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A long photoperiod relaxes energy management in *Arabidopsis* leaf six



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ABSTRACT

Plants adapt to the prevailing photoperiod by adjusting growth and flowering to the availability of energy. To understand the molecular changes involved in adaptation to a long-day condition we comprehensively profiled leaf six at the end of the day and the end of the night at four developmental stages on *Arabidopsis thaliana* plants grown in a 16 h photoperiod, and compared the profiles to those from leaf 6 of plants grown in a 8 h photoperiod. When *Arabidopsis* is grown in a long-day photoperiod individual leaf growth is accelerated but whole plant leaf area is decreased because total number of rosette leaves is restricted by the rapid transition to flowering. Carbohydrate measurements in long- and short-day photoperiods revealed that a long photoperiod decreases the extent of diurnal turnover of carbon reserves at all leaf stages. At the transcript level we found that the long-day condition has significantly reduced diurnal transcript level changes than in short-day condition, and that some transcripts shift their diurnal expression pattern. Functional categorisation of the transcripts with significantly different levels in short and long day conditions revealed photoperiod-dependent differences in RNA processing and light and hormone signalling, increased abundance of transcripts for biotic stress response and flavonoid metabolism in long photoperiods, and for photosynthesis and sugar transport in short photoperiods. Furthermore, we found transcript level changes consistent with an early release of flowering repression in the long-day condition. Differences in protein levels between long and short photoperiods mainly reflect an adjustment to the faster growth in long photoperiods. In summary, the observed differences in the molecular profiles of leaf six grown in long- and short-day photoperiods reveal changes in the regulation of metabolism that allow plants to adjust their metabolism to the available light. The data also suggest that energy management is in the two photoperiods fundamentally different as a consequence of photoperiod-dependent energy constraints.

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1. Introduction

Plants as light-dependent, autotrophic organisms have adapted to the regular light–dark cycles resulting from the rotation of the earth. The length of the light period, or photoperiod, depends on the latitude and time of the year. Plants must adjust to changes in day-length to optimize growth in varying photoperiod lengths. Although this requires tight control of physiological and molecular processes, the underlying regulatory mechanisms are still poorly understood. It is now well established that the circadian clock synchronizes metabolism with the changing photoperiods [1–4]. Photoperiod length affects net daily photosynthesis and starch metabolism [5,6] and adjusts seasonal growth [7–9]. However, the molecular integration of photoperiod, clock and metabolic control during leaf development remains a challenging problem.

Arabidopsis is a facultative long-day plant whose flowering is controlled by the photoperiod pathway [7,8,10,11] in concert with molecular, hormonal and environmental signals [10]. Interactions between the circadian clock and photoperiod length during vegetative growth affect leaf number and size, as well as their morphological and cellular properties [12–16]. Plants in which the vegetative to floral growth transition is accelerated by increasing day-length or repression of regulatory genes have fewer leaves, increased single leaf areas, and a higher epidermal cell number in individual leaves compared to late flowering plants [12,15,16]. While these adaptations to photoperiod are well documented at the phenotypic level, little is known about how concerted regulation of photoperiod-dependent gene expression and protein levels is achieved during diurnal cycles and at different stages of leaf development.

We therefore asked how phenotypic changes are related to molecular profiles in a single leaf of *Arabidopsis* plants growing in a long-day (LD; 16 h light, 8 h dark) or short-day (SD; 8 h light, 16 h dark) condition. These two photoperiods cause consistent phenotypic changes in the number and morphology of successive leaves on the rosette [12,16]. Because size and shape of successive leaves vary during *Arabidopsis* development [17] we decided to focus the analysis on leaf number 6, which is the first adult leaf of the *Arabidopsis* (Col-4) rosette in short-day conditions. Leaf 6 was used previously to generate molecular data for *Arabidopsis* grown in SD [18]. To gain insights into the molecular pattern underlying the phenotypic changes between photoperiods, we therefore analyzed

transcript and protein levels of leaf number 6 grown in LD at four developmental stages, both at the end of the day (EOD) and end of the night (EON). We then compared the data with the corresponding previously established molecular data for leaf 6 of *Arabidopsis* grown in SD either under optimal watering (SOW) or a 40% water deficit (SWD) [18]. Integration and comparative analyses of the quantitative proteomics and transcriptomics data revealed that fewer genes have significant diurnal transcript level fluctuations in LD than SD. Transcripts and proteins with significantly different levels in SD and LD validate the hypothesis that a short photoperiod requires a tight energy management, which is relaxed in a long photoperiod.

2. Material and methods

2.1. Plant material, leaf 6 and rosette growth measurements

Arabidopsis thaliana accession Col-4 (N933) plants were grown in a growth chamber equipped with the PHENOPSIS automaton [19] as described previously [18] with the exception that day length in the growth chamber was fixed at 16 h. In brief, seeds were sown in pots filled with a mixture (1:1, v/v) of a loamy soil and organic compost at a soil water content of 0.3 g water/g dry soil and just before sowing 10 ml of a modified one-tenth-strength Hoagland solution were added to the pot surface. After 2 days in the dark, day length in the growth chamber was adjusted to 16 h at $\sim 220 \mu\text{mol}/\text{m}^2/\text{s}$ incident light intensity at the canopy. Plants were grown at an air temperature of 21.1 °C during the light period and 20.5 °C during the dark period with constant 70% humidity. During the germination phase water was sprayed on the soil to maintain sufficient humidity at the surface. Beginning at plant germination, each pot was weighed twice a day to calculate the soil water content, which was adjusted to 0.4 g water/g dry soil by the addition of appropriate volumes of nutrient solution. The experiment was repeated independently three times and each leaf 6 sample was prepared by bulking material from numerous plants. The frozen plant material was sent to the MPI in Golm, where it was ground and aliquotted using a cryogenic grinder (German Patent No. 8146.0025U1).

Growth-related traits of leaf 6 at single leaf and cellular scales were measured as described [20]. Five rosettes were harvested and dissected every 2–3 days during each experiment. Leaf 6 area [mm^2] was measured after imaging with a binocular magnifying

($\times 160$) glass for leaves smaller than 2 mm^2 or with a scanner for larger ones. A negative film of the adaxial epidermis of the same leaf 6 as the one measured in surface was obtained after evaporation of a varnish spread on its surface. These imprints were analyzed using a microscope (Leitz DM RB; Leica) supported by the image-analysis software Optimas. Mean epidermal cell density [cells mm^{-2}] was estimated by counting the number of epidermal cells in two zones (at the tip and base) of each leaf. Total epidermal cell number in the leaf was estimated from epidermal cell density and leaf area. Mean epidermal cell area [μm^2] was measured from 25 epidermal cells in two zones (at the tip and base) of each leaf.

For rosette growth measurements, at each date of harvest all leaves with an area larger than 2 mm^2 from five rosettes were imaged with a scanner. The number of leaves was counted and total rosette area was calculated as the sum of each individual leaf area measured on the scan with the Image J software.

2.2. Carbohydrate determinations

Starch, glucose, fructose and sucrose content were determined by enzymatic assays in ethanol extracts of 20 mg frozen plant material as described in Cross et al. [21]. Chemicals were purchased as in Gibon et al. [22]. Assays were performed in 96 well microplates using a Janus pipetting robot (PerkinElmer, Zaventem, Belgium). Absorbances were determined using a Synergy microplate reader (Bio-Tek, Bad Friedrichshall, Germany). For all the assays, two technical replicates were determined per biological replicate.

2.3. Tiling array transcript data and quantitative iTRAQ proteomics data

Gene expression in leaves of the four developmental stages and at the two diurnal time points in the long day optimal water (LD) experiment and in a reference mixed rosette sample was profiled as described previously [18] using AGRONOMICS1 microarrays [23] and analyzed using a TAIR10 CDF file [24]. All \log_2 -transformed sample/reference ratios without p -value filtering were used in the analyses. Microarray raw and processed data are available via ArrayExpress (E-MTAB-2480).

Proteins in the same samples were quantified using the 8-plex iTRAQ isobaric tagging reagent [25,26] as described in detail previously [18] according to the labelling scheme in Supporting Table S5. The resulting spectra were searched against the TAIR10 protein database [27] with concatenated decoy database and supplemented with common contaminants with Mascot (Mascot Science, London, UK). The peptide spectrum assignments were filtered for peptide unambiguity in the pep2pro database [28,29]. Accepting only unambiguous peptides with an ion score greater than 24 and an expect value smaller than 0.05 resulted in 70 979 assigned spectra at a spectrum false discovery rate (FDR) of 0.07%. Quantitative information for all reporter ions was available in 50 947 of these spectra leading to the quantification of 1788 proteins based on 6178 distinct peptides (Supporting Table S6). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [30] with the dataset identifier PXD000908 and DOI 10.6019/PXD000908. The data are also available in the pep2pro database at www.pep2pro.ethz.ch

All proteome and transcriptome abundance measures for the LD experiment were integrated within the existing AGRON-OMICS database (LeafDB) [18]. A searchable web-interface containing these integrated data sets is available at <https://www.agronomics.ethz.ch/>

2.4. Statistical analyses of the protein and transcript changes

The statistical analytical methods were performed as described previously [18] subjecting the \log_2 -transformed sample/reference ratios to an analysis of variance (ANOVA) treating stage (S) and day-time (ND) as main effects followed by correction with Benjamini-Hochberg [31]. Transcripts and proteins with a pGlobal (p -value for an overall global change) < 0.05 and a maximum fold-change $> \log_2(1.5)$ were considered to change significantly (Supporting Tables S7 and S8). For a significant difference between EOD and EON we additionally required pND (p -value for the diurnal change) < 0.05 . The comparison of the protein and transcript levels between the LD and the two short day (SOW and SWD) experiments reported previously was performed with a paired t -test comparing the values for the 8 time-points between two experiments corrected with Benjamini-Hochberg [31] taking into account all non-plastid encoded transcripts without p -value filtering. All statistical analyses were performed using R [32].

2.5. GO functional classification

Assignment of protein and transcript functional categories was based on the TAIR GO categories from aspect biological process (ATH.GO.GOSLIM_20130731.txt) as described previously [18].

3. Results and discussion

3.1. LD accelerates Arabidopsis growth and increases individual leaf area but decreases rosette area

When plotted against time from leaf initiation to full expansion, leaf 6 area increased more rapidly and reached its final size earlier and was 50% larger in LD than in SD (Fig. 1A). The dynamics of cell production and expansion in the upper epidermis of leaf 6 indicates that both cell number and cell size increased more rapidly and reached their final values earlier in LD than in SD (Fig. 1B,C). Thus, photoperiod has a pronounced effect on the timing of leaf development because cell division, cell expansion and leaf expansion were faster in LD than SD and ceased earlier.

Similar to the faster growth of leaf 6 the whole rosette leaf area and leaf number initially increased faster in LD than SD (Fig. 2A,B). However, later in development and despite the increased individual leaf size at the fully expanded stage (Fig. 1A), the whole rosette area was smaller in LD than in SD. This was the result of a smaller final number of rosette leaves that were produced (Fig. 2A).

3.2. Successive cellular stages of leaf 6 development are a function of photoperiod

Because leaf 6 growth was accelerated in the long photoperiod and stages 2–4 of leaf development were reached earlier than in the short photoperiod (Fig. 1), biological samples of leaf 6 were harvested at four development stages corresponding to transitions associated with well-defined cellular processes [18]. The stage 1 leaf has maximum relative area and thickness expansion rates, stage 2 and 3 leaves have maximum and decreasing absolute area and thickness expansion rates, respectively, and in the stage 4 leaf expansion ends [18]. Sampling at defined stages allows a robust leaf scale comparison of photoperiod effects on leaf development despite different growth rates in different experiments. We found that stage 1 corresponds to the phase of rapid cell division around day 7 or 8 after leaf initiation in both photoperiods. Most of cell division had ceased at stage 2, which was around day 11 after leaf initiation in LD and day 14 in SD. Stage 3 is the phase of decreasing cell expansion rate around 14 days after leaf initiation in LD and day 21 in SD. At stage 4 cell and leaf expansion were nearly complete,

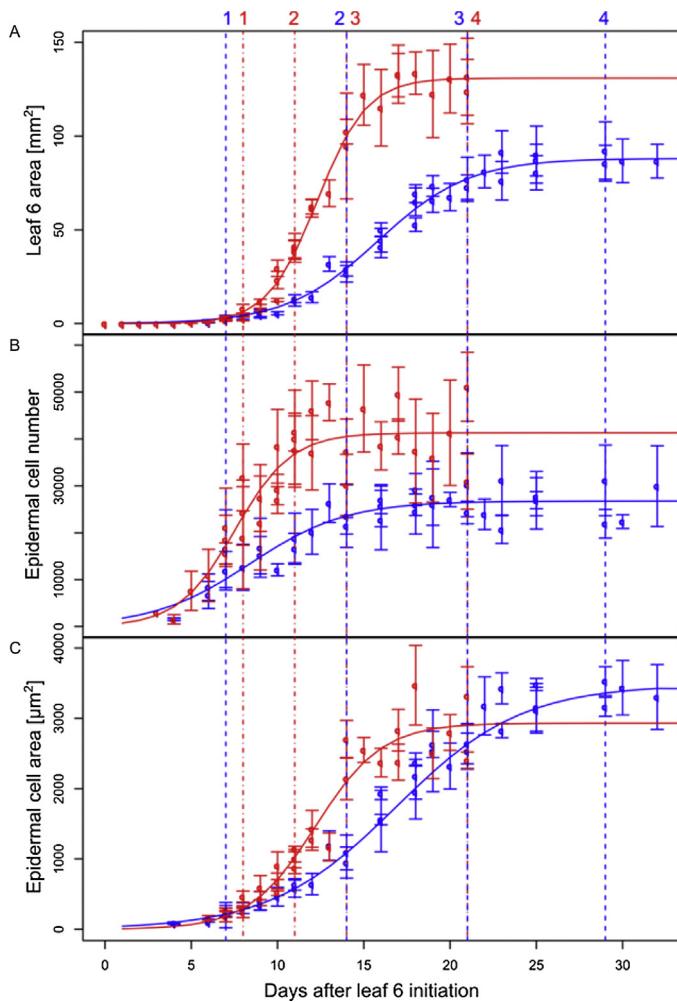


Fig. 1. Kinematic expansion phenotypes of leaves harvested for profiling in the SD (blue) and LD (red) experiments. Changes over time in leaf 6 area (A), mean cell number in leaf 6 adaxial epidermis (B) and mean cell area in leaf 6 adaxial epidermis (C). Data are mean and SD values, $n=5$. The increase of leaf area, cell number and cell area are described by sigmoid curves following the equations: $y = A/[1 + e^{-(X-X_0)/B}]$. The median date of the 4 harvest times are presented by vertical lines for the SD (blue dotted) and LD (red dot-dashed) experiments. Leaf 6 initiation occurred at around day 12 after sowing in SD and day 10 in LD.

corresponding to around day 21 after leaf 6 initiation in LD and day 30 in SD.

3.3. Photoperiod affects individual leaf expansion in the context of whole rosette development

Because photoperiod length affected both the progression of individual leaf stages and whole plant development, the four leaf 6 developmental stages did not have the same status with regard to whole rosette development in LD and SD plants. Leaf 6 expansion in SD was complete before the final number of rosette leaves was reached, whereas in LD more than 50% of leaf 6 expansion occurred after bolting. The floral transition at the shoot apex occurs several days before bolting, typically at 10–12 days after germination in LD [33]. Leaf 6 was initiated at 10 days after sowing, and therefore almost all its growth occurred after the floral transition at the shoot apex.

At stage 1 in LD, leaf 6 area represented approximately 5% of the whole rosette area. This proportion increased to 12–15% during stages 2 and 3 and at stage 4 declined to around 10%. In contrast, the proportion of leaf 6 area compared to whole rosette area at

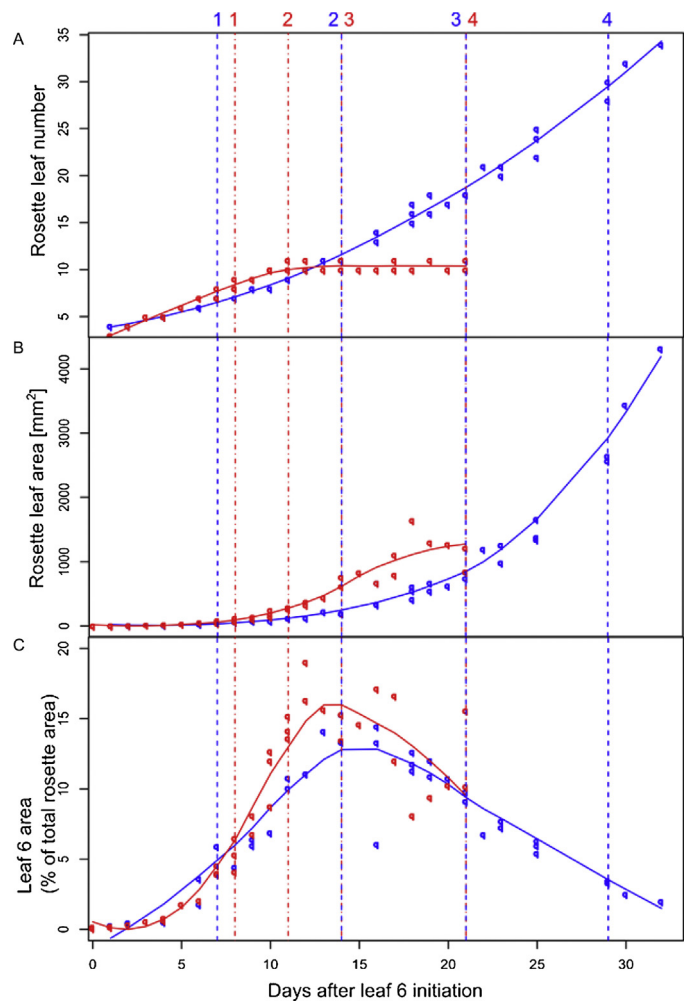


Fig. 2. Kinematic expansion phenotypes of whole rosette leaf growth of plants harvested for leaf 6 profiling in the SD (blue) and LD (red) experiments. Changes over time in the number of rosette leaves (A) and whole rosette area (B). Changes over time of the proportion of whole rosette area covered by leaf 6 area is presented in (C). The indicated trend lines represent predictions from a local polynomial regression fitting (loess). The 4 dates of harvest are presented by vertical lines for the SD (blue dotted) and LD (red dot-dashed) experiments.

stage 4 was less than 5% in SD, confirming that leaf 6 reaches its smaller final size in SD before whole rosette expansion was complete (Fig. 2C).

3.4. Experimental design for assessing molecular changes during leaf development

To quantitate protein and transcript levels during the growth of a single *Arabidopsis* leaf we harvested leaf 6 from plants grown in LD at the end of the day (EOD) and end of the night (EON) at the four successive stages of development defined above. Proteome and transcriptome profiling data, as well as the amounts of starch and soluble sugars were obtained from pooled samples of leaf 6 of three independent biological experiments. We then assessed how the molecular profiles in single leaves at precise stages of development from plants grown in LD differ from leaf 6 grown in SD by comparing them to the SD optimal watering (SOW) and 40% water deficit (SWD) experiments reported previously [18].

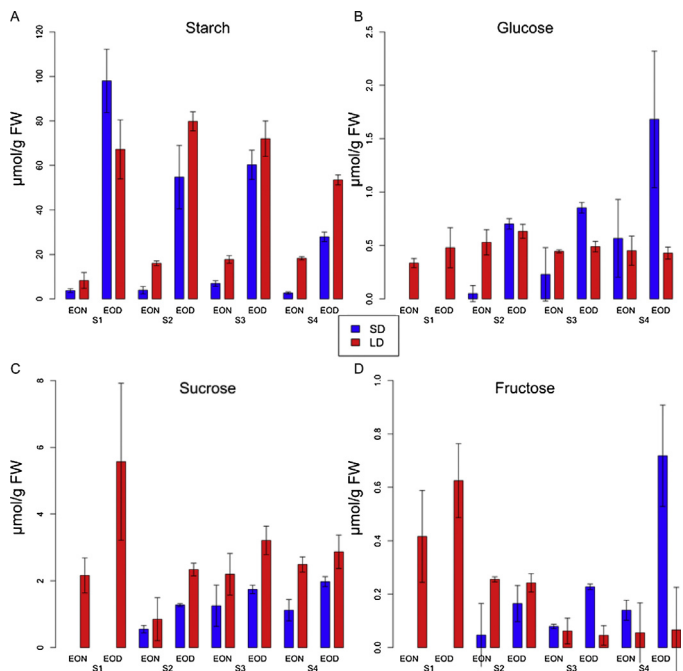


Fig. 3. The amounts of (A) starch and the soluble sugars (B) glucose, (C) sucrose and (D) fructose in $\mu\text{g/g}$ FW and their standard deviations at EON and EOD at the four leaf 6 developmental stages in SD (blue) and LD (red).

3.5. Photoperiod affects the amount and diurnal turnover of carbon reserves

Starch is the main carbon reserve for energy requirements during the night in *Arabidopsis* and represented about 80–93% of the carbohydrates measured at EOD in LD and SD (Fig. 3). In LD-grown plants, the amount of starch at EOD was similar at all four developmental stages. Although starch also decreased during the night in LD plants, considerably larger amounts of starch remained at EON, especially at stages 2, 3 and 4 (Fig. 3A). In SD a different pattern was found. The highest amount of starch at EOD was found for stage 1, with lower levels in stages 2, 3 and, especially, stage 4. Further, in SD, most of the starch that accumulated at EOD was consumed during the night at all developmental stages. In LD, the levels of glucose, sucrose and fructose were similar at EOD and EON

for all developmental stages, with the exception of stages 1 and 2 for sucrose, where the levels were higher at EOD than EON. Glucose levels in LD were similar at all developmental stages, but fructose and sucrose were highest for stage 1. In contrast, major differences were found in SD. First, glucose, fructose and sucrose levels in SD were consistently higher at EOD than EON, as previously reported for full rosettes [6]. Second, the highest levels of glucose, fructose and to some extent sucrose were determined for stage 4 at EOD. Third, sucrose levels for all developmental stages and harvest times were consistently lower in SD than LD, as previously reported for full rosettes [6] (Fig. 3B–D). Together, the data reveal that in *Arabidopsis* photoperiod length has a major influence on the metabolic status of the leaf during both development and the diurnal cycle.

3.6. Diurnal transcript level changes are less pronounced in a LD photoperiod

To account for the observed phenotypic and metabolic differences between SD and LD we analyzed quantitative protein and transcript data in detail. We first performed a Principal Component Analysis (PCA) to estimate the main factors that determine changes in transcript and protein levels in LD. The main contribution to the variance in the transcript data in the first two principal components is the difference between stage 1 and the later stages 2–4, which accounted for over 60% of the total variance (Fig. 4A). The EOD and EON samples are separated only in the third principal component, which accounted for about 8% of the total variance (Fig. 4B). This is in contrast to a PCA of the transcripts in SD conditions, where the time of harvest was the main contribution to the variation in the data in the first and second principal components [18]. Assessing the difference in transcript levels between EON and EON revealed that in LD only 21.2% of all transcripts showed significant diurnal transcript level fluctuations, in contrast to 50.3% in the SOW and 43.1% in the SWD conditions. Thus, in addition to metabolite changes, the LD photoperiod also has a considerable impact on diurnal mRNA expression patterns. For the protein data, the difference between the developmental stages contributes most to the variation in the data (Fig. 4C), as observed previously in SD [18].

3.7. Diurnal transcript fluctuations are shifted in LD and most pronounced for stress response

Transcripts that changed similarly between EOD and EON both in LD and SD included those encoding the central clock proteins

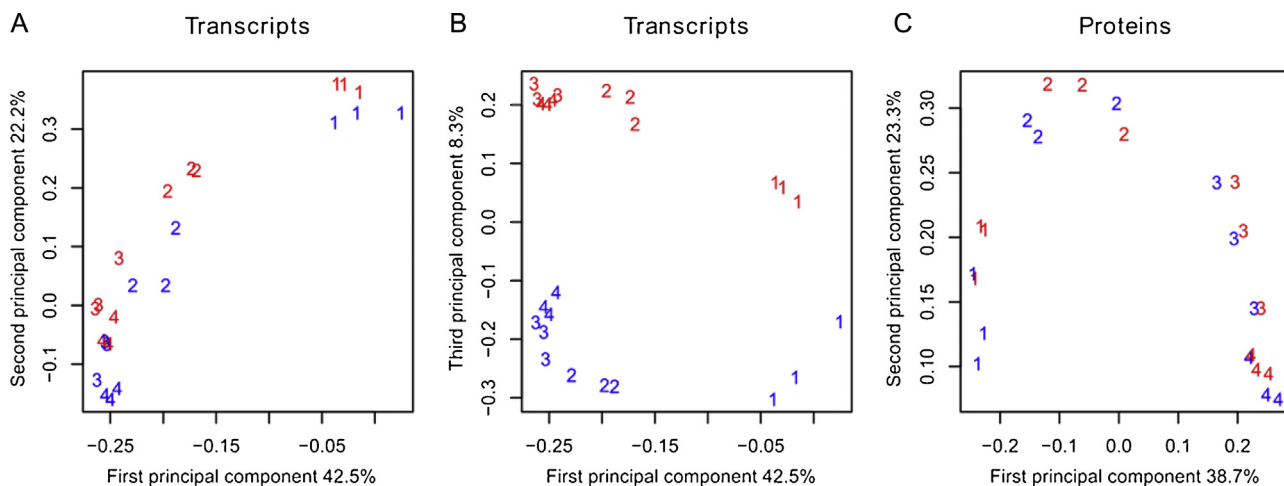


Fig. 4. Principal Component Analysis of transcript and protein profiles in leaf 6 grown in LD. (A) First and second principal component and (B) first and third principal component in the transcript data, and (C) first and second principal component in the protein data. The numbers indicate the growth stages 1 to 4 and are in blue for the EON samples and in red for the EOD samples.

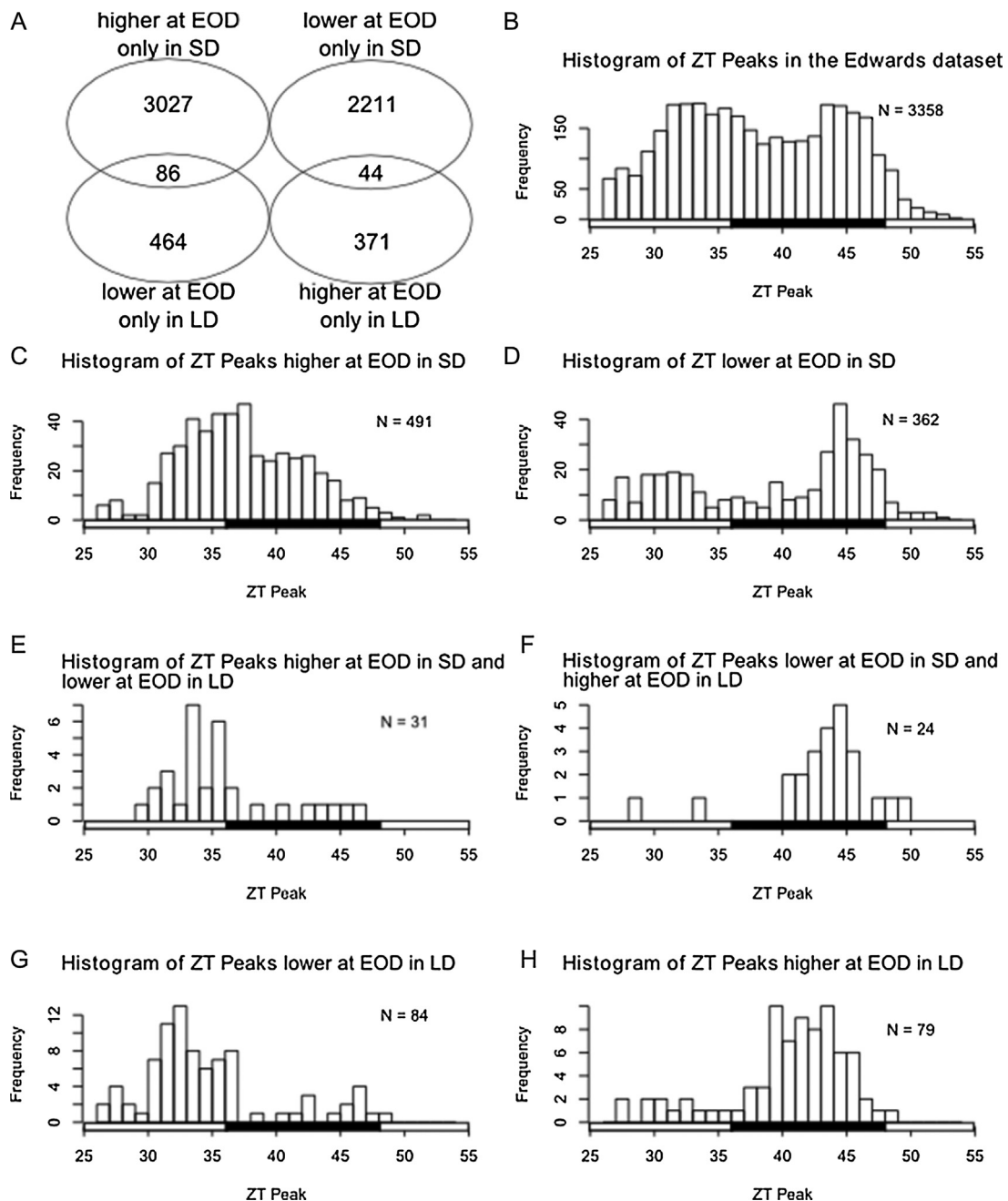


Fig. 5. (A) The number of transcripts with differential diurnal fluctuations between SD and LD. (B) For all the transcripts with differential diurnal fluctuations between SD and LD, and (C–H) for the transcripts in the different sub-categories depicted in (A), the histograms represent the frequency of the number of transcripts with an expression peak at a given ZT as determined in Edwards et al. [34]. The ZT here corresponds to the time in continuous light since the last dawn after plants had been entrained to 12 h/12 h light/dark cycles followed by one day in continuous light, and the expected light and dark periods are indicated by white and black bars, respectively.

LATE ELONGATED HYPOCOTYL 1 (LHY, AT1G01060), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1, AT2G46830) and TIMING OF CAB EXPRESSION 1 (TOC1, AT5G61380). However, as expected from the results of the PCA analysis, many more transcripts showed a significant change between EOD and EON in SD than in LD. We defined transcripts to change only in SD when they had significantly different levels between EOD and EON in SOW and SWD, but not in LD (5238 transcripts), and transcripts to change only in LD when they had significantly different levels between EOD and EON in LD, but not in SOW or SWD (835 transcripts) (Fig. 5A; Supporting Table S1). To further examine the differences in the diurnal fluctuations between SD and LD we used EON as reference point corresponding to Zeitgeber Time (ZT, hours after dawn)–1 in both experiments. We

then assessed which transcripts were significantly higher or lower at the respective EOD compared to the reference point only in SD, or only in LD (Fig. 5, Supporting Table S1). For all transcripts with differential diurnal fluctuations between SD and LD we examined whether they scored rhythmic by COSOPT in the free-running study conducted by Edwards et al. [34]. For those that were rhythmic we plotted the Zeitgeber Time (ZT) peaks determined in Edwards et al. [34] (Fig. 5B–H). The ZT peaks of the rhythmic transcripts that are lower at EOD only in SD and higher at EOD only in LD peak in the second half of the subjective night around ZT 43–44. Transcripts that are higher at EOD only in SD peak around ZT 33–37 corresponding to the subjective dusk, while those that are lower at EOD only in LD peak in the subjective afternoon around ZT 31–32. While the time of

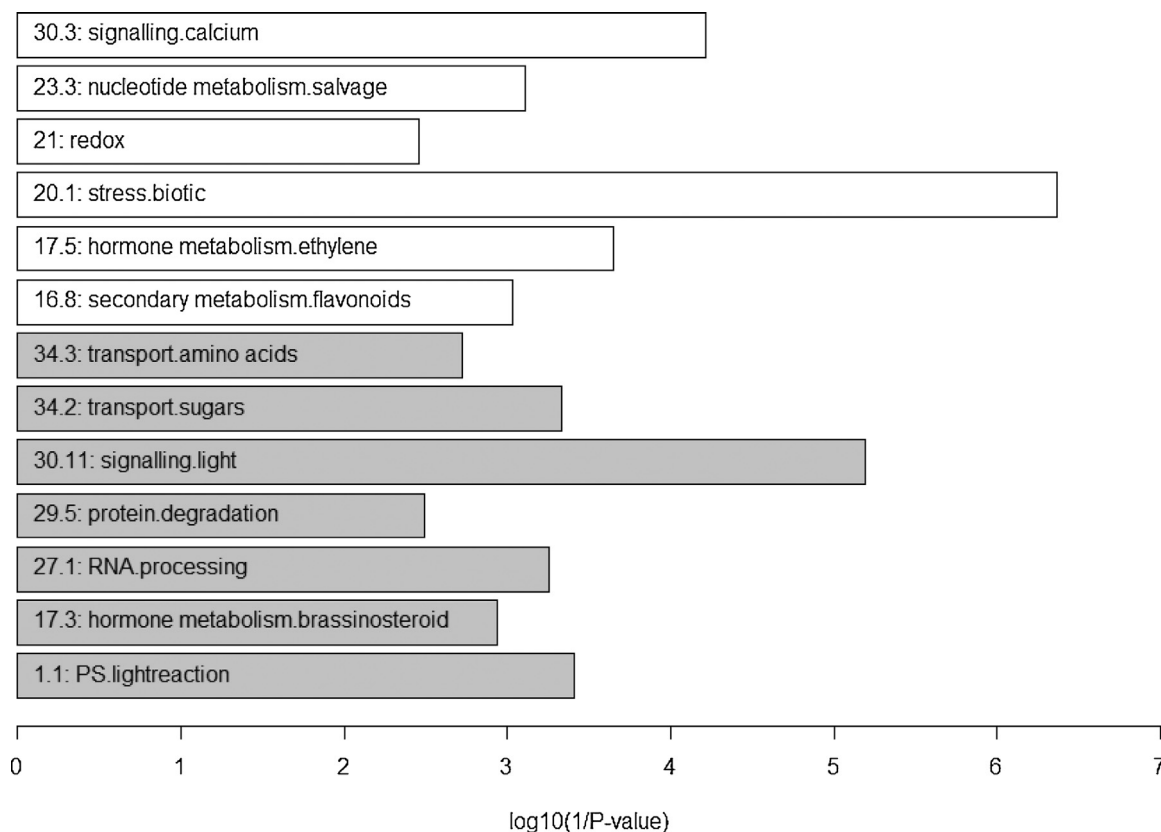


Fig. 6. MapMan categories that are over-represented in LD (white) or SD (grey). The MapMan bins with P-value < 0.01 are indicated and the length of the bar corresponds to the log-transformed MapMan categories that are over-represented in LD (white) or SD (grey). The MapMan bins with P-value < 0.01 are indicated and the length of the bar corresponds to the log-transformed p-value⁻¹.

harvest at the respective EOD in SD and LD photoperiods can affect the relative abundance difference between EOD and EON for transcripts peaking during the night, this is not the case for transcripts with ZT peaks in the afternoon or early night (Supporting Fig. S1). The different pattern of these transcripts therefore suggests a shift in their diurnal expression. The functional categorisation against GO Biological Process of the transcripts higher at EON only in LD gave as the top category *response to chitin* (p -value < 10^{-30}). The list of 23 transcripts that account for this over-representation contains 14 transcription factors according to the AGRIS website [35] (Supporting Table S2), and four of them are scored rhythmic with ZT peaks in the late afternoon. Together, this suggests that the expression patterns of specific transcripts, especially for transcripts linked to biotic stress response, are changed in response to light and the expected length of the night.

3.8. Photoperiod and growth behaviour have specific transcript signatures

The differences in the diurnal transcript accumulation between SD and LD prompted us to further examine the transcripts that are differentially expressed between LD and SD. We considered those transcripts to change in a photoperiod-specific manner that were significantly different (p -value < 0.05 in a paired t -test, average fold-change > 1.5) in the LD experiment compared to both the SOW and SWD experiments. A total of 3469 transcripts fulfilled these criteria with 1954 being higher in LD and 1515 higher in SD (Supporting Table S3).

As plants grow faster in LD than SOW and SWD conditions, it can be expected that some of the differences between the two photoperiods will be due to their different growth behaviours. Comparing

the two SD experiments we had already found that the transcript levels of proteins assigned to GO category *defence response to fungus* and those supporting fast growth, such as proteins involved in ribosome biogenesis and translation, are reduced in leaf 6 by water deficit [18]. To distinguish between effects caused by different growth rates and those specific for long day conditions, we defined sets of growth-specific transcripts based on the gradual increase in growth rate from SWD to SOW and the LD experiment. We hypothesised that transcripts, which accumulate to different levels between SD and LD and also show a significant difference in accumulation between the SWD and SOW conditions, are likely to be related to growth. Applying these criteria we found 134 transcripts that are most highly expressed in LD and 38 transcripts that are highest in SWD conditions (Supporting Table S3). Transcripts that are highest in LD and therefore might be associated with faster growth are over-represented in various response pathways, with *response to chitin*, *defence response to fungus* and *response to mechanical stimulus* as the top three categories. The GO processes that are over-represented in the transcripts highest in the SWD plants are *nitrile* and *proline biosynthetic process*, as well as *photosynthesis*, consistent with a tight energy management in a short photoperiod and reduced water condition.

3.9. Transcripts regulated by photoperiod belong to specific functional categories

Transcripts that were significantly higher in SD or LD (Supporting Table S3) were categorised using MapMan [36] and TAIR10 mapping (Ath.AGI.LOCUS.TAIR10.Aug2012). Over- and under-representation was assessed separately for the transcripts higher in SD and LD using a Fisher's exact test and by comparing the number

Table 1

Proteins with a significant change between the LD experiment and SOW. Proteins that were in addition significantly increased or decreased in the LD experiment compared to SWD are in bold.

Proteins significantly higher in long day conditions	
AT1G75040	pathogenesis-related gene 5, PR5
AT1G75750	GAST1 protein homolog 1
AT2G19730	Ribosomal L28e protein family
AT2G21660	cold, circadian rhythm, and rna binding 2, CCR2, GRP7
AT2G29350	senescence-associated gene 13
AT2G45790	phosphomannomutase, PMM
AT3G57260	beta-1,3-glucanase 2, ATBG2, ATPR2, BGL2, PR2
AT3G59760	O-acetylserine (thiol) lyase isoform C
AT4G17830	Peptidase M20/M25/M40 family protein
AT4G22670	HSP70-interacting protein 1
AT4G32915	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: regulation of translational fidelity
AT4G36810	geranylgeranyl pyrophosphate synthase 1, GGPS1, GGPPS11
AT5G39570	FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: cytosol
Proteins significantly lower in long day conditions	
AT1G54010	GDSL-like Lipase/Acylhydrolase superfamily protein
AT1G76100	plastocyanin 1, PETE1
AT2G22230	Thioesterase superfamily protein
AT2G42530	cold regulated 15b, COR15B
AT2G42540	cold-regulated 15a, COR15A
AT3G09260	Glycosyl hydrolase superfamily protein
AT4G29680	Alkaline-phosphatase-like family protein
AT5G10540	Zincin-like metalloproteases family protein
AT5G15970	stress-responsive protein (KIN2) / stress-induced protein (KIN2) / cold-responsive protein (COR6.6)
AT5G51720	2 iron, 2 sulfur cluster binding
AT5G54160	O-methyltransferase 1

of measured transcripts with the number that would be expected by chance. Fig. 6 shows the MapMan bins with p -value < 0.01 and the AGIs of the genes in these categories are listed in Supporting Table S4.

3.9.1. RNA processing mechanisms differ depending on photoperiod length

Among the genes for transcripts that have different levels between SD and LD we found fewer than expected that encode proteins for translation (bin 29.2) ($p < 2.05 \times 10^{-11}$ in a Fisher's exact test). This is in agreement with the finding that ribosome abundance does not change between SD and LD grown plants [6]. However, genes involved in RNA processing are over-represented in SD (Fig. 6), while genes for small nucleolar RNAs (snoRNAs) are over-represented in LD (4.25×10^{-6} in a Fisher's exact test) because 14 of 45 snoRNAs represented on the tiling array are significantly more highly expressed in LD. snoRNAs associate with proteins to form functional small nucleolar ribonucleoprotein complexes (snoRNPs), which are involved in the processing of precursor rRNAs in the nucleolus requiring exo- and endonucleolytic cleavages as well as modifications. These modifications are thought to influence ribosome function [37]. The differential expression of snoRNAs in SD and LD conditions might reflect a specific but currently unknown mechanism of adjusting translation to the prevalent photoperiod conditions.

3.9.2. Flavone biosynthesis is enhanced in the LD photoperiod

Transcripts that are higher in LD are over-represented in bin secondary metabolism.flavonoids (Fig. 6). Flavonoids are plant secondary metabolites with broad physiological functions [38]. Of the genes in this category, five encode enzymes in the KEGG [39] pathway *flavonoid biosynthesis*, namely TRANSPARENT TESTA 4 (CHS/TT4, AT5G13930), TT5 (AT3G55120), F3H/TT6 (AT3G51240), TT7 (AT5G07990) and FLAVONOL SYNTHASE (FLS, AT5G08640) (Supporting Fig. S2). These enzymes are required for the biosynthesis of the three major flavonols quercetin, kaempferol and myricetin, although the enzyme catalysing the last step of myricetin production has not yet been identified in *Arabidopsis* (Supporting Fig. S3). The transcript levels for these enzymes are all increased in

LD as compared to SD but generally decrease during leaf 6 development (Supporting Fig. S4). TT5 and TT6/F3H proteins were detected in LD. TT5 protein levels decrease significantly during development in LD but the protein was detected in all three experimental conditions (SOW, SWD and LD). Transcript levels of flavonoid pathway genes were reported to be up-regulated in leaves of sweet potato grown in LD that have high concentrations of kaempferol [40]. Kaempferol functions as an antioxidant in chloroplasts [41]. Higher transcript levels for the enzymes in the flavonol biosynthesis pathway in LD therefore correlate well with the over-representation of the bin redox in LD. The transcript levels for enzymes in flavonoid biosynthesis pathways involved in response to excess UV light or high light stress, such as anthocyanin biosynthesis, are not higher in LD as compared to SD. This confirms that under our experimental conditions the LD photoperiod is not triggering a stress response that would require enhanced photoprotection.

3.9.3. Light and hormone signalling differ between SD and LD

Plant hormones coordinate developmental processes and growth through converging pathways [42,43]. We therefore expected that several of the genes whose transcripts accumulate to different levels between SD and LD encode proteins involved in hormone metabolism and signalling (Supporting Fig. S5). The bin *hormone metabolism.ethylene* is over-represented in LD and the list of genes annotated to this bin that have increased transcript levels in LD includes 10 genes encoding different ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF) proteins. ERFs function in defence response and regulate chitin signalling [44,45]. Two of these ERFs, DREB AND EAR MOTIF PROTEIN 1 (DEAR1; AT3G50260) and ERF6 (AT4G17490), belong to the transcription factors that have higher transcript levels at EON only in LD and are assigned to *response to chitin* (Supporting Table S2).

Ethylene biosynthesis is restricted by the photoreceptor phytochrome B (PHYB; AT2G18790) [46]. PHYB transcript levels are decreased in LD as compared to SD, which correlates with increased ethylene biosynthesis in LD. In addition to PHYB, other genes encoding phytochromes such as PHYA (AT1G09570) and genes encoding phytochrome kinase substrates and phototropic responsive family

proteins are more highly expressed in SD, resulting in the over-representation of bin *signalling.light* (Supporting Table S4).

Photoperiod can be integrated with growth and time to flowering through regulation of the brassinosteroid hormone pathway [47]. It was therefore unexpected that bin *hormone metabolism.brassinosteroid* was over-represented in SD, as plants in SD grow more slowly and flower later. However, the mRNAs with higher levels in SD assigned to this bin also include the mRNA for cytochrome P450 CYP734A1 (AT2G26710). CYP734A1 converts active brassinosteroids into their inactive forms [48] and therefore acts as a negative regulator of brassinosteroid signalling. Thus, the over-representation of the bin *hormone metabolism.brassinosteroid* does not imply increased brassinosteroid signalling. In fact, the only brassinosteroid signalling-related mRNA with higher levels in LD encodes BES1/BZR1-LIKE PROTEIN 3 (BEH3, AT4G18890), which is a transcription factor that is homologous to BES1/BZR1, a positive regulator of brassinosteroid signalling [49].

3.9.4. SD increases transcript levels for sugar transport and photosystem proteins

Transcripts that are significantly higher in SD than LD encode twelve members of the monosaccharide transporter (MST)(-like) gene family [50] and the SUCROSE-PROTON SYMPORTER 9 (SUC9, AT5G06170). Accordingly, the bin *sugar.transport* is over-represented in SD (Fig. 6, Supporting Table S4). The members of the MST(-like) gene family are classified into seven distinct sub-families and have roles in both long-distance sugar partitioning and sub-cellular sugar distribution [50]. POLYOL/MONOSACCHARIDE TRANSPORTER 2 (PMT2, AT2G16130) and SUGAR TRANSPORTER 1 (STP1, AT1G11260) are located in the plasma membrane and were suggested to import monosaccharides into guard cells during the night and function in osmoregulation during the day [51]. The MST(-like) gene family members involved in sub-cellular sugar distribution include the plastid-localised PLASTIDIC GLC TRANSLATOR (PGLCT, AT5G16150), which contributes to the export of the main starch degradation products maltose and glucose from chloroplasts [52], and six proteins encoded by the ATERD6-like gene sub-family that are located in the vacuole membrane. ATERD6 homologs are thought to export sugars from the vacuole during conditions when re-allocation of carbohydrates is important, including senescence, wounding, pathogen attack, C/N starvation and diurnal changes in transient storage of sugars in the vacuole [50]. The increased transcript expression of genes for various sugar transporters in SD is consistent with the different amount and diurnal turnover of sugar levels in SD as compared to LD (Fig. 3) and indicates that long-distance and sub-cellular sugar partitioning is increased in shorter illumination periods.

The bin *PS.lightreaction* is significantly different between SD and LD and overrepresented in SD (Fig. 6; Supporting Figs. S5 and S6). Most of the transcripts assigned to this bin that are increased in SD encode photosystem I or II proteins (Supporting Table S4). Some of their genes seem to be linked to reduced growth, nevertheless, the SD compared to the LD photoperiod apparently increases photosystem abundance. This likely increases the rate of photosynthesis to use the light of the shorter illumination period most efficiently.

3.10. Proteins that differ between SD and LD can mainly be attributed to differences in growth

We next examined the proteins that are differentially expressed in the LD and SOW plants (p -value < 0.05 in a paired t -test, average fold-change > 1.5). A total of 24 proteins fulfilled the strict cut-off criteria that were also applied to the transcript data. Of the 13 proteins that were higher in LD, 5 were also increased in LD compared to SWD, and of the 11 that were lower in LD, 4 were also significantly decreased in LD compared to SWD. These proteins therefore

show a significant difference between LD and both SD conditions (Table 1).

The list of proteins that are more abundant in LD than in SD includes PATHOGENESIS-RELATED PROTEIN 5 (PR5, AT1G75040), PR2 (AT3G57260) and ribosomal L28e family protein (AT2G19730). This is consistent with our previous findings that most of the proteins that accumulated to higher levels in the faster growing SOW leaves than in the SWD leaves mainly comprised proteins involved in translation and that transcripts with higher levels in the SOW leaves are over-represented for GO categories *ribosome biogenesis*, *translation* and *defence response to fungus* [18]. Furthermore, MapMan bin *stress.biotic* was over-represented for transcripts that have higher levels in LD. The list of proteins that accumulate to significantly higher levels in LD also includes PHOSPHOMANNOMUTASE (PMM, AT2G45790), which is involved in the synthesis of GDP-mannose and is therefore required for ascorbic acid biosynthesis and N-glycosylation. Interestingly, the *pmm* mutant has a temperature-sensitive phenotype that was attributed to a deficiency in protein glycosylation [53]. The different abundance levels of PMM of in LD and SD might therefore suggest differential post-translational modifications in LD and SD. GERANYLGERANYL PYROPHOSPHATE SYNTHASE 1 (GGPPS11, AT4G36810), which is required for the biosynthesis of geranylgeranyl diphosphate (GGPP) [54], also accumulates to higher levels in leaf 6 grown in LD as compared to SOW conditions. In *Arabidopsis*, the chloroplast-localized GGPPS11 is the GGPPS isoform with the highest transcript level in rosette leaves and mainly responsible for the biosynthesis of GGPP-derived isoprenoid metabolites including chlorophyll and carotenoids [54]. The higher protein level of GGPPS11 in LD than in SD therefore suggests the increased production of these metabolites in LD.

The proteins that are significantly more abundant in SD than in LD are PLASTOCYANIN 1 (PETE1, AT1G76100) and the three cold response (COR) proteins COR15A (AT2G42540), COR15B (AT2G42530) and COR6.6 (AT5G15970) (Table 1). Although plastocyanins have been implicated in photosynthetic electron transport, their concentration is not limiting for electron flow in optimal growth conditions with 11 h light [55]. The increased PETE1 protein level in SD might therefore indicate a specific role for this protein in short photoperiods. The COR proteins are also significantly more abundant in leaf 6 grown in SWD as compared to SOW conditions and have been implicated in the adaptation response to the continuous 40% water deficit condition [18]. However, the LD data suggest that the accumulation of the three COR proteins may also be related to growth. We did not classify transcripts for these proteins as photoperiod-specific because they are significantly different between SWD and LD but not between SOW and LD. A crosstalk between cold response and flowering time regulation has been proposed previously, with SOC1 functioning as a negative regulator of CBFs that bind to the COR promoters [56]. Here, the situation is different, because SOC1 and CBF1 (AT4G25490) transcript levels are higher in LD as compared to SD and the COR transcripts show a different behaviour. Therefore, the levels of the COR proteins seem to be regulated differently and related to the growth rate of the leaves.

3.11. Flowering genes have photoperiod-specific transcript signatures in leaves

LD photoperiods that are characteristic of spring and early summer induce flowering in LD plants. The core photoperiodic flowering pathway comprises GIGANTEA (GI, AT1G22770), FLOWERING LOCUS T (FT, AT1G65480) and CONSTANS (CO, AT5G15840) [57,58]. Circadian clock regulation of CO transcript level and protein stability is key to monitoring changes in photoperiod length, and the biphasic regulation of CO ensures that flowering is induced in LD

[57]. The mRNA levels for the CO target *FT* were higher in LD compared to SD and increased during development (Supporting Fig. S7). Downstream of *FT*, the MADS-box transcription factors AGAMOUS-LIKE 20/SUPPRESSOR OF CONSTANS1 (*SOC1*, AT2G45660), *AGL24* (AT4G24540), *FRUITFULL* (*FUL*, AT5G60910) and *SHORT VEGETATIVE PHASE* (*SVP*, AT2G22540) function as floral integrator genes during the transition of the shoot apical meristem (SAM) to the floral meristem [59]. Notably, *AGL24* and *FUL* transcript levels were significantly higher in LD also in leaf 6. *SOC1* transcript levels were only higher in LD at early leaf 6 developmental stages, and *SVP* transcript levels were not significantly different between LD and SD (Supporting Fig. S7). In contrast, the mRNA levels for *FLOWERING LOCUS C* (*FLC*, AT5G10140), which is a key repressor of flowering [60], were significantly lower in LD as compared to SD (Supporting Fig. S7). *FLC* and *SVP* form heterodimers during vegetative growth to repress transcription of *FT* in leaves and *SOC1* in the SAM [61]. The reduced levels of *FLC* transcripts in LD together with the increased levels of *FT* transcripts are therefore consistent with an early release of flowering repression in LD.

SOC1 belongs to the group of genes that have a diurnal expression peak in the afternoon, with *SOC1* transcript levels being higher at EOD in SD, but higher at EON in LD (Fig. 5). Interestingly, this pattern was also found for transcript levels of the potential natural antisense RNA gene AT1G69572, whose genomic region overlaps with that of *CDF5*. According to data reported by Bläsing et al. [62], *SOC1* transcript levels were highest in the afternoon (ZT8) in a 12 h/12 h photoperiod. When compared to free-running conditions of continuous white light [63], *SOC1* transcript levels were highest at ZT8 during the first day but no subsequent circadian oscillation was detectable. *SOC1* therefore belongs to the group of genes whose transcript levels are not regulated by the circadian clock but directly by photoperiod.

3.12. *AtGRP7* protein, but not transcript, is more highly expressed in LD

The glycine-rich RNA-binding protein *AtGRP7* (AT2G21660) has an important role in flowering. Expression of *AtGRP7* is directly controlled by *CCA1* and *LHY*, and its transcript levels oscillate with a peak in the evening [64]. *AtGRP7* regulates the amplitude of the circadian oscillation of its mRNA through alternative splicing. *Arabidopsis* plants that constitutively over-express *AtGRP7* produce a short-lived mRNA splice form, which dampens *AtGRP7* transcript oscillations and influences the accumulation of other transcripts including *AtGRP8* (AT4G39260) [65]. As the result, *AtGRP7* promotes flowering, with a more pronounced effect in SD than in LD [66]. In LD we indeed observed a dampening of both *AtGRP7* and *AtGRP8* diurnal transcript level changes at all leaf 6 development stages, but the transcript levels of *AtGRP7* did not change significantly during development (Supporting Fig. S8). In contrast, *AtGRP7* protein levels were significantly higher in the LD experiment as compared to SOW (Table 1), did not display diurnal level changes, and decreased during development both in SD and LD (Supporting Fig. S8). The higher *AtGRP7* protein levels in LD as compared to SD provide an explanation for earlier observations that the effect of *AtGRP7* overexpression on time to flowering is stronger in SD than in LD.

4. Conclusions

In addition to photoperiod, which may act at multiple points in the circadian clock [67–69], the rhythmic, diurnal endogenous sugar signals can entrain circadian rhythms in *Arabidopsis* [70]. Furthermore, in an 18 h photoperiod considerable amounts of starch remain at EON while the rate of photosynthesis is decreased com-

pared to a 4-, 6-, 8-, and 12-h photoperiod. Consequently, in long photoperiods growth is not longer limited by the availability of carbon and the carbon conversion efficiency decreases [6]. By systematically investigating the molecular changes in a single leaf that are involved in the adaptation to different photoperiods in highly controlled conditions we demonstrated that fewer transcripts display significant changes between EOD and EON in LD than in SD. We previously discussed that different mRNA levels at specific times during the diurnal cycle might be required for the time-dependent regulation of the cellular energy status in prevailing environmental conditions [18]. If diurnal transcript level fluctuations are indeed required for efficient resource allocation, this might explain why plants grown in long days do not depend on a strict diurnal regulation of transcription to tightly economise their energy budget. We also established that transcripts regulated by photoperiod belong to specific functional categories that are important for adaptation to the prevailing photoperiod condition. In contrast, identified proteins that differ significantly between photoperiods are mainly related to the different growth rates of leaf 6. Together, changes in the complex molecular pattern underlying leaf growth in different photoperiods are tightly linked to the available energy.

Conflicts of interest

none.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cpb.2015.07.001>

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