent on phytochrome signalling [Hartmann et al. 2016]. The smaller number of red light-dependent AS changes detected in our data set may be explained by the presence of sucrose in the growth medium for our RNA-seq experiment, as this has been shown to suppress light signalling [Sheen 1990; Harter et al. 1993; Dijkwel et al. 1997]. Furthermore, red light intensities differed between the studies ($\sim$ 14 µmol m^{-2} s^{-1} vs. 8.3 µmol m^{-2} s^{-1} in Shikata et al. [2014]) possibly having influenced the numbers as well. Any putative stronger effect on AS by the higher light intensity used in our study, however, may well have been outweighed by the suppressive effect of sucrose.

6.3.2 Photoreceptors are not solely responsible for changing AS patterns in response to light

We found no experimental evidence that the PHYA/B photoreceptors are majorly involved in changing AS patterns under normal white light conditions, as splicing pattern changes were mostly unaffected in the *phyA phyB* double mutant. The same was true when the blue light receptor mutant *cry1 cry2* was examined [Hartmann et al. 2016]. When illuminating seedlings with monochromatic red light, we found that the *phyA phyB* mutant showed weaker responses in *SR30* splicing than the WT. Again, a similar effect, though mostly not statistically significant, was seen for the *cry1 cry2* mutant in monochromatic blue light [Hartmann et al. 2016]. The difference between WT and *phyA phyB* lines was most pronounced in far-red light, where *SR30* splicing did not change in the mutant upon illumination. Analysis of additional candidates in all light conditions confirmed the overall conclusion, but also highlighted differences between genes especially under red light, where differences between WT and mutant were found in two cases [Hartmann et al. 2016]. Shikata et al. [2014] have shown a weaker or even no response upon red light exposure of the *phyA phyB* mutant, and according to

the new analysis of their data with the pipeline used for this study, 97% of the detected AS changes are dependent on phytochrome signalling [Hartmann et al. 2016]. In addition, Shikata et al. [2014] have shown R/FR reversibility in one case. The different degrees of response for *phyA phyB* we and Shikata et al. reported can likely be explained by the light intensities used ($\sim$ 28 and 8.3 µmol m^{-2} s^{-1}, respectively). Higher light intensities as used by us may lead to stronger photoreceptor responses, but also could increase signalling through other pathways, like photosynthesis. It is therefore possible that a stronger activation of photosynthesis is responsible for the reduced phytochrome-dependency in our experimental setting. Increased signalling from the remaining phytochrome photoreceptors in the mutant in response to higher light intensities is unlikely contributing to the observed effects, as a quintuple *phyA-E* mutant was not affected in red light-dependent AS of *SR30* [Mancini et al. 2015]. Also, previous studies described metabolic repression of photoreceptor signalling [Sheen 1990; Harter et al. 1993; Dijkwel et al. 1997], which might limit the effect of photoreceptors in the light regulation of AS under photosynthesis-competent conditions. The lack of response of the *phyA phyB* mutant in far-red light clearly shows photoreceptor-dependency under these photosynthetically unfavourable circumstances. In summary, the data indicate that phytochromes and presumably cryptochromes can trigger AS changes upon light exposure but the effect is detectable only in monochromatic light and outweighed in normal white light conditions by a different signalling pathway.

6.3.3 Retrograde signalling could be a major contributor to light-dependent changes in AS

Light-dependent but photoreceptor-independent changes in AS have also been reported in other studies [Petrillo et al. 2014; Mancini et al. 2015]. In contrast to our study and the one by Shikata et al. [2014], those studies examined green leaves in their transition to and from darkness, and light-grown seedlings given a light pulse during the night, respectively. Petrillo et al. [2014] presented compelling evidence for the plastoquinone pool as the source of a white light-dependent retrograde signal by blocking electron transfer immediately up- and downstream of plastoquinone and finding opposing effects on light-dependent AS. They also reported testing the effect of glucose and sucrose on light-dependent AS, with only a mild effect of sucrose.

When we applied sucrose, however, we found sugar treatment of etiolated seedlings in darkness to result in very similar changes in AS patterns as light exposure did. Follow-up experiments also tested several other sugars, but sucrose had the maximum impact [Hartmann et al. 2016]. The variance in influence sucrose has on AS observed by Petrillo et al. [2014] and us could be due to the different materials that were examined; a metabolic signal connected to sucrose could be attenuated in green, sucrose-producing leaves, whereas etiolated seedlings may be more sensitive to it. Illumination and sucrose treatment together had an even stronger effect on AS than the single treatments in some

candidates that were tested additionally to the ones shown here [Hartmann et al. 2016]. An additive effect may also be present in our RNA-seq experiment, as seedlings were grown on sucrose-containing medium, yet our analysis identified many AS events that are regulated light-dependently on top of the possibly activated metabolic signalling pathway. This could be due to independent signalling pathways, but it is also plausible that single treatments do not saturate the response. By themselves, the treatments already resulted in strong AS shifts for most candidates, so biological relevance of an even stronger shift will have to be shown by future experiments. A gradual response may be necessary to integrate several signalling pathways, at least in some cases. The AS response could also differ between tissues or specific cells, both in intensity and specificity of the eliciting signal, possibly giving the appearance of an additive effect when examining whole seedlings.

We learnt from both our RNA-seq data and downstream analyses of select cases that red light can result in weaker AS changes compared to blue light. This could also be in line with a photosynthesis-dependent metabolic signal which reflects differences in photosynthetic output under different light conditions. It might be argued that weaker changes under blue light are to be expected, considering the higher photon fluence rate we applied of red light, and the higher efficiency of red light in driving photosynthesis [McCree 1972; Inada 1976; Evans 1987]. The wavelength-dependent photosynthesis rate is, however, typically determined in green leaves, and absorption by different pigments as well as the balance between the two photosystems factor into the photosynthetic efficiency [McCree 1972; Inada 1976; Evans 1987; Terashima et al. 2009; Hogewoning et al. 2012]. As the photosynthesis apparatus as well as the pigments are not yet fully established early in photomorphogenesis, the effect of different light qualities differs from what is expected in green tissue. Irradiation of etiolated barley seedlings with blue light resulted in photosynthetically active chloroplasts faster than upon exposure to red light [Lichtenthaler et al. 1980]. Cucumber leaves developing in blue light also showed a higher photosynthetic capacity than those developing in red light [Hogewoning et al. 2010]. In addition, a more recent publication reported different metabolite compositions in rice seedlings grown in red or blue light [Lakshmanan et al. 2015]. The authors attributed higher concentrations of secondary metabolites in seedlings grown under blue light to a higher photosynthesis rate. Interestingly, they also found genes involved in photosynthesis upregulated in blue light, and a downregulation of these in red light compared to white light exposure. As light quality effects on photosynthesis appear to be species specific to a certain extent [Inada 1976; Yorio et al. 2001], these results may not be immediately transferable to *A. thaliana*, but they point to the possibility that the different extents of AS changes under blue and red light could indeed be caused by a higher photosynthesis rate under blue light.

Based on our findings, we propose a photosynthesis-derived metabolic signal to mainly drive AS changes in early photomorphogenesis under normal light conditions. Under conditions allowing no or only inefficient photosynthesis, as in far-red or low intensity light, photoreceptors are the main

signalling sources. This could be the case at the initiation of photomorphogenesis generally, and in particular under dense canopies where far-red is the main wavelength available. Such an interpretation of our results is in line with the report by Petrillo et al. [2014]. However, unlike light-grown plants, etiolated seedlings do not possess a fully developed photosynthetic system. In order to affect AS early in photomorphogenesis through photosynthesis the system needs to be set up rapidly. To our knowledge, the onset of photosynthesis has not been reported in Arabidopsis, but experiments in barley (*Hordeum vulgare*) detected CO_2 assimilates after 1 h of light exposure [Biggins and Park 1966]. We therefore assume that photosynthesis is activated within the first hours of light exposure in Arabidopsis and quickly capable of generating a signal which affects AS patterns.

6.4 Interplay of Sugar and Light Signalling

Exactly how much each signalling pathway contributes to the final changes in AS, but also how this differs between tissues and possibly shifts during an ongoing response, will be interesting aspects of future studies. Unfortunately, separating the effects of photoreceptor and retrograde signalling is challenging, as signalling from chloroplasts can modulate light signals, and photoreceptor signalling affects chloroplast development. PHYA, PHYB, CRY1, and CRY2 all regulate key genes in chlorophyll synthesis [McCormac and Terry 2002; Fox et al. 2015], and *phyB* mutants have lower chlorophyll content and fewer chloroplasts [Reed et al. 1993]. As a result, experiments involving photoreceptor mutants to separate these two pathways become difficult to interpret, since the development in WT and mutants could be proceeding differently in crucial aspects. An alternative may be the application of photosynthesis inhibitors, like 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which has been successfully used by Petrillo et al. [2014] to influence splicing patterns. However, when applied to etiolated seedlings, changes in AS upon light exposure were weaker [Hartmann et al. 2016], but not clearly inhibited as published for green leaves [Petrillo et al. 2014]. The initial abscence of a fully assembled photosynthesis machinery in etiolated seedlings possibly explains the weak effect of DCMU, which has been reported before to have different effects on light- and dark-grown plants [Mancinelli 1994]. It is also conceivable that the role of photoreceptors in AS regulation becomes detectable in etiolated seedlings but not in light-grown plants upon inhibition of photosynthesis. The contributions of different pathways to adapting AS may be changing in the course of development, like it has been described for carbon and light signalling crosstalk between etiolated and green plants in regulating the expression levels of three genes involved in nitrogen assimilation [Thum et al. 2003].

Photoreceptor and sugar signalling crosstalk also modulates floral signal transduction [Matsoukas 2017], and light and metabolic signals are integrated by TARGET OF RAPAMYCIN (TOR) to activate stem cells at the shoot apical meristem [Pfeiffer et al. 2016]. We found expression patterns of SnRK1

targets *DIN1* and *DIN6* to correlate perfectly with changing splicing patterns in response to light and/or sucrose exposure, suggesting a role for SnRK1 in relaying the proposed metabolic signal to affect AS. The notion is further supported by experiments that showed similar shifts in AS upon application of a kinase inhibitor as light or sucrose caused for several genes [Hartmann et al. 2016]. While this does not prove involvement of SnRK1, the findings emphasize a role for phosphorylations upstream of light-dependent AS changes, possibly including phosphorylations on splicing regulators themselves. Whether these modifications are indeed SnRK1-dependent or involve different kinases remains to be shown.

SnRK1 adjusts metabolism by directly phosphorylating key enzymes [Sugden et al. 1999; Harthill et al. 2006], and by altering transcription [Polge and Thomas 2007; Baena-González and Sheen 2008; Mair et al. 2015]; effects on AS have not been described so far. However, AS and SnRK1 are linked by the production of different *SnRK1* isoforms [Williams et al. 2014], and via the indirect regulation of SnRK1 stability by splicing regulator SR45 [Carvalho et al. 2016].

Other than SnRK1, the PIF transcription factor family, with its prominent role in light signalling, could also play an important role in the described light and carbon crosstalk. Sucrose's effect on growth reportedly relies on PIF5 [Stewart et al. 2011], and sucrose was shown to affect both PIF transcription and their binding to promoters in feedback between circadian oscillators and metabolism [Shor et al. 2017]. PIF3 is involved in regulating the expression of photosystem I components as well as key enzymes of chlorophyll biosynthesis [Shin et al. 2009] and is therefore an important component in establishing photosynthesis in addition to its role in hypocotyl elongation in darkness and upon sugar feeding [Leivar et al. 2008; Shin et al. 2009; Liu et al. 2011b]. Interestingly, we discovered a light-dependent AS event in *PIF3* which alters the 5' UTR. Future work will have to address whether sucrose plays a role in this splicing regulation, as well as investigate the functional impact of this AS event, which could affect stability, localisation, or translation of the isoforms.

In conclusion, light alters AS, both through photoreceptor-dependent and -independent signalling. Photoreceptor-independent changes in AS are possibly initiated upon retrograde signalling from the chloroplast, and may involve energy sensors like SnRK1. Modifying the AS programme could be an important and powerful tool in adjusting the transcriptome and ultimately metabolism to changes in energy supply.

7 Methods

Methods from Hartmann et al. [2016] (www.plantcell.org, Copyright American Society of Plant Biologists) and Hartmann et al. [2018] (www.plantphysiol.org, Copyright American Society of Plant Biologists), were combined to give a coherent section. The published text is cited directly with small modifications like updated figure and primer references, plant lines, and spelling.

Plant cultivation and experiments "Generally, seeds were sterilised in 3.75% NaClO and 0.01% Triton X-100 and plated on ½ MS medium containing 0.8% phytoagar (Duchefa) with or without 2% sucrose added. Sucrose-containing medium was used for the RNA-seq and validation experiments (Figures 2, 3, 8, 9, 14, 19). The experiments with the photoreceptor mutants (Figures 17 and 18) were performed in parallel with seedlings grown on medium with and without sugar as indicated. Seeds were stratified for at least 2 days at 4°C, then germination was induced in white light for 2 h. Seedlings were grown in darkness for 6 days and then exposed to white, red, blue, or far-red light, or kept in darkness for the indicated period. Darkness samples were taken in green light.

For hypocotyl assays (Figures 13, 15, 16), seeds were plated singly on plates without sucrose. After the initial 2 h light exposure, plates were placed in red light or in darkness for 6 days. The lines to be compared were grown on the same plates. Seeds were the same age. Seedlings were scanned after transfer to ½ MS plates with 1.5% agar. The length of scanned seedlings was measured using ImageJ [Schneider et al. 2012]. All relative hypocotyl lengths are normalised to the average length of each line grown in darkness.

For transfer experiments and sugar treatments (Figure 20), seeds were plated densely on medium without sucrose and grown in darkness for 6 days after initial light exposure. Seedlings were transferred to liquid ½ MS medium without or with sugar supplementation under green light and incubated in white light or darkness for the indicated periods.

The following lines have been used in different experiments: *rrc1-2* (SALK_121526C, [Shikata et al. 2012b]), *lba1* [Yoine et al. 2006], *upf3-1* (SALK_025175), *lhy-null* [Yakir et al. 2009], *toc1-101* [Kikis et al. 2005], *prr7-3 prr9-1* [Farré et al. 2005], *phyA-211 phyB-9* (*phyA phyB*)."

From: Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. www.plantcell.org, Copyright American Society of Plant Biologists.

Light conditions "Continuous white light had an intensity of ~ 130 µmol m^{-2} s^{-1}. For monochromatic light LED fields (Flora LED, CLF Plant Climatics) were used. Specifications: blue 420-550 nm, maximum (max) 463 nm, Full Width at Half Maximum (FWHM) 22.2 nm; far-red 680-790 nm, max 742 nm, FWHM 23.8 nm; red 620-730 nm, max 671 nm, FWHM 25 nm. Light intensities are provided in figure legends and have been measured with a Skye SKR1850, using the far-red channel for far-red, and photosynthetically active radiation for blue, red, and white light. Red light intensity of ~ 10 µmol m^{-2} s^{-1} for hypocotyl assays were achieved by stacking plates with a layer of white paper between them."

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RNA-seq For one of the replicates, seeds were washed once in 70% ethanol before washing with bleach. "Seedlings were grown in darkness for 6 days, then sampled (0h), or exposed to light for 1 h or 6 h, or kept in darkness for 6 h before sampling (6D). RNA was extracted using the EURx GeneMATRIX Universal RNA Purification Kit. Starting from 4 µg total RNA, libraries were prepared using the Illumina TruSeq Kit v2, Box A mostly according to the manufacturer's instructions. The PCR step was performed using only half the template in a reaction volume of 34 µL, and the libraries were subsequently purified on a 2% agarose gel. After cutting a band of appropriate size from the gel for each library, the DNA was extracted using the Qiagen MinElute Gel Extraction Kit. Concentrations were determined using the Agilent 2100 Bioanalyzer with the DNA1000 kit. DNA Sequencing was performed on the HiSEQ2000, equipped with on-instrument HCS version 1.5.15 and Real time analysis (RTA) version 1.13. Cluster generation was performed on a cBot (recipe: SR_Amp_Lin_Block_Hyb_v8.0, Illumina) using a flow cell v3 and reagents from TruSeq SR Cluster Kits v3 (Illumina) according to the manufacturer's instructions. The libraries were sequenced single end with 101 bp read length and a 7 bp index read. The final DNA concentration was 7 to 8 pM on the flow cell. Samples were duplexed or quadruplexed using the adapters 012, 006, 019, and 005 (see Table 1). Each sample was run in biological duplicate. We used a previously established pipeline for alignment, splice event calling, and analyses (Rühl et al. 2012; Drechsel et al. 2013)."

From: Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy

Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. www.plantcell.org, Copyright American Society of Plant Biologists.

RNA-seq data analyses "In short, RNA-Seq reads were aligned to the TAIR 10 reference genome using PALMapper [Jean et al. 2010] in two steps. First, an alignment was performed to discover novel splice junctions. In the second step, the novel splice junctions were included in the alignment to obtain a splice-sensitive alignment. Subsequently, novel splice events were called using SplAdder [Kahles et al. 2015], as described in Drechsel et al. [2013]. Read counts and differential AS events were determined using rDiff [Drewe et al. 2013]. Differential gene expression was called, based on the read counts, using DESeq [Anders and Huber 2010]. For a detailed description of parameter settings, see Computational Parameter settings".

"To estimate the biological variance and thus determine accurate false discovery rates, the analyses of differential AS events and differential gene expression were performed jointly on all replicates of the samples that were to be compared."

"AS events with an FDR-value below a certain threshold were required to not show changes in the opposite direction in any other light condition (i.e. (B_up $<$ q and R_down $>$ q and W_down $>$ q) or (B_down $<$ q and R_up $>$ q and W_up $>$ q) for events changing significantly in blue light)." Similarly, genes changing their expression in opposite directions in different light colors were excluded from the differential gene expression analysis. "Data analysis was done using Excel (Microsoft) or Python (Anaconda distribution 2.1.0, Continuum Analytics) with SciPy [Jones et al. 2001], NumPy [van der Walt et al. 2011], Pandas [McKinney 2010], Matplotlib [Hunter 2007], and IPython [Perez and Granger 2007]".

"Functional clustering using the MapMan software [Thimm et al. 2004] was done as previously described [Rühl et al. 2012; Drechsel et al. 2013], and as detailed in Supplemental Data Set 5. Extraction of NMD features and analysis of intergenic regions were performed as described in Drechsel et al. [2013]."

"For determination of effect sizes, the splicing index (SI) was calculated for each event and light condition. SI is the ratio of the number of spliced alignments supporting the longer isoform, divided by all spliced alignments corresponding to this event. In case of intron retention events, the SI was determined as the average intron coverage divided by the average intron coverage plus the spliced alignments spanning the respective intron. As the reliability of the SI depends on the number of available alignments, no SI index was calculated when fewer than 10 isoform-specific reads were available. SI values for an event of the category 'old' were only computed when the event could be confirmed in the respective read library, that is there was a sufficient number of alignments present in the new libraries to call the event. For comparison of SI values of significantly changed AS events, the following filters were applied: all relevant replicates need to be assigned an SI value, and the variation in SI between

replicates needs to be less than 0.25. Furthermore, to exclude splicing variants of low abundance or with minor changes, only those events with SI changes greater than 0.05 were considered as having changed between dark and light samples. When combining data from different light qualities, events with opposite changes in SI > 0.05 were excluded."

From: Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. www.plantcell.org, Copyright American Society of Plant Biologists.

Computational Parameter Settings

"Demultiplexing Demultiplexing was performed with the FastX-toolkit using the default parameters.

Alignment Reads were aligned against the TAIR 10 reference genome in a two-steps approach with PALmapper [Jean et al. 2010]. In the first step, an alignment was performed to discover novel splice junctions. For this alignment the following parameters were used:

-M 3 -G 1 -E 4 -l 15 -L 25 -K 8 -C 35 -I 25000 -NI 2 -SA 100 -CT 10 -a -S -seed-hit- cancel-threshold 10000 -report-map-read -report-spliced-read -report-map-region - report-splice-sites 0.9 -filter-max-mismatches 0 -filter-max-gaps 0 -filter -splice-region 5 -qpalma-use-map-max-len 2000 -f bamp -threads 2 -polytrim 40 -qpalma-prb- offset-fix -include-unmapped-reads -min-spliced-segment-len 12 -junction- remapping-coverage 3 -fixtrimleft 4 -fixtrimright 4 -no-gap-end 10 -qpalma-indel- penalty 10

In a second step, the discovered splice junctions from the reads of the initial alignment were used for a sensitive realignment of the reads. For the second alignment the following parameters were used:

-M 4 -G 1 -E 4 -l 15 -L 25 -K 8 -C 35 -I 25000 -NI 2 -SA 100 -CT 50 -a -S -seed-hit- cancel-threshold 10000 -report-map-read -report-spliced-read -report-map-region - report-splice-sites 0.9 -filter-max-mismatches 0 -filter-max-gaps 0 -filter-splice-region 5 -qpalma-use-map-max-len 1000 -f bamn -threads 2 -polytrim 40 -qpalma-prb- offset-fix -min-spliced-segment-len 15 -junction-remapping-coverage 5 -junction- remapping-min-spliced-segment-len 15 -fixtrimleft 4 -fixtrimright 4 -qpalma-indel- penalty 1

Alignment post-processing The alignments were sorted using Samtools. Subsequently, reads that mapped to multiple locations were removed. Specifically, the best alignment of a read was kept only if the second best alignment had more than 1 mismatch more than the best alignment. Additionally, alignments with more than 2 mismatches were discarded.

Event extraction and quantification Alternative splicing (AS) events were extracted with SplAdder [Kahles et al. 2015], as described in Drechsel et al. [2013]. AS information from the 22 library alignments was integrated into a common reference graph using the SplAdder pipeline with confidence level 2 and otherwise default parameters. On this integrated splicing graph we performed AS event calling. This resulted in a total of 53,120 detected and confirmed AS events. In addition to that we also processed a set of 8 libraries that were generated for preliminary analyses. We will denote the 22 and 8 library data sets as 'new' and 'old' respectively. Based on the eight old libraries, the SplAdder pipeline was run with confidence level 3 (to only keep events of highest confidence) to generate a common splicing graph representation. From this common graph, we called and confirmed a total of 13,784 AS events. The two AS event lists generated from the old and new libraries, respectively, were then united into a common list of AS events. In this merged list 3,150 events were found only in the old analysis graph. These events have the prefix 'old_'. All other events have the prefix 'new_'. We then used SplAdder to also quantify the splicing graphs generated from the old data set with the alignments of the new libraries. Due to the graphs being generated from different data source the re-quantification resulted in a number of 14,412 AS events to be called and confirmed on the old graph using the new library data. Not all of the above 3,150 events could be confirmed using the new data. In this case, we give NA as quantification values."

"Read counting and testing for differential events For read counting and differential testing of events, we used scripts implemented in the differential testing toolbox rDiff [Drewe et al. 2013], as described in Drechsel et al. [2013].

Test for differential gene expression The differential gene expression was analyzed using the DESeq [Anders and Huber 2010] package for the R Bioconductor Suite, as described in Drechsel et al. [2013].

False discovery rate estimation The false discovery rate was estimated as described by Benjamini and Hochberg [1995].

Calling expressed genes We determined the expressed genes as described in Gan et al. [2011]."

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RNA extraction, RT, qPCR, and PCR product analyses "RNA was extracted using the Universal RNA Purification Kit (EURx) with an on-column DNase digest as instructed by the manufacturer. Reverse transcription was done with RevertAid Premium (Thermo Fisher) for etiolated seedling samples, or using AMV Reverse Transcriptase Native (EURx). The maximum volume of RNA template possible and a dT_{20} primer were used following the manufacturer's instructions. RT-qPCRs were performed as described previously [Stauffer et al. 2010]. In short, the Biorad CFX384 real-time PCR system and MESA GREEN qPCR Mastermix Plus (Eurogentec) were used. *PP2A (AT1G13320)* transcript levels or the total transcript levels of a gene were measured for normalisation. RT-PCR fragments were separated and visualised on ethidium bromide stained agarose or polyacrylamide gels. Isoform concentrations were measured using the Agilent 2100 Bioanalyzer with the DNA1000 Kit. Oligonucleotides used are listed in Supplemental Table 1. Gel pictures were enhanced using the Adobe Photoshop autocontrast function. Splice variants were subcloned using the pGEM-T Vector System I (Promega) or StrataClone PCR Cloning Kit (Agilent) and sequenced, or sequenced directly."

From: Hartmann, L., Drewe-Boß, P., Wießner, T., Wagner, G., Geue, S., Lee, H.-C., Obermüller, D. M., Kahles, A., Behr, J., Sinz, F. H., Rätsch, G., and Wachter, A. Alternative Splicing Substantially Diversifies the Transcriptome during Early Photomorphogenesis and Correlates with the Energy Availability in Arabidopsis. *The Plant Cell*, 28(11):2715–34, 2016. www.plantcell.org, Copyright American Society of Plant Biologists.

Cloning procedures "*SR30* overexpression constructs for immunoblots were based on the vector pBinAR [Höfgen and Willmitzer 1992]. All primers used for cloning are listed in Supplemental Table 1. For CDS constructs, inserts were amplified from cDNA using primers 163/211 (*.1*) or 163/212 (*.2*) omitting the STOP codon and cloned into a pBinAR containing HA_3-STOP via *Bam*HI/*Xba*I. The inserts of the cDNA constructs were amplified each in two parts inserting an HA-tag at the C-terminus and removing the STOP codon with 186/187 and 188/189 (*.1*) or 186/190 and 191/189 (*.2*). The respective parts were combined using the corresponding outer primers. Cloning into *Bam*HI/*Sal*I digested pBinAR was done via *Bam*HI/*Xho*I. To generate HA_3-tagged versions, the inserts both were amplified in two parts and HA_3 added using 186/312 and 311/189. Insertion into pBinAR was done as described above for the untagged cDNA constructs.

For constructs used for confocal microscopy, splice variants of *SR30* were amplified with primers 159/160 or 159/161, respectively, omitting the STOP codon, and recombined into pDONR201, then pB7CWG2 or pB7YWG2 [Karimi et al. 2002] using the Gateway system (Invitrogen).

The genomic reporter used in the splice assay (Figure 12) was amplified using primers 163/169 inserting the C-terminal HA-tag and cloned into *Bam*HI/*Sal*I digested pBinAR via *Bam*HI/*Xho*I. The splice

form-specific Flag-tagged CDS constructs were cloned similarly using primers 163/164 and 163/165, respectively."

The amiRNAs were designed "using the web tool WMD3 (http://wmd3.weigelworld.org; Ossowski et al. 2008) and cloned following the available protocol (http://wmd3.weigelworld.org/downloads/Cloning_of_artificial_microRNAs.pdf) using primers 47-62. After extension of the partial attachment sites with primers 65/66, the precursor was recombined into pDONR201, then pB7WG2 [Karimi et al. 2002] using the Gateway system (Invitrogen).

For expression of recombinant SR30 for immunisation, *SR30.2* CDS was amplified using 163/182, and cloned into pQE30 (Qiagen) via *Bam*HI/*Xho*I. Sequencing *SR30*, we discovered an insertion relative to the TAIR10 reference sequence. One G nt was inserted between positions 2926 and 2927 of the annotated gene in the 11^{th} intron. We found this insertion both in our WT line and the *lba1* mutant."

From: Hartmann, L., Wießner, T., and Wachter, A. Subcellular Compartmentation of Alternatively Spliced Transcripts Defines SERINE/ARGININE-RICH PROTEIN30 Expression. *Plant Physiology*, 176(4):2886–903, 2018. www.plantphysiol.org, Copyright American Society of Plant Biologists.

"*RRC1* overexpression constructs are based on the vector pGWB612 [Nakamura et al. 2010]. Oligonucleotide sequences are listed in Supplemental Table 1. CDS of the splicing variants, with the 3' UTR included were amplified from cDNA and the genomic sequence of *RRC1* was amplified from genomic DNA using primers 63/64 and recombined using the Gateway system (Invitrogen) into pDONR207, after PCR extension of the attachment sites with primers 65/66. Subsequently, *RRC1* sequences were recombined into pGWB612. For the complementation constructs under control of the endogenous promoter, an *RRC1* 1013 bp putative promoter fragment including the 5' UTR was amplified using primers 83/84 and exchanged with the 35S promoter of pGWB612 using *Hind*III/*Xba*I. Subsequently, the cDNA or genomic sequence was introduced as for the overexpression constructs."

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Antibody generation "*Escherichia coli* M15 expressing *SR30.2* in the vector pQE30 were grown in 3 L Terrific Broth medium to an optical density > 1 at 37°C. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside, and the culture further incubated at 37°C overnight. All following steps were done at 4°C or on ice unless specified otherwise. The cells were spun down and resuspended

in cold Lysis-Equilibration-Wash buffer (LEW; 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0)), then lysed using a cooled French pressure cell (Aminco, 3x 1000 p.s.i.). The lysate was treated with 50 µg/mL DNase for 20 min at room temperature under agitation, then centrifuged (10,000*g*, 30 min). The pellet was washed once with cold LEW, then resuspended in 25 mL Denaturing Solubilization Buffer (DSB; 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0), 8 M urea), incubated on a wheel shaker for 1 h, and spun at room temperature for 40 min at 10,000*g* or until supernatant was clear. The supernatant was added to Protino Ni-TED resin (Macherey-Nagel) prepared according to the manufacturer's instructions, and incubated on a wheel shaker for 1 h at room temperature. The column was drained by gravity at room temperature and the flow-through was collected. At room temperature, the resin was washed with 200 mL DSB, and protein was eluted three times with 3 mL 150 mM and three times with 3 mL 200 mM imidazole-containing DSB. Elution fractions were combined, diluted with 5 mL LEW per mL elution fraction, and incubated on a wheel shaker overnight. Precipitated protein was spun down, resuspended in 2x SDS sample buffer and denatured at 95°C for 10 min. Protein concentration was estimated by comparing band intensities on a gel to marker bands. Approximately 200 µg protein per lane was loaded on a 12% polyacrylamide gel. After Coomassie staining, the prominent band was excised excluding a slightly smaller band, and the gel pieces were washed in water until the pH was neutral. Rabbits were immunised six times with the gel-bound protein (BioGenes). The antibody was affinity purified from raw sera using membrane-bound antigen as described before [Rühl et al. 2012], but partly using a 1:1 dilution of 7 mL serum in one purification."

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Protein extraction, immunoprecipitation, and immunoblot analyses For SR30, "starting material from infiltrated *N. benthamiana* leaves was ~100 mg, and 200-300 mg Arabidopsis seedlings were used per extraction. For immunoblot analyses, proteins were extracted as described previously [Rühl et al. 2012], using an extraction buffer containing 65 mM KCl, 15 mM NaCl, 10 mM HEPES (pH 7.6), 10 mM Na_2EDTA, 5 mM DTT, 4 mM ATP, 1x phosphatase inhibitor mix (Serva) and 1x Complete (Roche) (after Zahler et al. 1992)."

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"RRC1 protein was extracted using a denaturing buffer as previously described [Shikata et al. 2012a] with Complete (Roche) as protease inhibitor." "All extracts were cleared by centrifugation at 4°C for

~20 min at ~15,000*g*. Using Protein G coupled Dynabeads (Life Technologies), α-SR30 was coupled to the beads in PBS-T by incubation under agitation for 10 min at room temperature. The beads were washed once with PBS-T. Protein extract was added to the beads and protein was allowed to bind to the beads for 1 h at room temperature on a wheel shaker. The beads were washed three times using the extraction buffer, and transferred to a fresh tube in a fourth washing step. Protein bound to Dynabeads was eluted at 95°C in 5x SDS sample buffer for 10 min while mixing. SDS-PAGE and semi-dry immunoblotting were performed according to standard protocols. For detection, the following commercial antibodies were used: rabbit α-histone H3 (Agrisera), rabbit α-UGPase (Agrisera), rabbit α-FLAG (Sigma), mouse α-HA (Sigma), α-mouse-peroxidase (Sigma), α-rabbit-peroxidase (Sigma). Chemiluminescence detection used Super Signal West Dura (Pierce)."

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Plant transformation "Heterotrophic cell culture protoplasts were transformed according to a previously published protocol [Schütze et al. 2009] with 2 µg of each plasmid, and kept in darkness for 2 d before microscopy. *Nicotiana benthamiana* leaves were transiently transformed by leaf infiltration as previously described [Wachter et al. 2007] using transformed *Agrobacteria* of an optical density 0.8 at 600 nm in water. Co-transformation of luciferase or one of the splicing variants with the reporter was achieved by mixing the respective bacterial suspensions 1:1 before infiltration. Co-transformation of the reporter with the luciferase control or one of the splicing variants was always done on corresponding leaf halves for normalisation purposes. Infiltrated plants were grown for additional 2 d before sampling. Arabidopsis plants were stably transformed by the floral dip method [Clough and Bent 1998]."

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Confocal microscopy "Microscopy was conducted with a TCS SP2 AOBS (Leica). The excitation (ex.) and emission (em.) settings were as follows: YFP 514 nm (ex.), 524-575 nm (em.) and DsRED 561 nm (ex.), 575-641 nm (em.) in Figure 11A and B; CFP 405 nm (ex.), 453-511 nm (em.) and YFP 514 nm (ex), 566-617 nm (em.) in Figure 11C; CFP 405 nm (ex.), 457-540 nm (em.) and YFP as for

Figure 11A in Figure 11D and E. The protoplasts were scanned in sequential mode, with the exception of the one shown in Figure 11C."

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Data Access Visualization of RNA-seq data is available at:
http://gbrowse.cbio.mskcc.org/gb/gbrowse/r403PAS/

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List of Supplemental Material

The supplemental material can be found on the accompanying CD.

Supplemental Figure 1. Sequences of Splicing Variants Identified.

Supplemental Table 1. Sequences of DNA Oligonucleotides.

Supplemental Data Set 1. Computational Analysis of Transcriptome-Wide AS.

Supplemental Data Set 2. Computational Analysis of Transcriptome-Wide Differential Gene Expression.

Supplemental Data Set 3. Splicing Index Analysis of AS.

Supplemental Data Set 4. AS event positions, extraction of NMD-eliciting features, and overlap between NMD- and light-regulated AS events.

Supplemental Data Set 5. Categorisation of Light-Regulated and Reference Gene Sets into Functional Subgroups.

Supplemental Data Set 6. Expressed Intergenic Regions.