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Loss of LEFKOTHEA Leads to Global Transcriptional and Post-Transcriptional Changes in Gene Expression During Early Light Response

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Abstract

In plants, the transition from heterotrophic to autotrophic growth is a critical developmental shift, tightly coupled to the establishment of photosynthesis. This process demands a precise interplay between the nucleus and chloroplasts, with communication schemes providing essential checkpoints to synchronize gene expression during seedling greening and establishment. While light response and photomorphogenesis are known to rely on transcriptional networks, recent evidence highlights a key role for alternative pre-mRNA splicing in facilitating plant adaptation to new light regimes, thereby enhancing transcriptome diversity. LEFKOTHEA, a dual-localized nuclear and chloroplast protein, has emerged as a potential integrator of these processes; it mediates the splicing of both chloroplast group II introns and nuclear introns via interactions with spliceosomal proteins. Here, we demonstrate that LEFKOTHEA is an active component of early light response signaling, regulating gene expression both transcriptionally and post-transcriptionally. Transcriptomic analysis reveals that LEFKOTHEA shapes the transcriptome both quantitatively and qualitatively by modulating alternative splicing, a mechanism essential for plant plasticity and adaptation. Furthermore, we show that the dynamic subcellular localization of LEFKOTHEA underpins its role in establishing a nucleus–chloroplast communication network that guides plant development.

Keywords: light response; seedling development; mRNA splicing; transcriptional regulation; Arabidopsis

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1. Introduction

Light is the source of energy for photosynthetic organisms such as plants. The continuous fluctuations of light, like diurnal rhythm and seasonal variations in photoperiod, define critical developmental processes in plant growth and development. Therefore, perception of light and activation of downstream signaling events are crucial for plant survival and reproductive success. Plants possess a sophisticated signaling network that controls light responses. This network influences a plethora of developmental processes, including seed

germination, dark-grown seedling de-etiolation, chloroplast ontogenesis and movement, stomatal physiology and development, and flowering time [1–4].

In higher plants, light quality perception is mediated through the activation of photoreceptors, which are categorized into at least five distinct classes such as phytochromes for red and far-red light [5], cryptochromes [6], phototropins [7], and members of the ZEITLUPE family [8,9] for blue and UV-A detection, and UV-B receptor for the detection of UV-B light [10,11]. Downstream of the photoreceptors, a cohort of signaling partners specifies and integrates the received light stimulus, leading to the adjustment of plant development. In addition, chloroplasts, as the main photosynthetic apparatus, perceive light stimuli and communicate light conditions to the nucleus through retrograde signaling to modulate the transcription and translation of genes acting in different cellular compartments [12]. Increasing evidence shows that photoreceptors and retrograde-mediated signaling act synergistically to fine-tune light response [13–16].

Response to alterations in light conditions relies on the activation of transcriptional networks mediated by photomorphogenesis-induced transcription factors expressed after the suppression of negative regulators, such as PHYTOCHROME INTERACTING FACTORs [17,18], DE-ETIOLATED1, and CONSTITUTIVE PHOTOMORPHOGENIC1 [19]. De-etiolation, the transition from skoto- to photomorphogenesis, also includes changes in chromatin organization, resulting in massive alterations in transcriptional patterns [20,21]. Besides the quantitative changes in gene expression, accumulating evidence shows that alternative precursor mRNA (pre-mRNA) processing assists plants in adapting to environmental changes by creating diverse protein isoforms from a single gene involved in photosynthesis, stress responses, and core clock functions, consequently increasing the degree of transcriptome complexity. It is worth mentioning that alternative splicing in plants is common and affects almost 60% of intron-containing genes in Arabidopsis [22,23]. Although the molecular mechanisms underlying alternative splicing remain mostly elusive, this regulation has been linked to chloroplast retrograde signaling, which leads to an increase in RNA polymerase II (Pol II) elongation speed in certain genes and consequently to alternate splicing patterns. Moreover, experimental data show that photosynthetic sugars affect alternative splicing events similarly to direct light, indicating the existence of intercellular mobile signals [24].

Chloroplasts evolved from a cyanobacterial ancestor, establishing an endosymbiotic relationship with eukaryotic cells. During evolution, many bacterial genes were lost from the genome and relocated to the host nucleus, while only a few genes were retained in the plastid genome [25]. In land plants, most of the chloroplast genes encode proteins that participate in the photosynthetic apparatus, such as photosystem I (PSI) and PSII subunits, the cytochrome b6f complex, the NAD (P)H dehydrogenase-like (NDH) complex, and some ATP synthase subunits or structural RNAs [26]. Recent reports suggest that the plastid genomes of angiosperms have highly conserved structures and gene content; many of the plastid genes contain introns, while the RNA processing is complex [27,28]. The plastid genomes contain about 20 genes with group II introns and a single gene with group I intron (within the pre-trnL-UAA region) [29]. Up to now, several nuclear-encoded splicing factors involved in intron splicing in plant organelles have been identified, including chloroplast RNA splicing and ribosome maturation (CRM) proteins, pentatricopeptide repeat (PPR) proteins, plant organellar RNA recognition, (PORR)-domain proteins, and members of the accumulation of photosystem one (APO) domain family [30]. Several reports suggest that the abnormal chloroplast group II intron splicing disrupts photomorphogenesis by affecting the synthesis of photosynthesis-related proteins, leading to defects in chlorophyll production and overall plant growth. Furthermore, loss-of-function mutants of the key splicing factors, like CRM, CAF1, and APO, result in severe developmental defaults like

chlorophyll deficiency and embryo lethality, underscoring the vital role of chloroplast protein synthesis in early plant development [30–32].

LEFKOTHEA is a nucleus-encoded protein that possesses a plant organelle RNA recognition (PORR) domain, promoting the splicing of both chloroplast group II introns and nuclear pre-mRNA introns. Although LEFKOTHEA is found in both the nucleus and the chloroplast, its nuclear compartmentalization is essential for promoting early embryonic development in *Arabidopsis* by repressing chloroplast biogenesis; disruption of this process leads to embryo lethality. Moreover, LEFKOTHEA was found to physically interact with spliceosomal proteins like U1-70K and SR45, as well as the RH3, a splicing component of chloroplast group II introns [33].

In this study, by using physiological, cell biology, and transcriptomic analysis approaches, we report that LEFKOTHEA actively participates in light response and photomorphogenic pathways during the early development of Arabidopsis seedlings. LEFKOTHEA influences gene expression both at the transcriptional and post-transcriptional levels. This is supported by the deregulated expression levels of genes involved in chloroplast function and structure, light response, and signaling pathways. Moreover, LEFKOTHEA significantly modulates the frequency and the type of alternative splicing events, affecting the production and the abundance of splicing variants, which in turn affect the transcriptomic plasticity required for the adaptation of the plant to environmental stimuli, such as light. Alteration in the subcellular distribution of LEFKOTHEA protein under different light conditions is also evident. Taken together, our results show that LEFKOTHEA functions as a molecular switch to synchronize gene expression in the nucleus and the chloroplast through the regulation of splicing events of nuclear genes. Thus, it is part of a plastid anterograde and retrograde signaling network that ensures appropriate communication between these two cellular compartments.

2. Results

2.1. lefko 1 Affects Photomorphogenesis

Previous studies have shown that the knockout mutant of LEFKOTHEA, *lefko 2*, exhibits an embryo developmental arrest at the early heart stage, resulting in a lethal phenotype (Figure 1a; [33]). On the other hand, the chemically induced mutant allele, *lefko 1*, exhibits an albino cotyledon phenotype, which can flower and set seeds (Figure 1b; [33]). In the *lefko 1* mutant, there is an amino acid substitution of glycine (G) 373 to aspartic acid (D) within the NES motif of the LEFKOTHEA protein, resulting in enhanced nuclear export and therefore abnormal nucleocytoplasmic partitioning [33]. To study the role of LEFKOTHEA protein in the early development of seedlings, we measured the hypocotyl and primary root length of etiolated seedlings. We showed that impaired function of LEFKO protein does not affect the development of dark-grown *lefko 1* seedlings, since no significant differences were observed in hypocotyl and root development (Figure 1c–e). On the contrary, measurements of primary root and hypocotyl length in 5-day-old seedlings grown in light conditions showed that *lefko 1* mutants have longer hypocotyls and shorter roots, showing an aberrant or delayed response during photomorphogenesis (Figure 1f–h), in agreement with the albino cotyledon phenotype.

To further support this notion, we studied stomatal development as a means of evaluation of photomorphogenesis [34]. Although our results showed that *lefko 1* mutants have increased stomatal density, accompanied by increased stomatal precursor density (Figure 1i–k), further investigation revealed decreased stomatal index and increased stomatal precursor index (Figure 1l,m). This inverse relationship between stomatal density and index suggest that the proliferation of epidermal cells exceeds the production of new stomata. Moreover, the increased stomatal precursor index indicates that although the

initial asymmetric divisions are proceeding at rapid rates, the differentiation of precursors to mature stomata does not proceed at the appropriate rate.

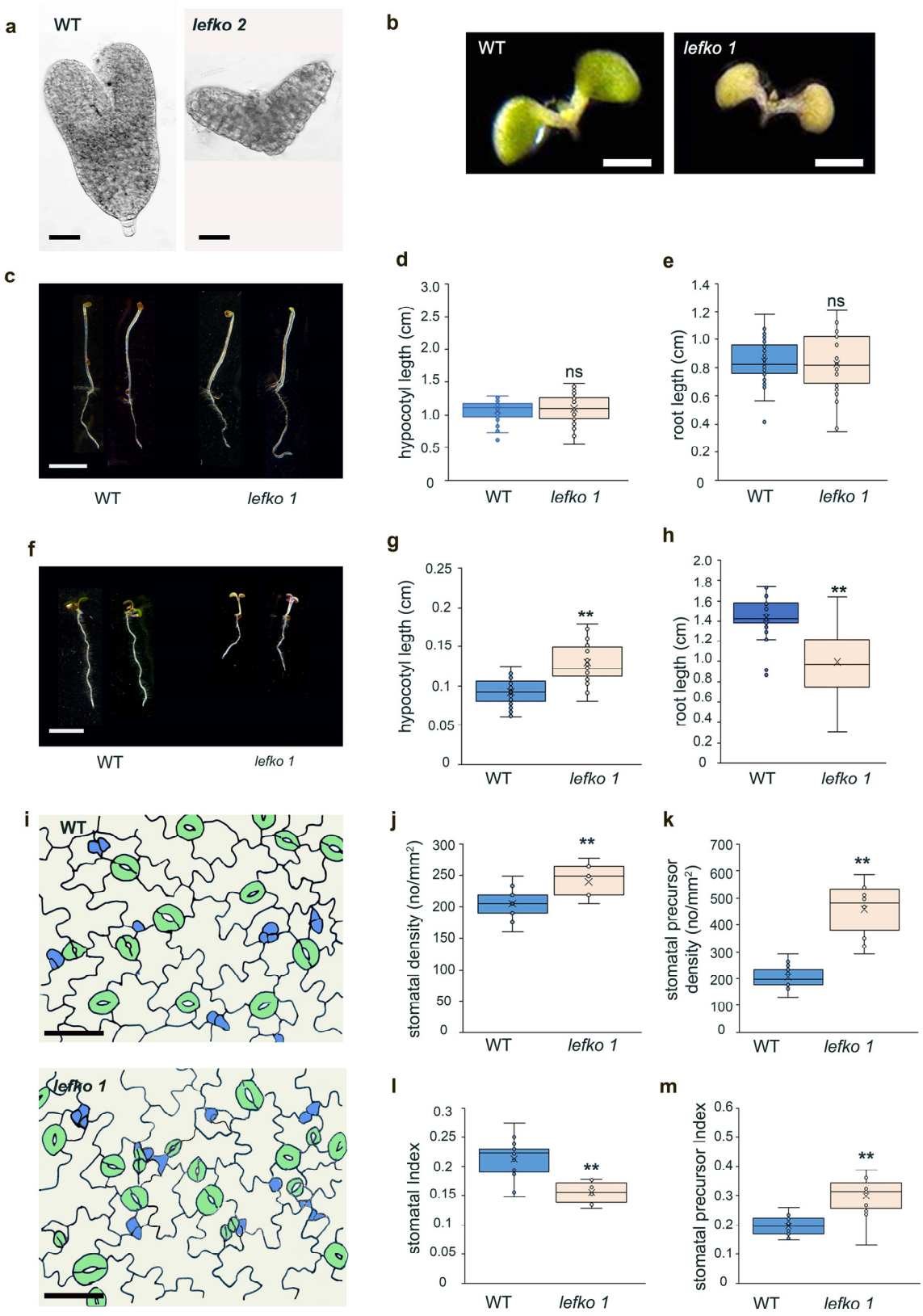


Figure 1. Impaired LEFKOTHEA function leads to aberrant light responses during early seedling development. (**a**) Images of wild-type and *lefko 2* embryos at the heart stage. Scale bar 50 µm. (**b**) Images

of 7-day-old cotyledons of wild-type and *lefko 1*. Scale bars, 0.5 mm. (**c**,**f**) Images of 5-day-old wild -type (**c**) and lefko 1 (**f**) etiolated seedlings. (**d**,**e**) Hypocotyl elongation (**d**) and primary root length (**e**) of 5-day-old etiolated seedlings of the indicated genotypes. (**c**) Images of 5-day-old wild-type and *lefko 1* light-grown seedlings. Scale bar 0.5 cm. (**g**,**h**) Hypocotyl elongation (**g**) and primary root length (**h**) of 5-day-old light-grown seedlings of the indicated genotypes. (**i**) Images of the epidermis of 6-day-old cotyledons of the indicated genotypes. Stomata are colored green, and stomatal precursors are marked with blue. Scale bar: 50 μm. (**j**–**m**) Quantification of stomatal density (**j**), stomatal precursor density (**k**), stomatal index (the ratio of stomata to the total number of epidermal cells; (**l**)), stomata; precursor index (the ratio of the stomatal precursors to the total number of epidermal cells; (**m**)) of the indicated genotypes. In box plots the middle line in the box represents the median, the × shows the mean, the bottom line depicts the 1st quartile, while the top line describes the 3rd quartile; the vertical lines (whiskers) extend to the minimum and maximum value within the 1.5× interquartile range (distance between the 1st and the 3rd quartile). Points outside of the whiskers mark outliers (values outside of the 1.5× interquartile range). Data were analyzed by *t*-test, ** $p < 0.01$, ns: non-significant.

2.2. LEFKOTHEA Loss of Function Leads to Reduced Biosynthesis of Photosynthetic Pigments During the Early Light Response

Chlorophyll biosynthesis is a key process in photomorphogenesis, profoundly influenced by light, that drives the conversion process of key precursors into chlorophyll and tightly regulates the genes and enzymes involved. This coordination is crucial for controlling the transition from heterotrophic growth in darkness to autotrophic growth when seedlings encounter light. This light-triggered reaction is widely considered a "master switch" that initiates chlorophyll production, converts the etioplast into a photosynthetically active chloroplast, and leads to the greening of the seedling [35]. Since our phenotypic analysis showed aberrant development of *lefko 1* seedlings under light, suggesting impaired photosynthesis, we evaluated the biosynthesis of photosynthetic pigments. Chlorophyll a and b showed a biphasic mode of accumulation. The first phase appeared between 20 and 30 min of light encounter, remained almost constant between 30 and 60 min, and then the second phase of gradual accumulation took place (Figure 2a–c). However, in *lefko 1* seedlings the first phase of chlorophyll a and b accumulation was almost obscure, showing a monophasic type of accumulation after 3 h of light encounter (Figure 2a–c), indicating that the response to light in *lefko 1* was delayed. Analysis of carotenoid content revealed a similar mode of accumulation; however, this was significantly reduced in *lefko 1* seedlings compared to the wild type (Figure 2d). Our results clearly indicate the significant role of LEFKOTHEA in photosynthetic pigment biosynthesis during early light response. Furthermore, wild-type plants showed the typical biphasic pattern of chlorophyll biosynthesis, which is characterized by an initial rapid surge that protects etiolated seedlings from photooxidative stress, followed by a secondary, more substantial accumulation phase as the plant transitions to photoautotrophic growth. In *lefko1* seedlings this pattern is disturbed and becomes monophasic. Given that the biphasic nature of chlorophyll synthesis depends on the successful transition from etioplast to chloroplast, the monophasic pattern in *lefko 1* indicates that the mutant has difficulties entering the second phase of chloroplast expansion, which is common in mutants defective in plastid-to-nucleus signaling, such as *gun1* [36].

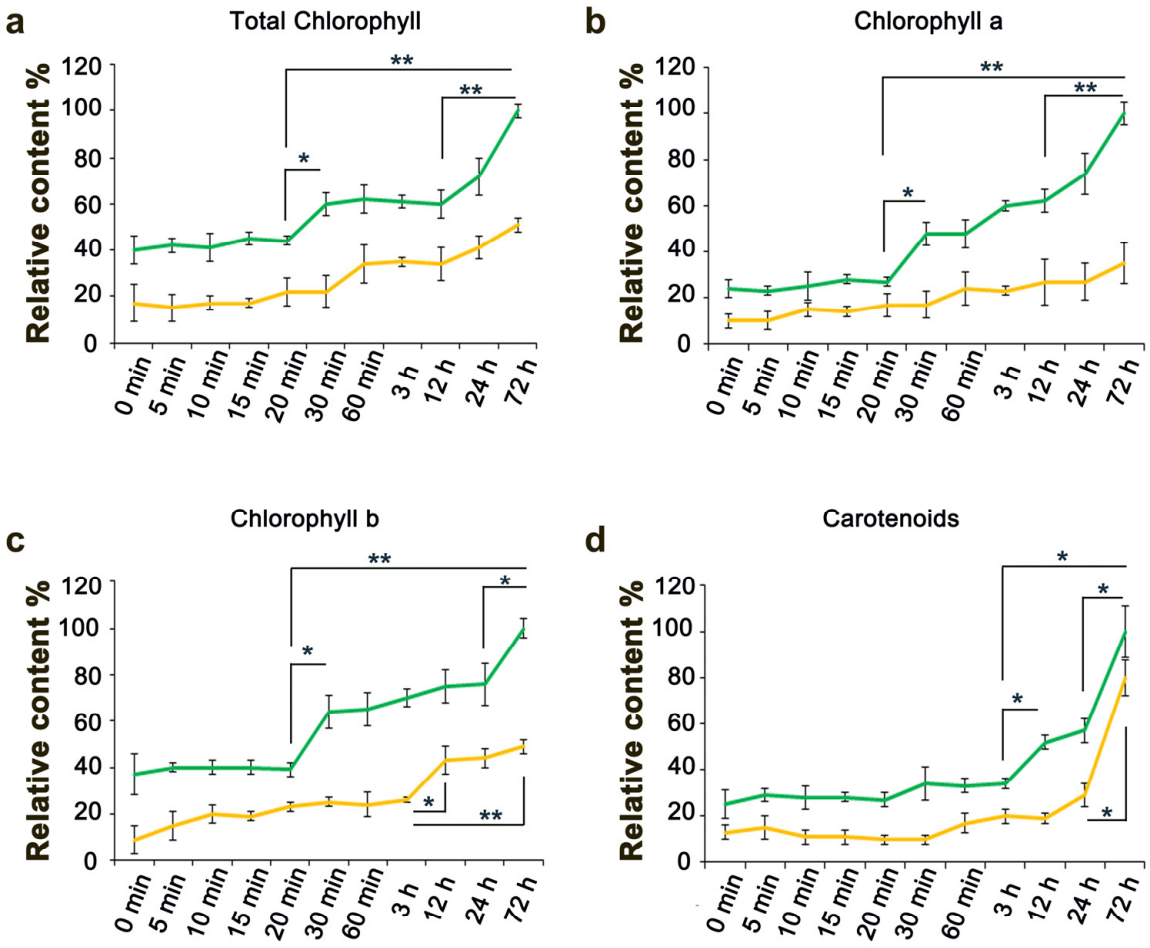


Figure 2. *lefko 1* seedlings exhibit lower and delayed biosynthesis of photosynthetic pigments. (**a**–**d**) Quantification of total chlorophyll (**a**), chlorophyll a (**b**), chlorophyll b (**c**), and carotenoid levels (**d**) in wild-type (green line) and *lefko 1* (yellow line) seedlings after time series exposure to light. Each value is the mean of three independent measurements, and standard deviation is shown by vertical bars. Asterisks indicate the statistically significant differences between the measurements (*t*-test, * $p < 0.05$, ** $p < 0.01$).

2.3. Transcriptomic Data Analysis Reveals an Aberrant Light Response of lefko 1

The development of photosynthetically competent chloroplasts is tightly connected to the exposure to light [37], and it is based on dramatic changes in the transcriptional program in the nucleus [36]. Since chloroplasts have evolved from cyanobacterium-like progenitors, they demonstrate complex development that is coordinated by plastid and nuclear genomes. During the early stages of chloroplast development, the nucleus exerts anterograde control as many of the chloroplastic genes are encoded in the nucleus [38]. Later in development, chloroplasts employ retrograde signaling to regulate nuclear gene expression in an appropriate and coordinated manner [39,40]. Mutations in the signaling from the chloroplast to the nucleus lead to reduced carotenoid heme, chlorophyll biosynthesis, or photosynthesis-associated nuclear genes (PhANGs; [41,42]).

To investigate the response to light of *lefko 1* mutants, we performed transcriptomic analysis. To this end, RNA-seq analysis was performed on 5-day-old dark-grown wild-type and *lefko 1* seedlings exposed to light for 10 min, 1 h, 3 h, 24 h, and 72 h (Figure 3a). In total, 36 RNA samples (i.e., the triplicates of six sampling time points of Col-0 and *lefko 1*) were sequenced using DNBseq platform, generating 13.53 Gb data on average per sample. After read filtering, the clean reads were mapped to the Arabidopsis reference genome with an

average alignment ratio of 99.02%. Similarly, the average alignment ratio to the gene set was 93.87% and in total, 27,035 genes were identified. The uniformity of the mapping result for each sample suggested that the samples were comparable (Supplementary Table S1). Moreover, evaluation of the quality and reproducibility of RNAseq data was performed, as well as PCA analysis for sample clustering and replicate consistency, in addition to mapping statistics per sample (Figures S1 and S2; Supplementary Table S1). The above quality control showed that the RNAseq data can lead to robust patterns of gene expression.

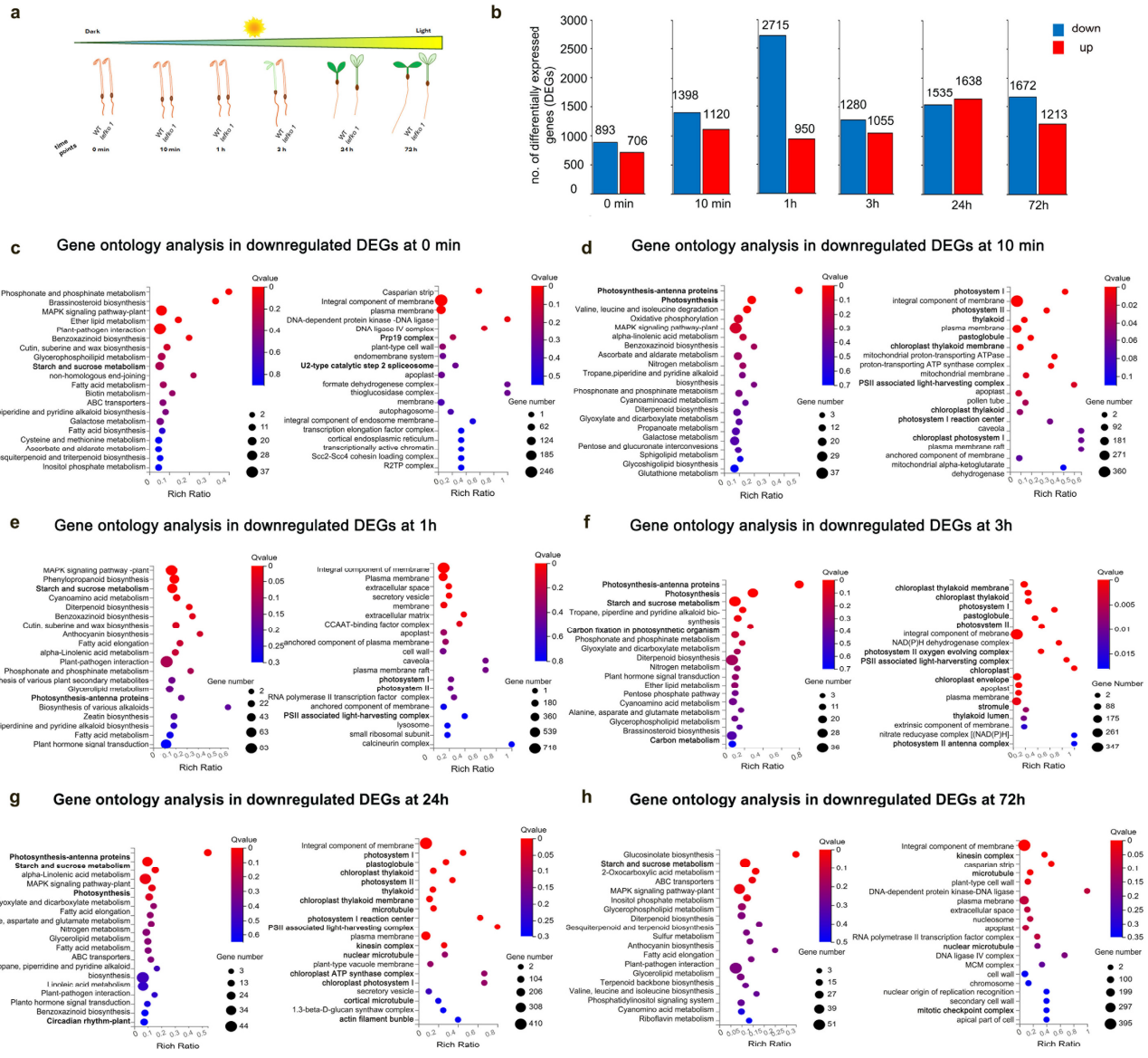


Figure 3. Impaired function of LEFKOTHEA affects the transcriptional output during early light response. (**a**) Experimental design. (**b**) Numbers of upregulated and downregulated differentially expressed genes (DEGs) in the time series transcriptomic analysis. Data are presented as bars showing the DEGs in the pairwise comparison of *lefko 1* versus wild-type seedlings exposed to light for the indicated time. |log2FC| > 1. (**c**–**h**) Bubble charts of the top 20 KEGG pathways and cellular compartment enrichment analysis of downregulated DEGs for time 0 min (**c**), time point 10 min (**d**), time point 1 h (**e**), time point 3 h (**f**), time point 24 h (**g**) and time point 72 h (**h**).

Pairwise comparisons at each time point between wild type and *lefko 1* showed a high number of differentially expressed genes (DEGs) in *lefko 1* at each time point compared to wild type. In detail, at darkness (t = 0 min) and after 10 min exposure to light, we identified 893 and 1398 downregulated genes, while 706 and 1120 genes were upregulated,

respectively (Figure 3b, Supplementary Table S2). After 1 h of light, the number of downregulated genes was significantly increased (2715 versus 950 upregulated), suggesting an increased variance in gene expression between lefko 1 and wild-type seedlings (Figure 3b, Supplementary Table S2). At 3 h, there were 1280 downregulated and 1055 upregulated genes, whereas at 24 h, the upregulated genes in *lefko 1* were slightly increased (1535 downregulated and 1638 upregulated genes; Figure 3b, Supplementary Table S2). Finally, at 72 h of light, our analysis showed 1672 downregulated and 1213 upregulated DEGs in the *lefko 1* versus wild type (Figure 3b, Supplementary Table S2).

To investigate the gene's function, we performed KEGG and GO enrichment analysis of DEGs. The analysis revealed that most of the downregulated DEGs were mainly associated with primary and secondary metabolism pathways at all time points, and with signaling transduction pathways in some cases (Figure 3c–h). In the dark (t = 0 min), while most of the DEGs showed membrane localization; some DEGs are components of the splicing machinery in the nucleus (Figure 3c). During the first 24 h of light exposure (i.e., t = 10 min, 1 h, 3 h, and 24 h), the downregulated DEGs in *lefko 1* were found to be primarily involved in photosynthesis and photosynthetic apparatus (Figure 3d–g). Interestingly, photosynthesis-related terms were not enriched among the downregulated DEGs at the last time point, while in the cellular compartment analysis, membrane and nuclear subcellular parts were overrepresented (Figure 3h).

KEGG analysis showed that upregulated DEGs were enriched in primary and secondary metabolism as well as in signaling pathways at all time points. According to cellular compartment analysis, the cell wall, apoplast, extracellular space, and vacuole were overrepresented in most cases (Figure S3a–f). However, a significant enrichment of pathways involved in photosynthesis and photosynthetic apparatus, as well as in carbon and porphyrin metabolism, was found at the first time point (t = 0), together with chloroplast localization of many upregulated DEGs (Figure S3a). Interestingly, photosynthesis-related terms were absent at the next stages (t = 10 min, 1 h, and 3 h, Figure S3b–d) but were found enriched, along with plant hormone signaling pathways, only at the two last time points (24 h and 72 h, Figure S3e,f). Altogether, our transcriptomic analysis indicates that the need for genes' up- or downregulation depends on the physiological and metabolic processes taking place during light encounter.

2.4. LEFKOTHEA Loss of Function Impacts the Mode of Alternative Splicing Events

In agreement with previous studies, our results showed that light induces a massive reprogramming of gene expression, enabling the transition of the seedling to the autotrophic state after germination. Adaptation of etiolated seedlings to the new light regime is controlled at different levels, including post-transcriptional mechanisms such as alternative splicing that regulates gene expression and protein synthesis, contributing to the diversification of proteins. Since LEFKOTHEA protein is actively involved in the splicing of nuclear pre-mRNA introns as well as chloroplast group II introns [33], we investigated the regulatory potential of alternative splicing in association with transcriptomic changes during the light response of wild-type and *lefko 1* seedlings. In this context, we identified and thoroughly analyzed alternative splicing events that emerged from the transcriptomic analysis.

In plants, several modes of alternative splicing have been identified, such as 3′ splice sites (3′SS), 5′ splice sites (5′SS), mutually exclusive exons (mxe), intron retention (IR), and exon skipping (ES), generating transcript variants encoding for protein isoforms with distinct function, localization, or stability (Figure 4a; [43]). In plants, the most prevalent type of alternative splicing is intron retention [23,44].

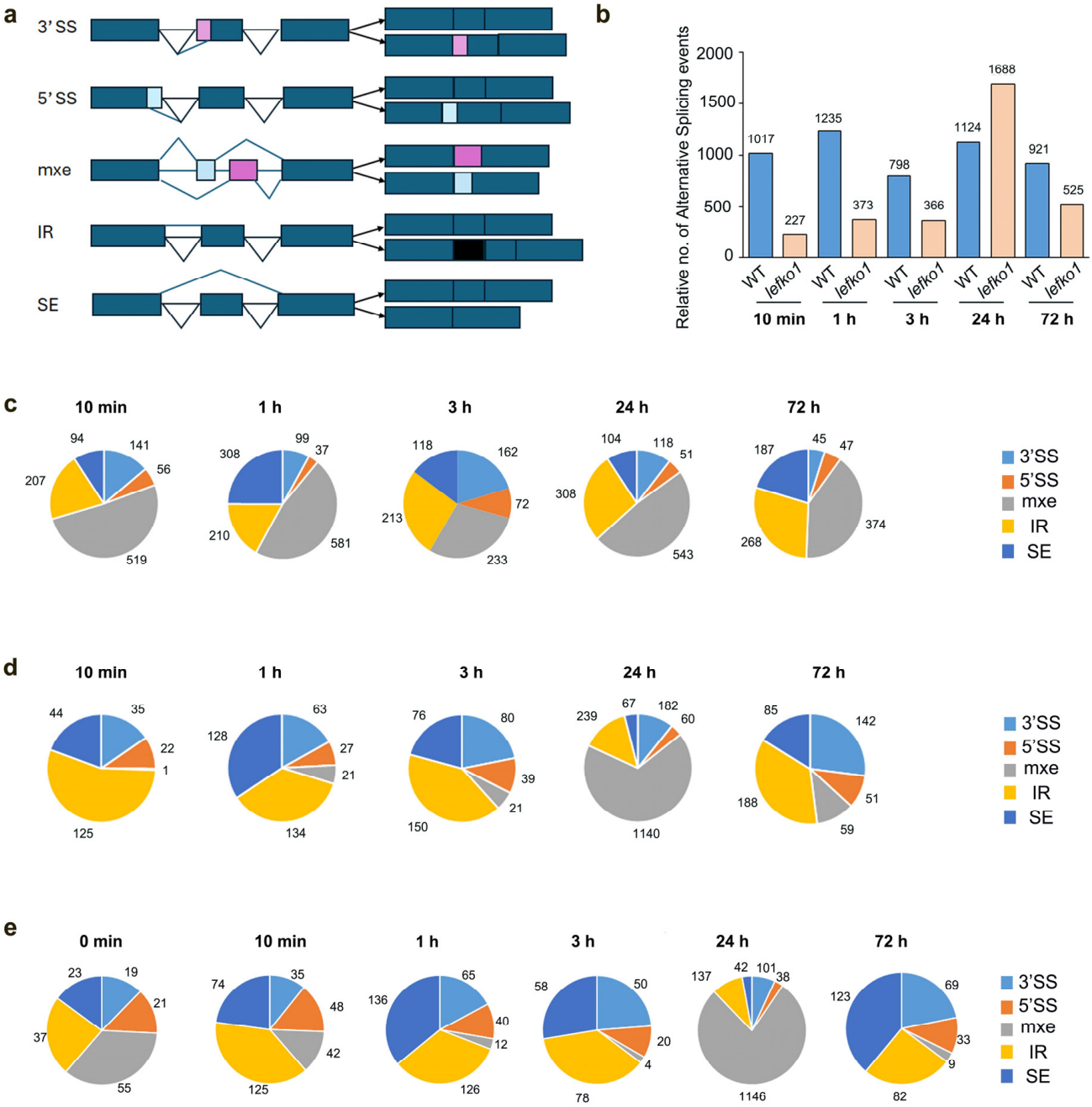


Figure 4. Light and LEFKOTHEA affect the mode of alternative splicing during early seedling development. (**a**) Types of alternative splicing events, arrows show the structure of the produced transcripts. (**b**) Number of alternative splicing events in wild-type and *lefko 1* seedlings in the time series light exposure experiment. FDR < 0.05. Each time point is compared to time point 0 min (dark-grown seedlings). (**c**,**d**) Pie charts illustrating the number of alternative splicing events during the time series exposure to light in wild-type (**c**) or *lefko 1* (**d**) seedlings. The five modes of alternative splicing events, including "3′SS", "5′SS", mxe, IR, and SE, are shown. Each time point is compared to time point 0 min (etiolated seedlings). (**e**) Pie charts showing the types of alternative splicing defects deriving from the pairwise comparison of wild-type versus *lefko 1* seedlings at the indicated time points upon light exposure. FDR < 0.05.

Analysis of the total alternative splicing events at the different time points after exposure to light showed that *lefko 1* seedlings exhibit fewer splicing events compared to wild type at all time points except after 24 h exposure to light (Figure 4b), which agrees with the reported role of LEFKOTHEA in the splicing machinery. Next, we analyzed the modes

of alternative splicing for each genotype at different time points. Our analysis revealed that in wild-type seedlings, mxe and IR are the prevalent types of alternative splicing at all the tested time points compared to the first time point (Figure 4c). Changes in the frequency of the different types of alternative splicing were observed among the tested time points compared to time point 0 (Figures 4c and S4a). Analysis of the alternative splicing modes in *lefko 1* seedlings revealed that the IR mode was the prevailing one with the exception of the 24 h time point, where the mxe mode was the most abundant (Figure 4d). It is worth mentioning that the frequency of the mxe subtype increased from 0.44% after 10 min to almost 6% after 1 h and 3 h exposure to light, abruptly to 68% at 24 h, and then decreased to 11% at 72 h (Figures 4d and S4b).

Next, we compared the modes of alternative splicing at the different time points between wild-type and *lefko 1* seedlings. We observed that the differences in the alternative spliced version of transcripts, representing splicing defects of the *lefko 1* mutant, increased gradually during the time course of the experiment. Specifically, over this series of time points, 155, 324, 379 and 210 splicing differences were detected after 0 min, 10 min, 1 h and 3 h exposure to light, respectively. These differences peaked at 1464 at 24 h and then decreased to 316 at 72 h (Figure 4e). The comparison of wild type versus *lefko 1* at the time point of 0 min showed that most of the differences involved the mxe mode, while the next defective mode of alternative splicing was IR (Figures 4e and S4c). The IR defects increased up to 3 h exposure to light, where there was a significant decrease (Figures 4e and S4c). In contrast, the mxe defects were gradually minimized up to 3 h and then were abruptly increased at 24 h, becoming the major aberrant splicing mode, being minimized again at 72 h (Figures 4e and S4c). Overall, the most notable changes in the number of splicing defects were observed at the 24 h time point (Figures 4e and S4c). Interestingly, a similar analysis of transcriptomic data from 7-day-old cotyledons of wild-type and *lefko 1* light-grown seedlings revealed high levels of IR splicing defects [33]. Many splicing defects were also observed in the 3′SS, 5′SS, and ES alternative splicing modes (Figure 4e). Altogether, we provide evidence that the LEFKOTHEA protein functionally influences light-induced alternative splicing by modulating both the number of splicing events and their specific modes. Moreover, our analysis showed that although the mxe mode is a rare splicing subtype, it is the most significantly affected in the current experimental setup.

2.5. Alternative Splicing Events in Genes Are Distinct from DEGs in lefko 1

Several recent reports underscore the importance of coordination between alternative splicing and transcription in regulating gene expression [45,46]. To shed light on the biological function of genes undergoing alternative splicing during light response, we performed GO analyses of all the genes exhibiting alternative splicing from time point 0 to 72 h (Supplementary Table S3). In wild-type seedlings, pathway GO enrichment revealed overrepresentation of primary and secondary metabolic pathways, like carbon fixation or the citrate cycle. However, spliceosome-related and mRNA surveillance pathways were also present (Supplementary Table S3). Significantly over-represented biological processes included circadian rhythm establishment, as well as mRNA splicing, which agrees with the overrepresentation of the nucleus in the GO enrichment of cellular compartments (Supplementary Table S3).

Next, we performed similar analyses with the specific modes of alternative splicing. Genes undergoing 3′SS alternative splicing were enriched in biological pathways and functions, such asmRNA splicing, RNA processing, and circadian rhythm establishment, and in cellular components including the nucleus and plastids (Supplementary Table S3). Genes with 5′SS alternative splicing were enriched in protein polyubiquitination pathways and in ubiquitin-related complexes (Supplementary Table S3). On the other hand, genes

undergoing mxe alternative splicing were enriched in primary and secondary metabolism and spliceosome pathways, with strong presence of chloroplast/plastid compartments, plasmodesmata and cell junctions (Supplementary Table S3). The RI alternatively spliced genes were overrepresented in ubiquitin-dependent protein degradation, circadian rhythm and post-embryonic developmental processes, and signaling pathways (Supplementary Table S3). Finally, t SE alternatively spliced genes were enriched in spliceosome and RNA splicing processes (Supplementary Table S3).

A similar GO analysis on alternatively spliced genes from light-exposed *lefko 1* seedlings was performed. The analyses revealed a significant enrichment for pathways involved in primary and secondary metabolism, spliceosome function, and RNA editing. Most of the alternatively spliced gene products were localized in the chloroplast or the vacuole (Supplementary Table S3). To fully appreciate the regulatory potential of the specific alternative splicing subtypes in *lefko 1* seedlings under our experimental setup, we also performed GO enrichment analysis for each splicing mode. Interestingly, our analyses revealed almost the same GO terms regarding the pathways, biological functions, and cellular compartments as the wild-type seedlings (Supplementary Table S3), indicating a conserved regulatory mechanism even in the presence of defects in splicing.

Moreover, we examined whether the defective alternatively spliced genes in *lefko 1* seedlings at the different time points of light exposure exhibit differential expression as well. To this end, we classified the splicing defects into two groups: (i) the ones that showed FDR < 0.5 and Inclusion Level Difference > 0 in the comparison of WT versus *lefko 1*, meaning higher splicing frequency in WT than the mutant, and (ii) the ones that showed FDR < 0.5 and Inclusion Level Difference < 0 in the comparison WT versus *lefko 1*, meaning higher splicing frequency in the mutant than WT (Supplementary Table S4).

Subsequently, we isolated a subset of differentially expressed genes (DEGs) that also exhibited significant splicing changes, as defined by a positive or negative Inclusion Level Difference. At the time point of 0 min, we identified 94 splicing defects with Inc. Level Difference > 0, referring to 78 genes from which only 2 belonged to downregulated or upregulated DEGs. The analysis of the defective spliced genes with Inc. Level Difference < 0 showed 61 splicing events referring to 47 genes, from which 4 were downregulated and 3 were upregulated (Figure S5a). Similarly, at time point 10 min, the analysis showed 132 splicing events with Inc. Level Difference > 0 referring to 104 genes, from which 1 was downregulated and 6 were upregulated, while there were 192 splicing events with Inc. Level Difference < 0 referring to 165 genes from which 9 were downregulated and 3 were upregulated (Figure S5b). At the time point of 1 h, the analysis showed the existence of 131 splicing events with Inc. Level Difference > 0, referring to 114 genes, among which 6 were downregulated and 8 were upregulated, while there were 258 splicing events with Inc. Level Difference < 0 concerning 217 genes, from which 20 were downregulated and 4 were upregulated (Figure S5c).

A similar pattern was observed at the time point of 3 h, as we observed 132 splicing events with Inc. Level > 0 affecting 114 genes. Among them, 8 were upregulated and none were downregulated. Similarly, we identified 78 splicing events with negative Inc. Level Difference referring to 64 genes, among which 5 were downregulated and none were upregulated (Figure S5d). At time point 24 h, our analysis showed 1324 splicing events with Inc. Level Difference > 0, referring to 1059 genes, among which 32 were downregulated and 20 of them were upregulated. We identified 160 splicing events with a negative Inc. Level Difference, corresponding to 147 genes, of which 5 were downregulated and 8 were upregulated (Figure S5e). Finally, at time point 72 h, we identified 118 splicing events with a positive Inc. Level Difference affecting 96 genes, including 1 downregulated and 7 upregulated DEGs. In contrast, there were 198 splicing events with a negative

Inclusion Level Difference affecting 169 genes, which included 5 downregulated and 4 upregulated DEGs (Figure S5f). Altogether, our results reveal that genes targeted for expression regulation and genes defective in RNA splicing do not overlap, suggesting a dual mode of regulation of gene expression: at the transcriptional level per se and at the post-transcriptional level—splicing.

2.6. Aberrant Function of LEFKOTHEA Modulates the Interconnection Between Light Response and Alternative Splicing

The alternative splicing process is considered an important regulatory mechanism for plant adaptation to environmental and developmental stimuli. Since in the pairwise comparison of wild-type seedlings versus *lefko 1*, genes targeted for defective splicing events are enriched for GO terms like mRNA splicing via the spliceosome and RNA metabolic processes, we investigated the relationship between defective splicing and light response in *lefko 1*. It is known that many splicing factors are subjected to splicing, adding another level of regulation to alternative splicing mechanisms [47,48]. Indeed, our data showed that among these genes, there are U ribonucleoproteins (U-snRNPs), subunits of the spliceosome complex, as well as SR proteins (Supplementary Table S5). Previous studies have shown that splicing isoforms of SR proteins also regulate alternative splicing [49]. Our results also showed that RNA splicing-related genes exhibit differential expression in *lefko 1* light-exposed seedlings compared to wild type (Figure S6a). Interestingly, the majority of them were downregulated at most time points tested in *lefko 1* seedlings. Therefore, RNA splicing defects in splicing-related genes, in combination with the differential and deregulated expression of other genes involved in RNA editing and metabolism, could be responsible for the aberrant light response of *lefko 1* seedlings.

To test this hypothesis, we checked whether, among the genes with splicing defects in the pairwise comparison of *lefko 1* light-exposed seedling versus wild type, we could identify genes related to light signaling. Our analysis revealed enrichment for GO terms such as circadian rhythm and response to light stimulus, exhibiting defective splicing, among which there were transcription factors like PIF4 and LHY and the negative regulator of light-mediated development DET1 (Supplementary Table S5). Analysis of the differential expression of genes related to light stimulus showed deregulated transcriptional activation of many transcription factors related to light response, like HY5, HYH, PIFs, and BBXs (Figure S6f). Detailed gene expression analysis in wild-type and *lefko 1* seedlings showed downregulation of the transcript levels of *HFR1* and *PIF4*, essential regulators of light signaling [17,18], at the tested time points of light exposure (Figure S6b,c). There was upregulation of *PIL2* at almost all the time points (Figure S6e), while the expression of HY5 was increased at the first time points in *lefko 1* compared to wild-type seedlings; it decreased 1 h after light exposure and increased again later on (Figure S6d). In wild-type plants, HY5 accumulates to activate the transcription of photosynthetic genes [19]. However, in *lefko 1*, *HY5* is upregulated but the photosynthetic genes are downregulated, suggesting that the retrograde signal from the dysfunctional chloroplasts is overriding the positive signal from HY5. The abnormal expression of light-related transcription factors indicates that loss of LEFKOTHEA function likely disrupts the light-signaling pathway that normally degrades PIFs or suppresses their transcription upon light exposure. Under these conditions, the *lefko 1* seedlings remain trapped in a state of skotomorphogenesis even in the light.

Notably, among the genes exhibiting multiple splicing defects, we identified members of the family GLYCINE-RICH PROTEINs (*GRPs*), like *GRP9* and *GRP3* (Supplementary Table S5). GRP9 or PSEUDO-RESPONSE REGULATOR9 (PRR9) is a part of a regulatory loop with LIGHT-REGULATED WD1 (LRWD1) in the plant's circadian clock, involved in the light response and the regulation of the circadian clock [50]. While GRP3 is not directly linked to light response, its expression is modulated by light. In Arabidopsis,

the mitogen-activated kinases (MAPKs) directly regulate alternative splicing by phosphorylating splicing factors such as SCL30 and SKIP [51], highlighting the way that MAPK signaling influences gene expression not only at the transcriptional level but also post-transcriptionally by modifying splicing events. Interestingly, among the genes with many defects in splicing, we identified MEKK1 (MAPK/ERK kinase 1) (Supplementary Table S5), while our transcript expression analysis showed significant enrichment of the MAPK signaling term (Figure 3). We also observed significant transcriptional deregulation of genes related to MAPK signaling, among which we identified several genes involved in stomatal development, such as *SPCH*, *EPF1*, *EPF2*, and *ERL1* (Figure S6c). The fact that *SPCH* is upregulated in *lefko 1* seedlings at most of the time points of our experiment may explain the higher abundance of stomatal precursors in the cotyledons of the mutant (Figures S6g and 1j,k,m).

Photosynthesis was one of the main enriched terms of DEGs in our pathway analysis (Figure 3). Nuclear genes encoding for components of photosystems I and II and the light-harvesting mechanism, such as LHCA1, LHCA4, and PsbP-like proteins, were found to undergo alternative splicing (Supplementary Table S5). Interestingly, the expression of both the nuclear- and the chloroplast-encoded photosynthesis-related genes in *lefko 1* was upregulated at the first time point (t = 0) but immediately downregulated after 10 min of light exposure. The decreased expression level in the mutant was observed until the last time point of the experiment (72 h) (Figure S7). Detailed analysis of gene expression in specific genes related to chloroplast organization such as *PSAD-2* and *PSAO* that encode for photosystem I subunits or *PSB27* and *PSBP-1* encoding for photosystem II subunits showed decreased transcript levels in almost all tested time points in *lefko 1* seedlings (Figure S7b–e). In addition, the expression levels of genes encoding for antenna proteins in light-harvesting complexes, such as *LHCA3*, *LHCB3*, *LHCB6* and *LHCB2.1*, were also reduced at almost all the tested time points in *lefko 1* seedlings (Figure S7f–i). Collectively, our results are in agreement with previous reports [33] and suggest that LEFKOTHEA loss of function leads to arrested chloroplast biogenesis, which is responsible for the low chlorophyll levels as well as for the observed monophasic pattern in chlorophyll biosynthesis in *lefko 1* seedlings.

2.7. Localization of LEFKOTHEA in Root Tips During Early Seedling Development

Since our phenotypic analysis showed defects in the primary root elongation of light-grown but not etiolated seedlings, we checked the expression pattern of the LEFKOTHEA protein using complemented *lefko 1* plants expressing the *pLEFKO::LEFKO-YFP* construct [33]. In 4-day-old etiolated seedlings, LEFKOTHEA protein was detected at the root tips showing a uniform distribution (Figure 5a,b). In light-grown seedlings, the expression of LEFKOTHEA protein was more evident in the root epidermis and the cortex, (Figure 5a,b). In 8-day-old light-grown seedlings, LEFKOTHEA protein level decreased in the root meristematic zone, which was more evident in the central cylinder and the lateral root cap (Figure 5a,b). Quantification of fluorescent signal intensity in two different cell files, such as the epidermis (i) and cortex (ii), showed that LEFKOTHEA protein levels are increased in 4-day-old light grown seedlings and decrease again in 8-day-old light-grown seedlings, suggesting that LEFKOTHEA expression is regulated by the light regime as well as age.

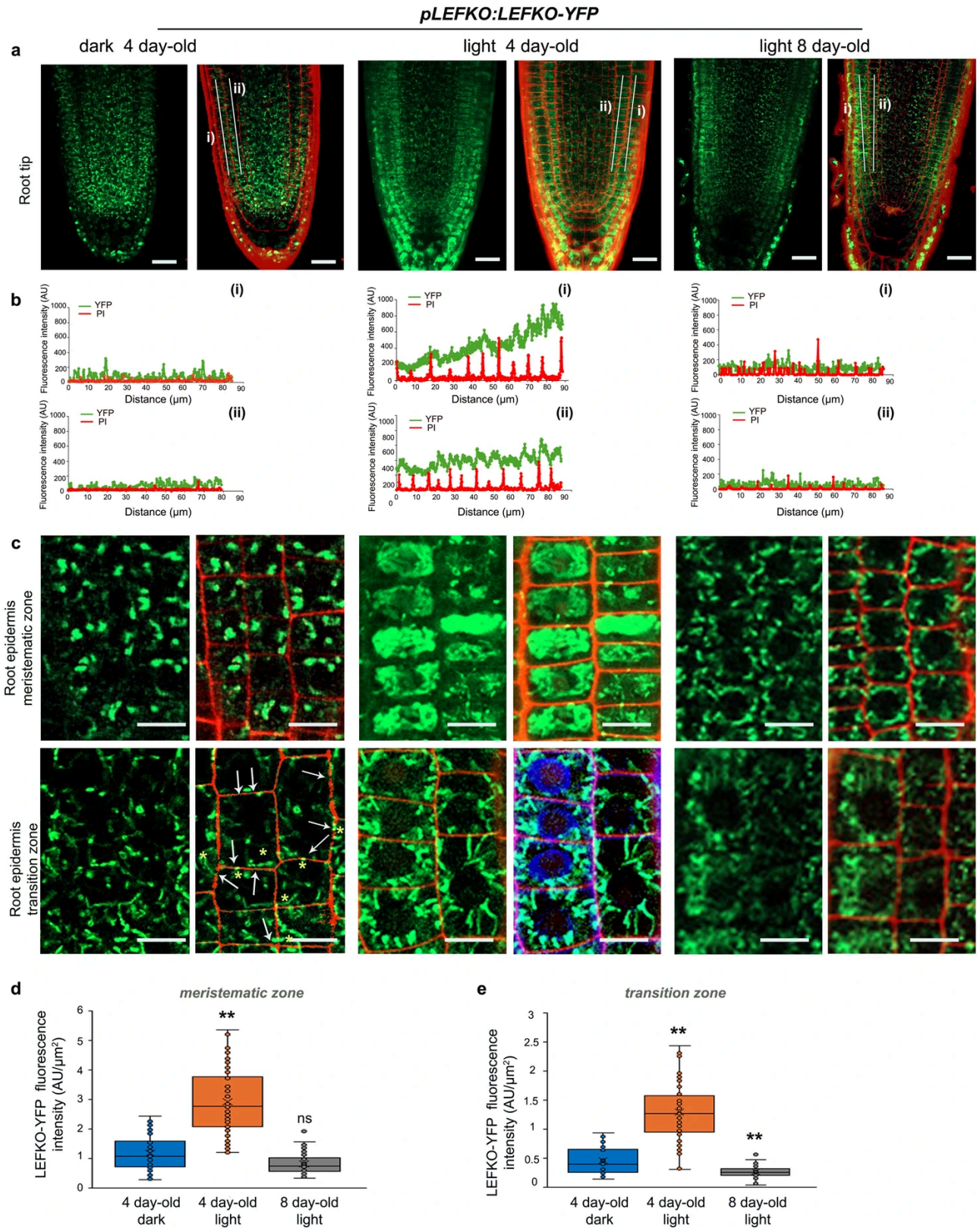


Figure 5. Light does not affect plastid localization of LEFKOTHEA in root tips. (**a**) Expression pattern of LEFKOTHEA in the root tips of transgenic *lefko 1* seedlings expressing *pLEFKO:LEFKO-YFP* grown under the indicated light conditions. Scale bar: 20 μm. (**b**) Diagrams presenting the quantification of the fluorescent signal intensity of the cells marked with white lines in (**a**). The diagrams are prepared in ZenBlue. (**i**) epidermis cell file; (**ii**) cortex cell file. (**c**) Subcellular localization of LEFKOTHEA in

epidermal cells of the meristematic zone and transition zone. Scale bar: 10 μm. (**d**,**e**). Quantification of the fluorescent signal in epidermal cells of the meristematic zone (**d**) or transition zone (**e**) of seedlings grown under the indicated light conditions. Box plots (**d**,**e**) display the first and third quartiles, split by the median; whiskers extend to include the maximum/minimum values. Data were analyzed by one-way ANOVA followed by Tukey test for multiple comparison. *, $p < 0.05$, **, $p < 0.01$, ns: non-significant. Statistical analysis is presented in Supplementary Materials. Propidium iodide (PI) was used for the staining of cell walls to delineate the cell patterns, and in some images, 4′,6-diamidino-2-phenylindole (DAPI) was used for the staining of the nucleus. White arrows indicate plasmodesmata at the cell wall of the respective cells; yellow asterisks indicate the stromule-like tubular extension ending up at the place of plasmodesmata. All the images were produced under the same microscope settings.

Subcellular analysis of the LEFKOTHEA protein expression pattern in the epidermal cells of the root meristematic zone revealed accumulation of the protein in leucoplast-like structures with tubular extensions in 4-day-old etiolated seedlings (Figure 5c). On the contrary, in 4-day-old light-grown seedlings, we observed prominent signal at the cytoplasm (Figure 5b). In 8-day-old light-grown seedlings, LEFKOTHEA protein was mainly localized in leucoplasts-like structures (Figure 5b). Next, we analyzed the expression of LEFKOTHEA in epidermal cells of the transition zone of the root. In etiolated seedlings, LEFKOTHEA abundance was relatively low and localized in leucoplast-like structures with tubular extension ending up at plasmodesmata at the cell wall (Figure 5c). Interestingly, these structures were more evident in 4-day-old light-grown seedlings, where these structures created a network connecting the nucleus to the periphery of the cell (Figure 5c). Later in development, in 8-day-old light-grown seedlings, the protein was still detected in similar structures, but it was less prominent (Figure 5c). Quantification of signal fluorescence in epidermal cells of the meristematic and the transition zone showed induced expression of LEFKOTHEA protein in light and reduction with age (Figure 5d,e). The extraordinary subcellular localization of the LEFKOTHEA protein—characterized by leucoplast-like structures with tubular extensions—facilitates a network between the nucleus and the cell periphery. This spatial arrangement, enhanced by light during early development, aligns with its proposed function as a molecular communication device that synchronizes gene expression in the nucleus and plastid.

2.8. LEFKOTHEA Is Dynamically Distributed in the Nucleus and Chloroplasts During Light Response

In hypocotyl lying under the apical hook of etiolated seedlings, LEFKOTHEA was mainly found in the cytoplasm and stromule-like structures (Figure 6a). After exposure to light for 4 h, live cell imaging showed that LEFKOTHEA accumulated in chloroplasts and stromule-like structures. Magnification of etiolated hypocotyl cells beneath the apical hook revealed LEFKOTHEA protein localized in stromule-like structures. These structures terminated at cell wall sites associated with plasmodesmata (Figure 6d), suggesting a role for the protein in intercellular communication. In light-grown seedlings, the protein was mainly localized in chloroplasts, while its localization at stromule-like structures was less profound (Figure 6a). In petioles of etiolated seedlings, LEFKOTHEA protein accumulated in the nucleus (Figure 6b). While analyzing light impact on LEFKOTHEA localization, we found that exposure to light resulted in depletion from the nucleus to the cytoplasm and its accumulation in chloroplasts and stromule-like structures (Figure 6b,e). In petioles of light-grown seedlings, the protein was localized in the cytoplasm and in chloroplasts surrounding the nucleus (Figure 6b).

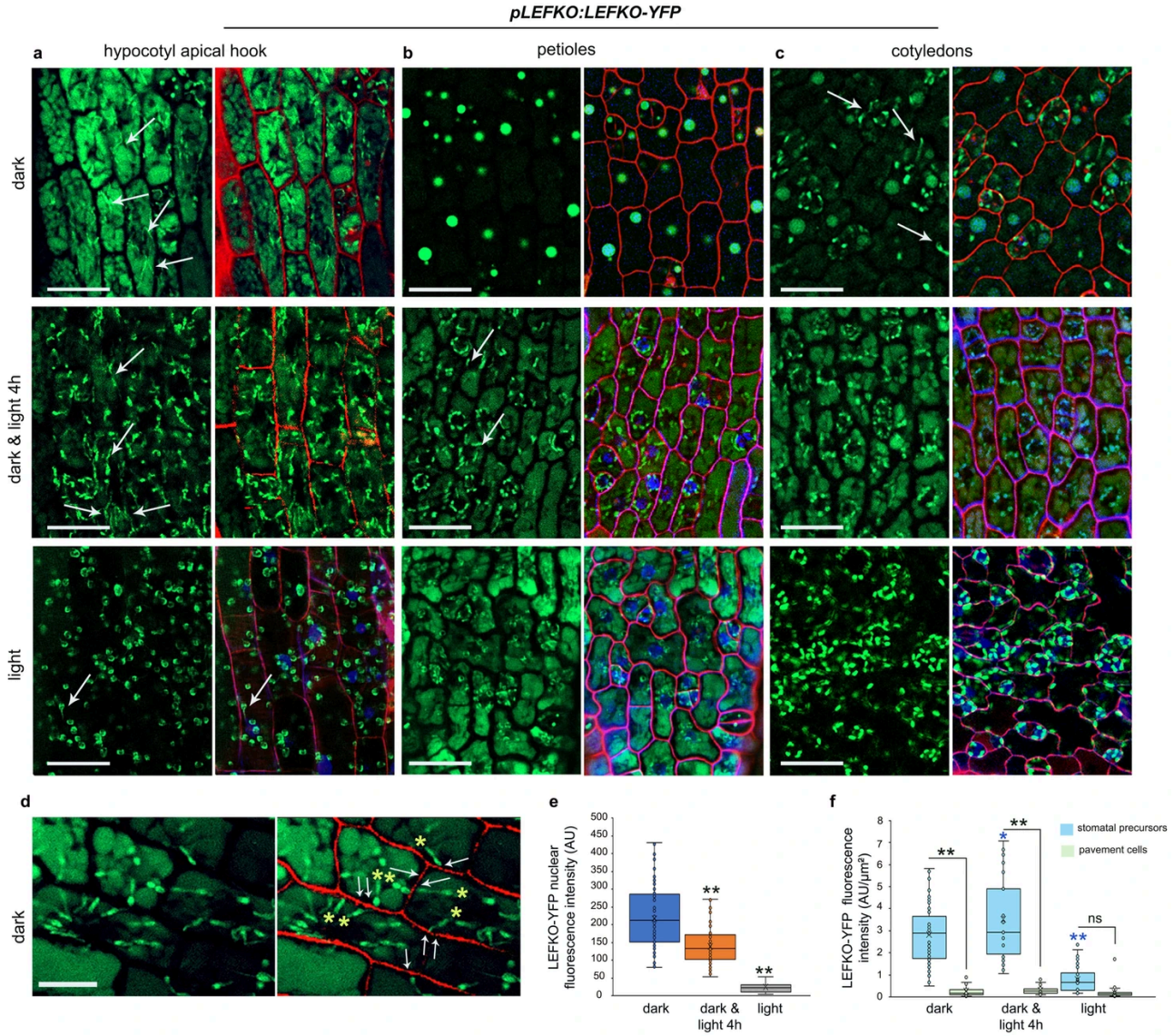


Figure 6. The subcellular distribution of LEFKOTHEA is dynamically regulated by light in aboveground tissues. (**a**–**c**) Subcellular distribution of LEFKOTHEA cells residing at the apical hook (**a**), the petioles (**b**), and the epidermis of cotyledons (**c**) of transgenic *lefko 1* seedlings expressing *pLEFKO:LEFKO-YFP* grown under the indicated light conditions. Arrows indicate the localization of LEFKOTHEA in stromule-like structures. Scale bar: 20 µm. (**d**) Magnification of hypocotyl apical hook cells of dark-grown seedlings. White arrows indicate plasmodesmata at the cell wall; yellow asterisks indicate stromule-like structures ending at plasmodesmata sites. Scale bar: 10 µm. Propidium iodide (PI) was used for the staining of cell walls to delineate the cell patterns, and in some images, 4′,6-diamidino-2-phenylindole (DAPI) was used for the staining of the nucleus. (**e**) Quantification of the nuclear fluorescence signal in petioles cells of seedlings grown at the indicated light conditions. (**f**) Quantification of fluorescence signal in stomatal precursors and pavement cells of seedlings grown under the indicated light conditions. Box plots (**d**,**e**) display the first and third quartiles, split by the median; whiskers extend to include the maximum/minimum values. Data were analyzed by one-way ANOVA followed by Tukey test for multiple comparison. *, $p < 0.05$; **, $p < 0.01$, ns: non-significant. Statistical analysis is presented in Supplementary Materials. All the images were produced using the same microscope settings.

Finally, analysis of LEFKOTHEA subcellular localization in the cotyledon epidermis of etiolated seedlings showed nuclear localization in pavement cells but not in guard cells or stomatal precursor cells (Figure 6c). Exposure to light led LEFKOTHEA protein to the cytoplasm and to chloroplasts. However, in the epidermis of light-grown

seedlings, LEFKOTHEA protein was found to localize at the chloroplasts of cells belonging mainly to the stomatal lineage and less in pavement cells (Figure 6c,f). The localization of LEFKOTHEA in stromule-like structures prompted us to analyze expression of gene coding for stromule-related protein in our transcriptomic data. Our analysis showed a significant transcriptional deregulation in genes coding for proteins localized in stromules (Figure S8). Among them we identified nuclear genes encoding for proteins responsible for plastid division and development, such as CPN60 [52], genes encoding for RNA-binding proteins, like CP29 [53] or CRB which link circadian rhythm to plastid function [54], and many genes related to the photosynthetic apparatus or photosynthesis-related metabolic processes, like HCEF1, SHM, GAPB, RCA [55–58]. Taken together, these results demonstrate that light is a crucial regulator of LEFKOTHEA subcellular localization in a cell-type and differentiation-dependent manner. Our data collectively suggests that the nuclear localization of LEFKOTHEA is linked to skotomorphogenesis in specific above-ground tissues. However, when these tissues are exposed to light for at least 4 h, LEFKOTHEA is transiently absent from the nucleus and accumulates in chloroplasts.

Stromules are dynamic, plastid stroma-filled tubular extensions that increase the surface area of the envelope and extend the reach of the plastids within the plant cell. They are involved in plant stress responses and signaling pathways, forming connections to other organelles like the nucleus, mitochondria, and endoplasmic reticulum. Thus, stromules act as communication conduits, allowing chloroplasts to effectively signal their physiological status and coordinate responses with the rest of the cell, especially during stress or developmental changes [59,60]. In this respect, LEFKOTHEA protein localization in stromules is in line with its proposed role as a molecular communication device that synchronizes gene expression in the nucleus and chloroplast.

3. Discussion

Light acts both as an energy source controlling photosynthesis but also as an environmental signal shaping plant growth and development. In addition to extensive transcriptional control by modulating the expression of thousands of genes, light also governs post-transcriptional regulation by shaping the alternative splicing profiles of numerous genes [61]. Previous studies support the essential role of alternative splicing in photomorphogenic processes occurring during early seedling development [61–65]. The regulation of transcription and pre-mRNA splicing is essential for transcript diversity and the expression of light-regulated genes. However, while many transcription factors controlling these processes have been identified, only a handful of splicing factors have been linked to photomorphogenic pathways.

Alternative splicing is a post-transcriptional regulatory mechanism that provides transcriptome plasticity, necessary for optimal plant development in response to environmental cues [66]. In addition, environmental stimuli modulate the function of splicing factors and regulators, leading to the production of diverse splicing variants [66]. Recent studies have highlighted the role of specific splicing factors or regulators in the adaptation of plants to changing light regimes by modulating the production, abundance, and levels of splicing variants [64].

Here, we describe the role of LEFKOTHEA in the regulation of transcriptional and post-transcriptional mechanisms controlling early light response during the development of Arabidopsis seedlings. LEFKOTHEA, a nuclear-encoded RNA-binding protein, coordinates chloroplast group II intron and nuclear pre-mRNA splicing through its physical interactions with components of the chloroplastic group II intron complex and factors involved in the nuclear splicing mechanisms. Nucleocytoplasmic partitioning and chloroplast allocation enables LEFKOTHEA to ensure successful chloroplast maturation and

seedling development [33]. While light promotes rapid chlorophyll accumulation during chloroplast structure establishment, followed by sustained increase during chloroplast proliferation [35]; in this study, the *lefko2* mutant showed defects in chlorophyll levels relative to WT. Since the development of photosynthetic capacity relies on efficient chloroplast biogenesis, it is anticipated that disruptions in chlorophyll biosynthesis stall chloroplast differentiation during de-etiolation in *lefko2* seedlings.

Our analysis reveals that the alternative splicing defects in the *lefko 1* mutant are observed in the nucleus immediately after light exposure, showing that it modulates the splicing of splicing factors via a feedback loop of splicing events that occur when LEFKOTHEA undergoes an enhanced nuclear export. At the same time, its constant localization to chloroplasts upon light exposure ensures the normal splicing of chloroplastic group II introns. Further, our results demonstrate that LEFKOTHEA regulates the frequency and the mode of alternative splicing events which, in turn, affects the transcript levels of genes involved in photomorphogenic processes, while its subcellular localization and distribution depend on the cell type and light conditions.

To understand the landscape of alternative splicing regulation in response to light, and specifically the role of LEFKOTHEA in this regulation, we identified the differentially spliced genes in a time series experiment in wild-type and *lefko 1* seedlings gradually exposed to light. While intron retention (IR) is typically the dominant mode of alternative splicing in plants, we observed a high prevalence of mutually exclusive exons (mxe) during light exposure in wild-type seedlings. The high percentage of mxe splicing mode was evident, with almost stable frequency at all the tested time points in wild-type seedlings. In *lefko 1* mutants, however, mxe frequency fluctuated significantly, peaking abruptly at 24 h before declining. This suggests that LEFKOTHEA regulates splicing factors through light-induced redistribution; notably, at the 24 h mark, the protein is no longer detectable in the nucleus, coinciding with the observed splicing defects. This influence is transient, as mxe events become nearly undetectable by 72 h and in 7-day-old cotyledons [33].

Mxe is considered a rare subtype of alternative splicing in which two or more splicing events are not independent but are executed or disabled in a coordinated manner, resulting in the retention of one exon and the splicing out of the other [67]. The mxe splicing mode results in protein isoforms with the same size, given that the introduced sequence has the same size and a stop codon is not introduced. Through mxes, proteins can acquire highly specific functional alterations without compromising their structural scaffold. This is achieved via minor sequence changes that preserve the spatial fold, a mechanism that provides critical advantages in proteins like ion channels [68]. In animals, mxe mode is more common and highly regulated, particularly in invertebrates like *Drosophila melanogaster*, where *Dscam* genes have clusters with dozens of mutually exclusive exons [69]. Intriguingly, in plants, while rarer, mxe impacts genes involved in crucial processes such as stress responses and developmental processes, supporting the role of alternative splicing in plant adaptation to environmental conditions [70–72].

The subcellular localization and distribution of a protein are tightly connected to its function, since they provide the correct cellular environment and molecular partners. Analysis of LEFKOTHEA's subcellular localization in different tissues, cell types, and under different light regimes was indicative of the function of the protein. Our results showed that in the underground part of the plant, LEFKOTHEA is expressed in the root meristematic zone, which is in line with the fact that knockout mutants exhibit embryo lethality as they fail to develop the embryonic root [33]. In the primary root, LEFKOTHEA is localized in leucoplast-like structures with tubular extensions. Since LEFKOTHEA rapidly exports from the nucleus, its localization in this specific compartment is difficult to detect. Light does not seem to have a significant impact on the subcellular distribution of the protein in

the primary root, although LEFKOTHEA protein levels increase under light conditions. In contrast to what was observed in the root, in the above-ground tissues, light seems to be a significant determinant of LEFKOTHEA subcellular localization, particularly in specific cell types. The most prominent change in the subcellular localization of LEFKOTHEA was observed in young petiole cells of etiolated and light-grown seedlings, where the protein was found to localize at the nucleus or at the chloroplasts, respectively. The nuclear localization of LEFKOTHEA protein in the dark indicates that the protein acts as a repressor of photomorphogenesis or as a regulator of chloroplast division/differentiation, keeping the plant in a "dark" developmental program. The nuclear depletion of LEFKOTHEA after light exposure removes the repression, allowing the transcriptional activation of photosynthetic genes.

Stromules, which are dynamic, tubular extensions of chloroplasts, form in response to light or under stress and play a role in signal transmission from the chloroplast to other parts of the cell, such as the nucleus and plasmodesmata [59,60]. The detection of LEFKOTHEA, an RNA splicing factor, in stromule-like structures raises key questions for its functional role within these unique compartments. In addition, the localization of LEFKOTHEA in stromule-like structures which aligns with plasmodesmata at the cell wall was evident, especially after exposure of dark-grown plants to light. This localization points toward a potential function in cell-to-cell signaling. Based on our results, we propose that LEFKOTHEA—targeted to both the nucleus and chloroplast—acts as a molecular module that synchronizes gene expression by modulating nuclear pre-mRNA and plastid group II intron splicing. In *lefko 1* mutants, the disruption of this dual targeting causes a post-transcriptional bottleneck and creates incorrect or confusing signals between organelles. This uncoupling of nuclear transcription from chloroplast assembly prevents the plant from accurately responding to light stimuli during early development to become photosynthetically efficient.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Arabidopsis thaliana wild-type ecotype Columbia-0 (Col-0), mutant *lefko 1*, and transgenic *lefko 1*; *pLEFKO:LEFKO-YFP*-expressing seeds were surface-sterilized and sown on Petri dishes containing 0.5x Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands), pH 5.7, supplemented with 1% sucrose, and solidified with 0.4% phytagel (Sigma, St. Louis, MO, USA). After 24 h of stratification at 4 °C, seedlings were positioned to grow vertically at 22 °C in a Phytotron growth chamber (Weiss, Buchen, Germany) under a long photoperiod with 16 h of light and 8 h of darkness per day and 100 mmol m^{-2} s^{-1} light intensity. For dark-to-light transition experiments, the seedlings were grown vertically in the dark for the specified time. Plates were digitally photographed, and the ImageJ software(ImageJ -win64) was used for hypocotyl and root measurements.

4.2. Extraction and Quantification of Photosynthetic Pigments

Photosynthetic pigments were extracted from Arabidopsis seedlings at the mentioned time points by incubation with DMSO for 30 min at 65 °C. The O.D. of each extract was determined, and pigments concentrations (mg/gr fresh weight) were calculated using the equations given in [73]. Three independent experiments were performed for each sample.

4.3. Transcriptomic Analysis

Wild-type and *lefko 1* mutants were germinated in the dark for 5 days, and total RNA was isolated at various time points (i.e., 0 min, 10 min, 60 min, 3 h, 24 h and 72 h) after cotyledons encounter light by using the phenol-sodium dodecyl sulfate (SDS) extraction

method. RNA concentrations were determined spectrophotometrically and verified by ethidium bromide staining on agarose gels. DNA was eliminated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). Afterwards, the cDNA libraries were constructed and sequenced in the DNBSEQ platform (BGI, Hong Kong, China). Raw sequencing data were filtered using SOAPnuke software (version 1.5.6) to exclude low-quality reads, and the clean reads were mapped onto the Arabidopsis reference genome (TAIR10) by means of HISAT2 software (version 2.2.1). Clean reads were aligned to the reference gene set using Bowtie2 (version 2.4.5). Gene expression level was calculated with RSEM (version 1.3.1), and differential expressed genes (DEGs) were identified with DESeq2 (version 1.34.0) using $|\log2\text{Fold Change}| > 1$ and Qvalue < 0.05 as thresholds. Alternative splicing and differential alternative splicing were detected by using rMAT (version 3.2.5). To further explore gene functions, GO (http://www.geneontology.org/) and KEGG (https://www.kegg.jp/) enrichment analysis was performed on differential expressed genes and differential alternative spliced genes.

4.4. Confocal Microscopy

Transgenic lines carrying the GFP fluorescent marker were imaged with a Zeiss LSM880 inverted confocal microscope(Zeiss, Oberkochen, Germany) in Airyscan mode, using a dry 20X/0.8 M27 Plan-Apochromat objective. Seedlings of the indicated age growing under specific conditions were mounted in MS after staining with Propidium Iodine (PI, Thermo Fisher Scientific, Waltham, MA, USA) and imaged. Settings were constant between samples. Laser excitation for the PI channel was set to 568 nm with emission at 600 nm, and the laser excitation for the GFP channel was set at 488 nm with emission at 509 nm. The processing of the images was performed using Zen 3.1 Blue software (Zeiss, Oberkochen, Germany).

4.5. Quantification and Statistical Analysis

Hypocotyl, root length, stomatal density, and stomatal and precursor indexes of seedlings were measured with ImageJ2 software. All experiments were performed three times. For tests that involve pairwise comparisons, Student's *t*-test was used to assess the statistical significance of the differences. The statistical analysis was performed using R (R version 4.2.2). Graphs were created in Microsoft Excel 16. Heatmap samples were generated by using the Perseus software (version 1.6.15.0).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants15050772/s1, Figure S1: Quality evaluation of RNAseq data. Figure S2: Assessment of RNAseq sample similarity, normalization effectiveness, identification of potential outliers, and assessment of sample clustering and quality. Figure S3: Gene ontology enrichment analysis of the upregulated DEGs in the time series transcriptomic analysis of wild-type and *lefko 1* seedlings. Figure S4: Analysis of the frequencies of the alternative splicing events in wild-type and *lefko 1* seedlings. Figure S5: Splicing events in genes are distinct from DEGs in *lefko 1* seedlings. Figure S6: Impaired function of LEFKOTHEA affects the expression of genes involved in RNA splicing, MAPK signaling, and light response pathways. Figure S7: LEFKOTHEA affects the expression of genes involved in photosynthesis and the structure of the photosynthetic apparatus. Figure S8: LEFKOTHEA regulates the expression of genes encoding proteins that localize at stromules. Table S1. RNAseq data statistical analysis. Table S2: Differentially expressed genes (DEGs) between *lefko 1* and wild-type Col-0 plants in each of the six sampling time points. Table S3: KEGG and GO pathway analysis of total alternative splicing events identified in all *lefko 1* and Col-0 samples. Table S4: The alternative splicing events identified in all *lefko 1* and Col-0 samples and in the pairwise *lefko 1* to Col-0 comparisons at each time point. Table S5: The alternative splicing events in the *lefko 1* to Col-0 pairwise comparison at each time point.

Author Contributions: A.A., D.S., L.R., K.P. and G.D. performed the experiments; A.A. and D.S. analyzed the data; A.A., D.S. and P.H. wrote the paper; P.H. conceptualized the work; S.R., D.M. and K.H. reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Next-generation RNA sequencing raw and processed data has been deposited into the Gene Expression Omnibus (GEO) with accession number GEO: GSE313266.

Conflicts of Interest: The authors declare no conflicts of interest.

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