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Quantitative Circadian Phosphoproteomic Analysis of Arabidopsis Reveals Extensive Clock Control of Key Components in Physiological, Metabolic, and Signaling Pathways*[S]

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The circadian clock provides adaptive advantages to an organism, resulting in increased fitness and survival. The phosphorylation events that regulate circadian-dependent signaling and the processes which post-translationally respond to clock-gated signals are largely unknown. To better elucidate post-translational events tied to the circadian system we carried out a survey of circadian-regulated protein phosphorylation events in Arabidopsis seedlings. A large-scale mass spectrometry-based quantitative phosphoproteomics approach employing TiO2-based phosphopeptide enrichment techniques identified and quantified 1586 phosphopeptides on 1080 protein groups. A total of 102 phosphopeptides displayed significant changes in abundance, enabling the identification of specific patterns of response to circadian rhythms. Our approach was sensitive enough to quantitate oscillations in the phosphorylation of low abundance clock proteins (EARLY FLOWERING4; ELF4 and PSEUDORESPONSE REGULATOR3; PRR3) as well as other transcription factors and kinases. During constant light, extensive cyclic changes in phosphorylation status occurred in critical regulators, implicating direct or indirect regulation by the circadian system. These included proteins influencing transcriptional regulation, translation, metabolism, stress and phytohormones-mediated responses. We validated our analysis using the *elf4–211* allele, in which an S45L transition removes the phosphorylation herein identified. We show that removal of this phosphorylatable site diminishes interaction with EARLY FLOWERING3 (ELF3), a key partner in a tripartite evening complex required for circadian cycling. *elf4–211* lengthens period, which increases with increasing temperature, relative to the wild type, resulting in a more stable temperature compensation of circadian period over a wider temperature range. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.047183, 2243–2260, 2015.***

The timing of many physiological and developmental processes in most eukaryotes is under the control of a circadian clock. This endogenous, self-sustaining oscillator maintains a 24 h rhythm that coordinates a wide range of processes in organisms as diverse as cyanobacteria, fungi, algae, plants, and metazoans. Circadian rhythms allow an organism to anticipate the onset of environmental changes, providing an adaptive advantage that can result in increased fitness and survival (1–3). Classical and molecular genetics have helped to identify genes (clock genes) in Drosophila, Arabidopsis, *Neurospora* and mammals that establish a central oscillator that forms the core of the circadian system. In all these systems both the mRNA and protein products of key genes very often cycle in abundance and/or activity, creating two or more interlocked transcription-translation feedback loops (4–8).

Many studies of eukaryotic clock systems have shown the fundamental importance of post-transcriptional processes, particularly proteolysis and phosphorylation (9–15). In plants, TIMING OF CAB EXPRESSION1 (TOC1)[1] and other pseudo-

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[1] The abbreviations used are: TOC1, TIMING OF CAB EXPRESSION1; FDR, false discovery rate; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LD, 12 h white light/12 h dark cycle; LL, constant white light; PTM, post-translational modification; RAE, relative amplitude error; RT-qPCR, Reverse transcription and quantitative polymerase chain reaction; SEM, standard error of the mean; SOTA, self-organizing tree algorithm; WT, wild type.

response regulator (PRR) proteins are differentially phosphorylated over the course of the circadian cycle (16) and both CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) are phosphorylated by casein kinase 2 (CK2) (17, 18). In some cases the significance is known (16, 19, 20). For example, TOC1 phosphorylation enhances dimerization with PRR3 and binding to ZEITLUPE (ZTL), the F-box protein that mediates TOC1 ubiquitination (16, 21). CCA1 phosphorylation by CK2 controls CCA1 access to key oscillator promoters (22, 23).

Global analyses of transcriptome dynamics during circadian oscillations using microarrays have shown that ~30% of the plant transcriptome is regulated by the circadian system (24–27), including a substantial number of kinases and phosphatases (28, 29). Similar studies have been performed in metazoan systems (30–32). However, there have been few global examinations of proteins and/or phosphoproteins controlled by the eukaryotic clock and these have generally been limited to specific tissues (33–36).

Protein phosphorylation and dephosphorylation is a highly controlled biochemical process that responds to various intracellular and extracellular stimuli. Phosphorylation status modulates protein activity, protein stability, influencing the structure of a protein, controlling subcellular distribution, and regulating interactions with other proteins; thereby regulating crucial processes such as metabolism and development. Advances in phosphoproteomics, including phosphopeptide-enrichment methods, high accuracy MS, and associated bioinformatic tools, have made it feasible to obtain an unbiased view of phosphoproteomes at a systems level (37–39). Recent progress in phosphoproteomics technology paved the way for the identification of a few thousand phosphorylation sites from unfractionated plant cells by simple one-step phosphopeptide enrichment methods (40–43). Deciphering specific signaling pathways in response to external cues, however, not only requires the identification but also the quantification of phosphorylated peptides.

In this study, we applied phosphoproteomic technologies to cellular phosphorylation events and obtained for the first time a systems-wide view of circadian-phase dependent phosphorylation-site dynamics in Arabidopsis. By quantitative comparison of the level of phosphopeptides at different time points we gain a first look into the circadian-regulated phosphorylation responses of Arabidopsis proteins. Additionally, we validate the value of this approach by demonstrating the role of a novel phosphorylation site in EARLY FLOWERING 4 (ELF4 (S-45)), a key component of a tripartite evening repressor complex (EC) in the Arabidopsis circadian oscillator.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Arabidopsis seedlings (Col) were entrained under 12 h white fluorescent light (120 μmol/m^2/s)/12 h dark cycles for 10 days on MS (Murashige and Skoog) plates with 3% sucrose and 1% agar at 22 °C. The seedlings were then maintained under constant light and temperature for 24h before harvesting. The tissues were harvested in tubes containing Zirconia beads at the indicated time points (LL25, LL29, LL 33, LL37, LL41, and LL45) during the 12th day of growth and frozen immediately. *elf4–211* and *elf4–209* seed were obtained from the Arabidopsis Biological Resource Center (ABRC; Ohio State University) and validated by sequencing. Generation of *ELF4pro:ELF4-HA* seed has been described previously (44).

Phosphopeptide Preparation From Arabidopsis Seedlings—The frozen Arabidopsis seedlings (~0.2 g) were disrupted with a Shake Master Neo (Bio Medical Science, Tokyo, Japan) and extracts were kept on ice during all subsequent manipulations. The disrupted seedlings were suspended in 8 M Urea, 0.1 M Tris-HCl (pH 9.0), containing protein phosphatase inhibitor cocktails 1 and 2 (Sigma, St. Louis, MO). The homogenate was centrifuged at 1500 × *g* for 10 min and the supernatant collected. Protein concentration was determined with a bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA). The protein solution was reduced with 10 mM dithiothreitol for 30 min, alkylated with 50 mM iodoacetamide for 30 min in the dark, at room temperature and digested with Lys-C (1:200, w/w) for 3 h at room temperature, followed by fourfold dilution with 50 mM ammonium bicarbonate and digestion with trypsin (1:100, w/w) overnight at room temperature. These digested samples were acidified with the addition of trifluoroacetic acid (TFA) and were desalted using StageTips with C18 Empore disc membranes (3M, St. Paul, MN) (45) as described previously (41). Phosphopeptides were enriched from the desalted samples by the lactic acid-modified titania-utilized hydroxy acid-modified metal oxide chromatography (Ti-HAMMOC) (40) as described previously (41). Briefly, the desalted tryptic digest from 400 μg of Arabidopsis protein was loaded to a custom-made HAMMOC tips with 3 mg of bulk titania (particle size, 10 μm; GL Science, Tokyo, Japan) for the phosphopeptide enrichment. The enriched fraction was acidified with TFA and desalted using C18 StageTips and dried in a vacuum evaporator. The desalted peptides were dissolved in 9 μl of 5% acetonitrile containing 0.1% trifluoroacetic acid for subsequent LC-MS/MS analysis.

LC-MS Analysis—An LTQ-Orbitrap XL (Thermo Fisher Scientific) coupled with a Dionex Ultimate3000 pump and an HTC-PAL auto sampler (CTC Analytics) was used for nano-LC-MS/MS analyses. A self-pulled needle (150 mm length × 100 μm i.d., 6-μm opening) packed with ReproSil C18 materials (3 μm; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) was used as an analytical column with "stone-arch" frit (Ishihama *et al.*, 2002). A spray voltage of 2,400 V was applied. The injection volume was 6 μl, and the flow rate was 500 nL min-1. The mobile phases consisted of 0.5% acetic acid and 2% acetonitrile (A) and 0.5% acetic acid and 80% acetonitrile (B). A three-step linear gradient of 5% to 10% B in 10 min, 10% to 40% B in 120 min, 40% to 95% B in 5 min, and 95% B for 10 min was employed. The MS scan range was *m/z* 300 to 1400. The top-10 precursor ions were selected in the MS scan by Orbitrap with resolution = 100,000 and for subsequent MS/MS scans by ion trap in the automated gain control mode, where automated gain control values of 5.00e + 05 and 1.00e + 04 were set for full MS and MS/MS, respectively. The normalized collision-induced dissociation was set to 35.0. A lock mass function was used for the LTQ-Orbitrap XL to obtain constant mass accuracy during gradient analysis (46). Multistage activation was enabled upon detection of a neutral loss of phosphoric acid (98.00, 49.00, or 32.66 amu) (47) for further ion fragmentation. Selected sequenced ions were dynamically excluded for 60 s after sequencing.

Mass Navigator version 1.3 (Mitsui Knowledge Industry, Tokyo, Japan) with the default parameters for LTQ-Orbitrap XL was used to create peak lists on the basis of the recorded fragmentation spectra. The m/z values of the isotope peaks were converted to the corresponding monoisotopic peaks when the isotope peaks were selected

as the precursor ions. In order to improve the quality of MS/MS spectra, Mass Navigator discarded all peaks of less than 10 absolute intensity and with less than 0.1% of the most intense peak in MS/MS spectra (48). Peptides and proteins were identified by means of automated database searching using Mascot version 2.3.02 (Matrix Science, Tokyo, Japan) in The Arabidopsis Information Resource database (TAIR10_pep_20101214, ftp://ftp.arabidopsis.org/home/tair/Sequences/blast_datasets/TAIR10_blastsets/) with a precursor mass tolerance of 3 ppm, a fragment ion mass tolerance of 0.8 Da, and strict trypsin specificity (49), allowing for up to two missed cleavages. Carbamidomethylation of Cys was set as a fixed modification, and oxidation of Met and phosphorylation of Ser, Thr, and Tyr were allowed as variable modifications.

Quantitative Analysis—MS peaks were detected, normalized, and quantified using an in-house 2DICAL software package, as described previously (50). A serial identification (ID) number was applied to each of the MS peaks detected (1 to 22619). The stability of LC-MS was monitored by calculating the correlation coefficient (CC) and coefficient of variance (CV) of every measurement. The mean CC ± S.D. and CV ± S.D. for all 22,619 peaks observed in the 36 runs were as high as 0.850 ± 0.122 and as low as 0.321 ± 0.075, respectively. The normalized peak intensities of MS chromatograms were used for quantitative values. For each time point, six samples, technical triplicates from biological duplicates, were analyzed and the significance of time-dependent changes was tested. Statistical relevance was determined using one-way ANOVA with a Benjamini Hochberg FDR multiple testing correction (p value <0.05).

Bioinformatics—We used the Motif-X algorithm (51) to extract significantly enriched phosphorylation motifs from circadian regulated phosphopeptide data sets whose phosphorylation sites were confidently identified with a more than 90% score. The width of the peptides was set to 15 amino acids with the phosphorylated site centered by retrieving the sequence context from the IPI Arabidopsis proteome data base or by filling up the required number of "X" (X stands for "any amino acid"), if necessary. The default IPI Arabidopsis Proteome data set was used as the background data set. The centered peptides were aligned and used to extract the motif with the probability threshold $p < 10^{-4}$, and the occurrence threshold was set to 10.

For Gene Ontology (GO) enrichment information for differentially expressed significant phosphoproteins data sets, the three GO vocabularies, biological processes, cellular component, and molecular function were searched using Cytoscape version 3.1.1 (52) along with its plugin Bingo version 2.44 (53). The AGI accession numbers for Arabidopsis were uploaded and ontology of GO cellular component, biological process, and molecular function was chosen with other settings as default (significance level 0.05). GO terms were represented as nodes, and the size of each node was proportional to the number of proteins in the query set with that term. The yellow and orange nodes represent terms with significant enrichment, whereas the darker orange represents a higher degree of significance.

A functional network of circadian modulated phosphoproteins was predicted using STRING version 9.05 (54) (available at http://string-db.org). Functional protein-association networks were visualized with medium confidence (0.4), with *Arabidopsis thaliana* set as the organism.

To show the co-expression pattern of differentially expressed significant phosphoproteins self-organizing tree algorithm (SOTA) clustering (55) was used. The clustering was performed on log-transformed fold induction expression values across six time points using Multi Experiment Viewer (MEV) software (The Institute for Genomic Research). The clustering was performed with the Pearson correlation as distance with 10 cycles and a maximum cell diversity of 0.9 (56).

Constructs—The S45L amino acid substitution (corresponding to the *elf4–211* allele) was introduced by site-directed mutagenesis of the pENTR223-ELF4 entry vector. Both the wild type and the S45L alleles were introduced into the pCsVMV1300-HA vector between the KpnI and XmaI sites. The ELF3 coding sequence was subcloned into the pENTR2B vector between the BamI and NotI sites. After confirmation by sequencing, the destination vector of 35S:TAP-ELF3 was generated by LR recombination with pCD3–696. The 35S:GI-GFP vector has been described previously (57).

Coimmunoprecipitation Assay—Agrobacteria containing both CsVMV:ELF4-HA and 35S:TAP-ELF3 or 35S:GI-GFP were coinfiltrated into 4-week-old *Nicotiana benthamiana*. Leaf material was ground to a fine powder in liquid nitrogen, and protein extracted with ice-cold IP buffer (57). The cleared supernatant was used for immunoprecipitation using human IgG agarose (Sigma, St. Louis, MO, A6284), or GFP antibody (Invitrogen, Carlsbad, CA, 11120) as described previously (57). GFP-tagged GI protein and HA-tagged ELF4 protein were detected by Western blotting using GFP antibody (Abcam, Cambridge, UK, 6556) and HA antibody (Roche, Basel, Switzerland, 3F10). PAP antibody (Sigma, St. Louis, MO, P1291) was used to detect TAP-ELF3 protein. Protein quantification was conducted with Quantity one software (BioRad, Hercules, CA).

Luminescence Measurement and Rhythm Analysis—Seedlings containing a *CCA1::LUC* reporter were entrained under 12-h-light/12-h-dark white fluorescence light (60 μmol m^{-2} s^{-1}) for 7 days at 22 °C. Luminescence measurement was taken at the temperatures as noted under constant red light (20 μmol m^{-2} s^{-1}). Images were collected using a digital CCD camera and processed using NightOwl software (58). Data were imported into the Biological Rhythms Analysis software system (BRASS Ver. 2.14, available from http://www.amillar.org) and analyzed with the FFT-NLLS suite of programs. Period lengths are reported as variance-weighted periods ± S.E., which were estimated using bioluminescence data with a time window from 36–108 h unless otherwise noted.

Hypocotyl Length and Flowering Time Measurements—Stratified seeds were grown under constant red light (10 μmol m^2/s) for 7 days at 22 °C and hypocotyl length was determined with Scion image software. Flowering time was determined from seeds sown directly on soil and grown under short days (S.D., 8 h light/16 h dark) or long days (LD, 16 h light/8 h dark). Flowering time was measured by scoring the number of rosette and cauline leaves on the main stem at the time of first flower emergence. Hypocotyl length and flowering time data are presented as means ± S.E. from one of three (flowering time) or two (hypocotyl length) biological replicates with similar results.

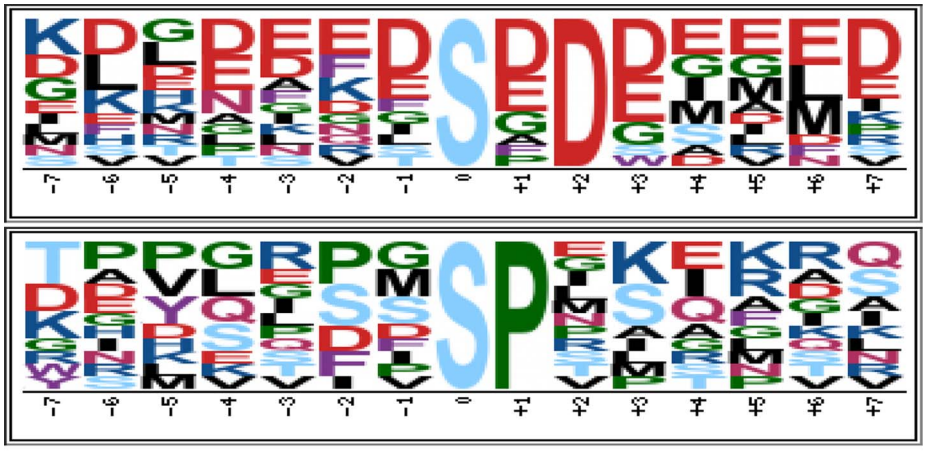
RT-QPCR—Reverse transcription and quantitative real-time PCR (RT-qPCR) analysis was performed to assess *PIF4*, *PIF5*, *PRR7* and *PRR9* transcript levels in Col. WT, *elf4–211* and *elf4–209* using a Bio-Rad CFX96 real-time PCR system with SYBR GREEN I (Stratagene) and *ACTIN2* as the control gene. Total RNA was isolated using TRIzol reagent (Invitrogen, La Jolla, CA) followed by treatment with RNase-free DNase I at 37 °C for 30 min. The absolute values were obtained using the delta Ct method and the average value of all the time points from the wild type was used for normalization. Each single time point value from *elf4–209* and *elf4–211* mutants was then normalized using the wild type average value to make them comparable. Data are represented as means ± S.E. from three biological replicates.

Primers—All primers used are listed in supplemental Table S5.

Accession Numbers—ELF4(At2g26330), *ELF3*(At2g25930), *GI*-(At1g22770), *ACTIN2* (At3g46520).

RESULTS AND DISCUSSION

Identification of Phosphopeptides Under LL in Arabidopsis—To understand the extent to which protein phosphoryla-



Motif	Motif Score	Foreground Matches	Foreground Size	Background Matches	Background Size	Fold Increase
.......s.D.....	4.97	13	60	52870	1013104	4.15
.......sP.....	4.59	11	47	50594	960234	4.44

FIG. 1. **Motif-X-extracted motifs from the circadian regulated phosphopeptide data set.** The peptides window is 15 amino acids with the phosphorylated site centered by retrieving the sequence context from the IPI Arabidopsis proteome database or by filling up the required number of "X" (X stands for "any amino acid"), if necessary. The centered peptides were then aligned and used to extract the motif with the probability threshold was set to $p < 10^{-4}$, the occurrence threshold was set to 10, and the default IPI Arabidopsis Proteome data set was used as the background data set. Note that only those phosphorylated amino acids that were confidently identified (90% cutoff) as the exact site of phosphorylation were used for the analysis.

tion patterns are under circadian regulation, we characterized the phosphoproteome of Arabidopsis seedlings under free-running, constant light conditions. Arabidopsis seedling were entrained for 10 days under 12 h light and 12 h dark cycles (LD) and further maintained under constant white light for 24 h and sampled at four hour intervals over the subsequent 24 h. Phosphorylation sites were identified using HAMMOC-TiO2 phosphopeptide enrichment strategies in combination with high-accuracy mass spectrometric phosphopeptide detection in an LTQ-Orbitrap mass spectrometer (supplemental Fig. S1). A large-scale data set of Arabidopsis phosphorylation sites was obtained using lactic acid-modified titaniaphosphopeptide enrichment methods, as previously detailed (40). The 2.2 version of the MASCOT software was used to simultaneously identify and quantify phosphoproteins. Using this strategy, we identified a total of 2104 unique phosphosites, spanning 1586 phosphopeptides on 1080 proteins from unfractionated Arabidopsis seedling lysates (supplemental Table S1). These numbers are similar in size to previously reported phosphoproteome data sets (40, 59). When compared with other global phosphoproteome studies we found that 159 phosphopeptides are newly reported (supplemental Table S2) (59). The distribution of phosphorylation events was Ser (89%), Thr (10%), and Tyr (1%) (supplemental Fig. S2), which is comparable to the distribution reported by Sugiyama *et al.*, (2008).

Dynamic Phosphoproteome Profiling Under Constant Light/Statistical Cluster Analysis—Using the six point time series we identified 102 phosphopeptides that significantly oscillated. This was determined first using One-way ANOVA Benjamini-Hochberg (FDR Threshold value ≤ 0.05) followed by a visual inspection of the time series. These results were double plotted and paired with mRNA abundance levels of the corresponding gene over the same free run conditions using data from the open transcriptome database of the Plant DIURNAL Project (http://diurnal.mocklerlab.org/) (60) (supplemental Table S3).

The co-expression profile of the significantly oscillating phosphopeptides was represented by an expression graph (Fig. 1) and a heat map (supplemental Fig. S3) using the Multi Experiment Viewer (MEV) software (The Institute of Genomic Research (TIGR) (61). To achieve a comprehensive overview of the expression profile of the circadian regulated phosphoproteins that are co-expressed at different time points during constant light, SOTA clustering was performed whereby proteins with similar patterns in expression level are grouped together. Using SOTA analysis we grouped the 102 proteins into eight distinct clusters (ranging from 6 to 27), allowing a maximum diversity of 0.9 within a single cluster (Fig. 1).

We also grouped the cycling phosphoproteins according to their phase of peak phosphorylation (Fig 2*A*). Peak expression for ca. 80% of the peptides fell either just after subjective dawn or subjective dusk, with about an equal number at each phase. These results reflect a similar phasing of global mRNA expression of cycling transcripts and the two major peaks are 12 h apart. However, our results differ in that the phosphoprotein peaks came just after subjective dawn and dusk rather than being phased slightly before (27). This difference could

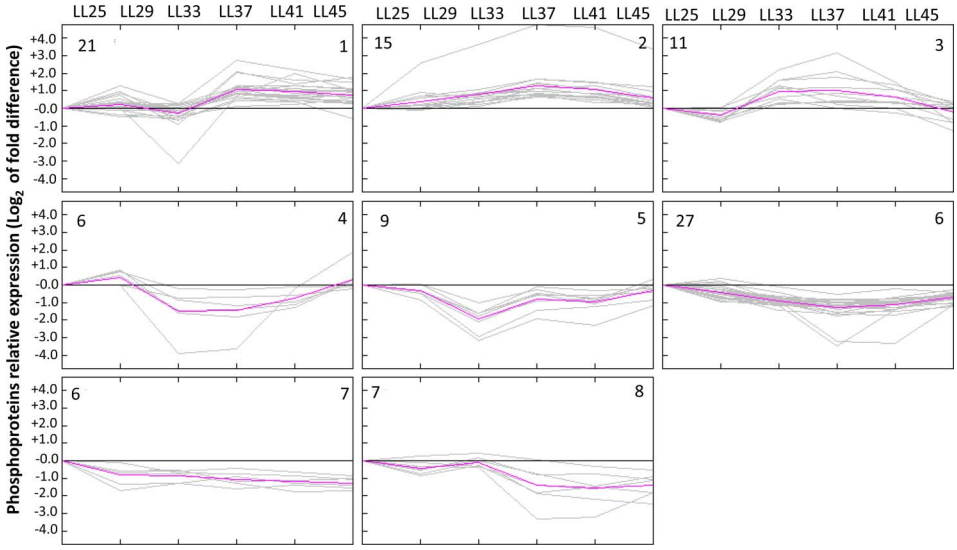


FIG. 2. **Clustering analysis of expression profiles of 102 phosphoproteins under constant light.** SOTA algorithm (MEV software ver 4.9.0) was used for cluster analysis. The phosphoproteins were grouped into eight clusters based on their expression profiles during a circadian cycle. The expression profile of individual proteins in each SOTA cluster is normalized to LL25 and shown in gray; the mean expression profile is marked in pink for each cluster. Vertical axis is in $\log_2$ scale. For each distinct cluster the cluster number is in the upper right corner, and the number of proteins present is in the left upper corner. Detailed information on the proteins within each cluster is in supplemental Fig. S3.

reflect a delay in rhythmic protein accumulation that follows mRNA accumulation.

When the oscillating phosphopeptides were compared with changes in mRNA abundance levels of the corresponding gene (supplemental Table S3), more than half of the rhythmic phosphopeptides exhibited no cycling in message levels (Fig. 2*B*). However, this group showed the same post-subjective dawn and post-subjective night maxima as observed for those genes that were both phospho-rhythmic and mRNA rhythmic (Fig. 2*C*). Thus, post-translational processes control these phospho-oscillations, through phase-specific effects on protein abundance and/or phase-specific protein phosphorylation.

Conversely, 19% of the nonoscillating phosphopeptides were associated with rhythmic mRNA, perhaps reflecting a relatively high stability of the respective proteins.

PTM Motifs—To address the question of which kinases might control circadian phospho-oscillations we used the Motif-X algorithm (51) to identify significantly enriched motifs, using the *Arabidopsis thaliana* database as a reference and a 15 amino acid sequence window surrounding the phosphorylated residues (S, T, and Y). At significance level $p < 10^{-4}$, two phosphorylation motifs were enriched in our data set (Fig. 3 and supplemental Table S4). We further assessed the kinases specific for each motif of the 102 rhythmic phosphopeptides using the NetPhosK 1.0 algorithm (62), and found that casein kinase II (CKII) and proline directed kinases predominate at circadian-regulated phosphosites (supplemental Table S4; supplemental Fig. S4*A*). CKII is known to phosphorylate Ser/Thr residues surrounded by acidic regions, and the P-sP, sP and tP motifs are known targets for proline-directed kinases, which include mitogen activated kinases, cyclin dependent kinases and GSK3 (63). A detailed list of probable kinases involved in circadian regulation based on the NetPhosK1.0 algorithm is presented in supplemental Table S4. We also examined the phase-specific frequency of each of the probable kinases (supplemental Fig. S4). CKII and proline-directed kinases are most likely to be involved in cyclic phosphorylation at subjective dawn (LL25) and subjective dusk and early night (LL 37–41), similar to the times of peaks in phospho-oscillations (Fig. 3*A*; supplemental Fig. S4*A*). In contrast, the AGC kinase family (PKA, PKC and PKG) tend to predominate at the remaining circadian phase times (supplemental Fig. S4*B*).

Previous whole phosphoproteome studies in Arabidopsis have identified enrichment in motifs specifying CKII and proline-directed kinases (64). We support these findings as CKII and proline-directed kinases are predicted to account for 56.0% of the cyclic phosphorylations at all time points. Similarly, for the noncyclic peptides these same kinases are associated with 55.5% of the phosphosites identified. Hence, although motif enrichment within the entire group of cycling phosphopeptides shows no difference from noncyclic ones, there are phase-specific differences as noted above (supplemental Fig. S4). As CKII is currently the only identified protein kinase involved in the plant circadian system (17, 22), these results implicate the involvement of a much wider range of kinases.

GO Analysis—To further characterize the broader relationships of oscillating phosphopeptides, we performed gene ontology (GO) enrichment analysis. Three vocabularies were used to classify the cellular compartments, biological processes and molecular functions of phosphoproteins overrep-

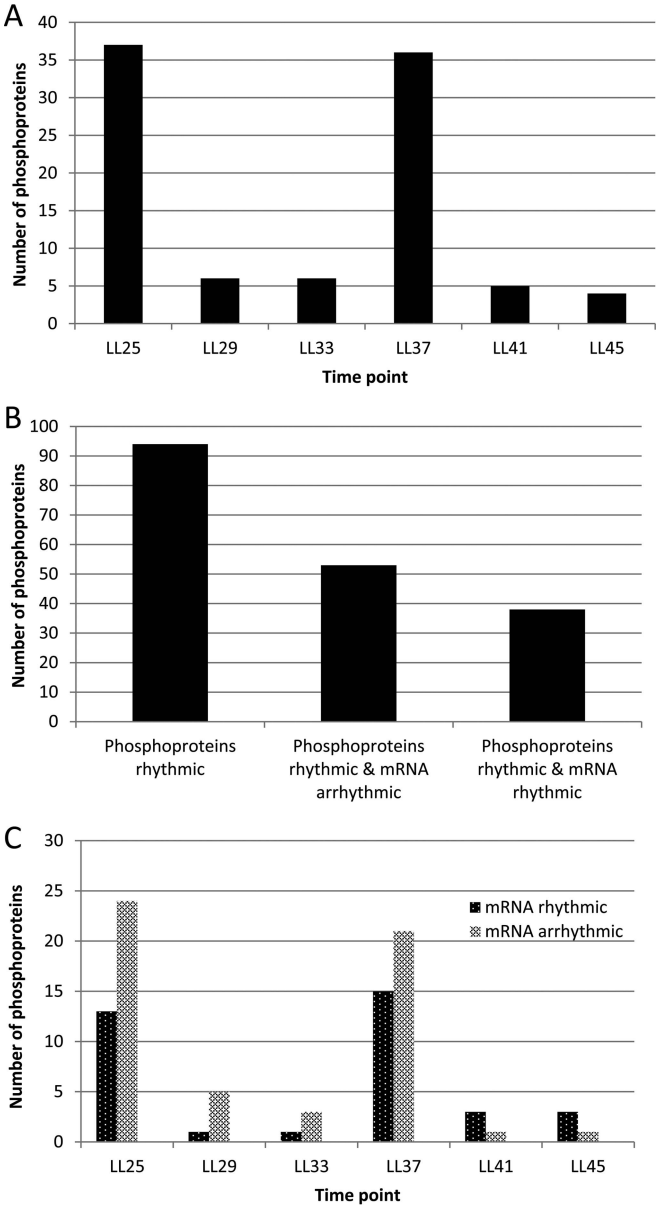


FIG. 3. **Phosphoprotein distribution by circadian phase and mRNA rhythmicity.** (*A*) and (*z*) Number of rhythmic phosphoproteins are shown relative to phase of peak occurrence (A) and relative to whether the respective mRNA is also rhythmic (*B*). *C*, Peak phasing of rhythmic phosphoproteins are shown according to whether the respective mRNA is rhythmic (dark stipple) or whether the respective mRNA is arrhythmic (light stipple).

resented in our data set (Fig. 4). In the cellular component GO, there was an over-representation of cycling phosphopeptides in the cytosol, membrane-associated proteins, and in photosynthesis-related complexes (Fig. 4*A*). In biological process GO categories responses to abiotic stress, including elements of abscisic acid (ABA) mediated signaling, and cellular metabolic processes were notably overrepresented (Fig. 4*B*). This analysis also revealed a significant enrichment of proteins involved in the molecular functions of substrate specific channel activity and carbon metabolism, suggesting that these classes of proteins are preferential targets for circadian regulated phosphorylation (Fig. 4*C*). A previous report of a global Arabidopsis phosphoproteome showed enrichment of chloroplast-related functions and molecular functions concerning RNA metabolism, intracellular trafficking and protein phosphorylation (64). However, at least part of the difference may be because of the much older, leaf-rich tissue in the Reiland study (25 day old rosette leaves)(64), compared with the 12 day-old seedlings used here. Consistent with this, a GO enrichment analysis of the total (cycling and noncycling) phosphopeptide collection of our study showed the nucleus as the overrepresented cellular organelle, whereas in the Reiland study it is the chloroplast.

Functionally Related Proteins Have Similar Phosphorylation Patterns—We next used STRING, a database which allows accumulated protein-protein interaction data to be assembled and viewed as interaction networks, for further analysis of known or predicted interactions between the significantly circadian regulated phosphoproteins (54). Here we identified six different groups of processes, all of which demonstrated the common feature of phosphorylation rhythms present within their pathway or process. These processes include photosynthesis-related proteins, the ribosomal network, the abscisic acid (ABA) pathway, nitrogen metabolism, chaperones, and the circadian system (Fig. 5). This clustering of oscillating phosphopeptides helps to reveal that functionally related proteins may follow similar kinetics of phosphorylation over a circadian time course. For example, the two plastid ribosomal proteins (RPL4 and PSRP4) show identical subjective dusk phases in peak phosphorylation, whereas cytosolic ribosomal proteins RPS6A, RPS6B and the 60S ribosomal protein family are maximally phosphorylated just after subjective dawn (supplemental Table S3). Using SOTA analysis, the phosphorylation status of proteins in cluster 1 (Fig. 2; supplemental Fig. S3) is slightly biphasic, increasing in relative expression to LL29 followed by a decrease and then a gradual increase and leveling off from LL37. This cluster is enriched in proteins involved in the ABA signaling pathway such as ABF2; SNRK2.2; SNRK2.3 and COR78/RD29A. Clusters 2 and 3, which contain phosphoproteins peaking near LL37, were enriched in chaperones and Hsp-related phosphopeptides (Fig. 2; supplemental Fig. S3 and supplemental Table S3). All five of the Hsp and chaperone proteins identified were phased to this time. There are little to no mRNA oscillations for these genes suggesting significant post-translational control of their phosphorylation state. It is also possible that circadian regulation of translation could cause protein cycling and concomitant phospho-oscillations despite constant levels of mRNA.

Phospho-oscillations in Key Elements of Carbohydrate and Nitrogen Pathways—Circadian oscillations in enzyme activity can be conferred in various ways, including cycling protein levels, cycling substrate concentrations, changing levels of

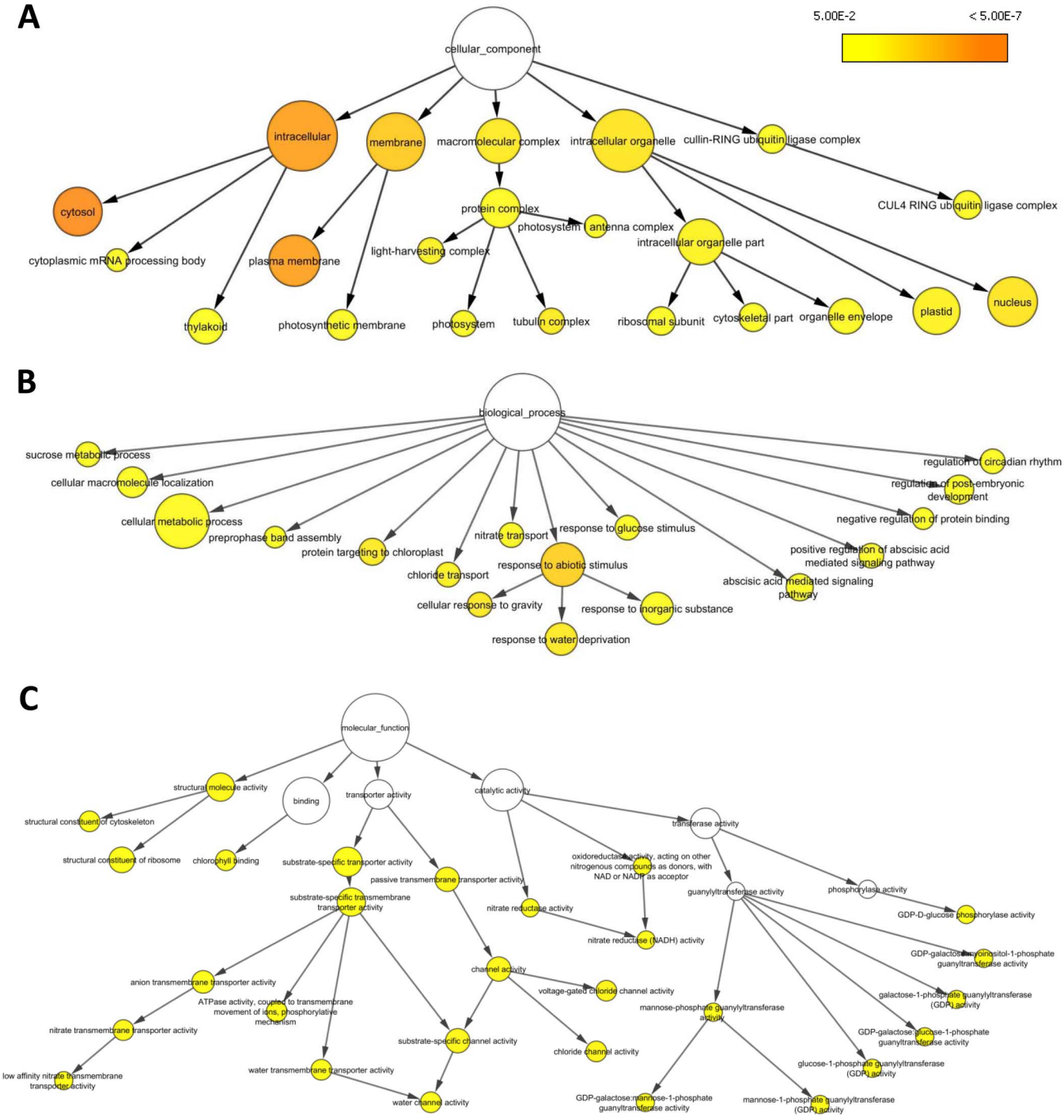


FIG. 4. **Functional classification of the identified circadian-regulated phosphoproteins by GO analysis.** Over-represented GO terms were displayed graphically as yFiles hierarchical trees for three GO vocabularies: *A*, cellular component, *B*, biological process, and *C*, molecular function. The size of the node is proportional to the number of molecules within the group, and the color of the node represents the significance of enrichment (see color scale). Cytoscape version 3.1.1 (http://www.cytoscape.org/) with plugin Bingo version 2.44 were used in this analysis.

allosteric effectors, or changing protein phosphorylation or redox state. Sucrose phosphate synthase 1F (SPS; EC 2.4.1.14) is a central enzyme in photosynthetic metabolism and catalyzes a rate-limiting step in sucrose biosynthesis. SPS is subject to multiple levels of control, including regulation by the allosteric effectors Glc-6-P, phosphate (65, 66) and regulation by protein phosphorylation (67, 68). SPS activity exhibits diurnal and circadian rhythms in tomato, which are the result of corresponding oscillations in SPS protein phosphorylation. In turn, the SPS phosphorylation state is the

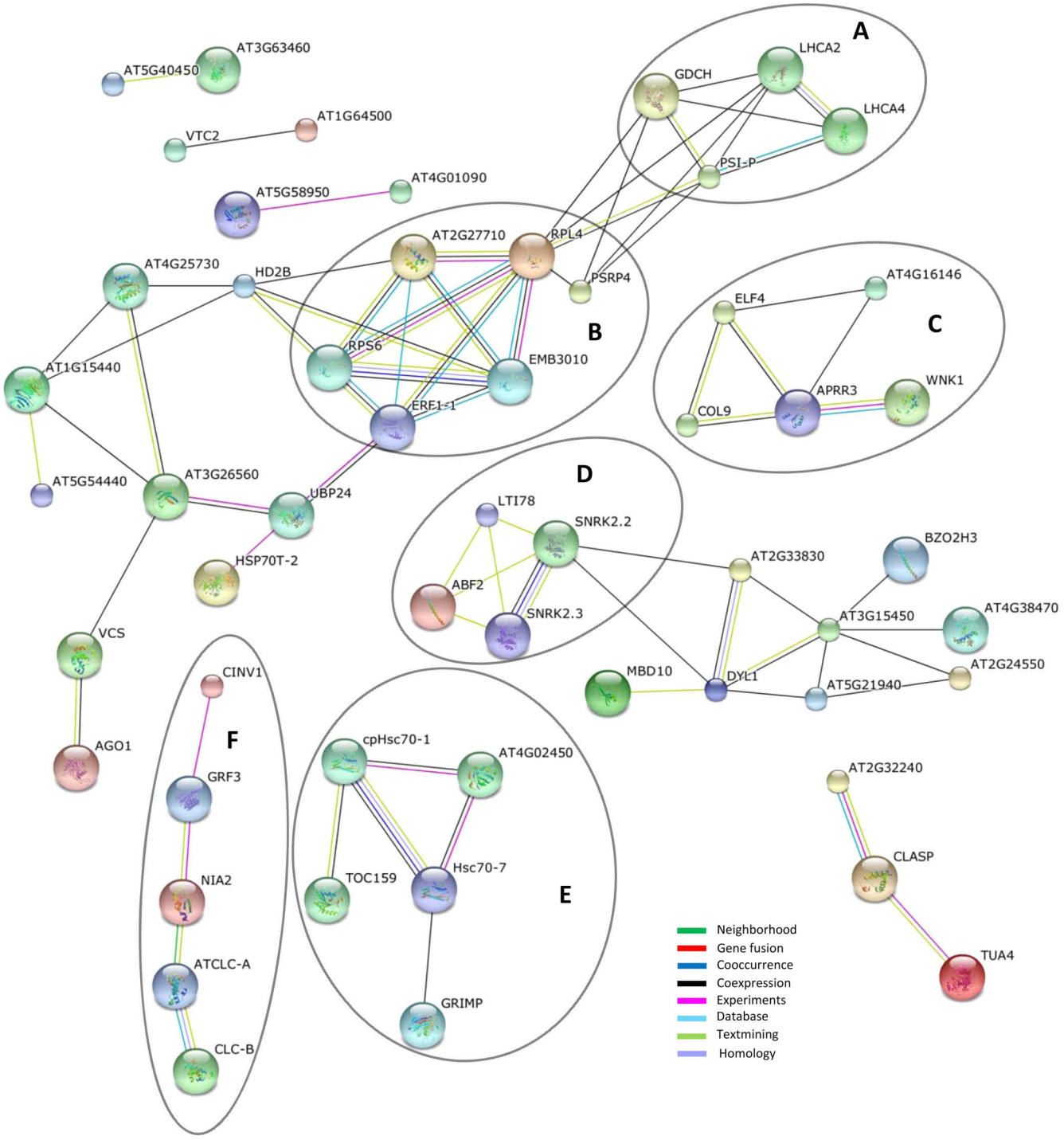


FIG. 5. **Interaction networks of the identified circadian-regulated phosphoproteins.** Network mapping performed by the STRING protein interaction algorithm based on the STRING database (51). *A*, Photosynthesis. *B*, Ribosomal proteins network. *C*, Circadian regulated. *D*, ABA pathway. *E*, Chaperones. *F*, Nitrogen metabolism. Proteins lacking interactions were removed from this display. Line colors indicate the types of evidence for each association.

result of circadian-regulated transcription of a protein phosphatase (69).

Although SPS mRNA is arrhythmic (supplemental Table S3), we identified three differentially phosphorylated SPS peptides that display circadian oscillation. Two are in phase (S121 and S125) and peak at LL25 whereas the third peptide is maximally phosphorylated at subjective dusk (LL37) (S706) (supplemental Table S3). Their anti-phasic nature correlates well with findings from spinach where two sites bring about opposite effects in SPS activity (68, 70). Taken together these

results suggest a mode of post-translational regulation of SPS in Arabidopsis that is similar to previously studied species, but now implicating additional circadian-mediated phosphoregulation of enzyme activity.

Starch degradation and accumulation are under control of light, metabolites, day length, and circadian rhythms (71, 72). The protein levels of many of the enzymes involved in starch degradation are constant throughout the day/night cycle, indicating that post-transcriptional regulation is important in controlling the amount of enzymes and their activities *in vivo* (73).

We identified circadian oscillations at two residues (S55 and S59) of the plastid-targeted β-amylase (BAM1) that is specifically involved in starch degradation in stomata (74) (supplemental Table S3). BAM1 is proposed to trigger diurnal starch degradation, with increased stomatal starch accumulation and reduced diurnal stomatal opening in *bam1* mutants (74). With decreasing BAM1 phosphorylation during the subjective day, our findings suggest that phosphorylation at these sites inactivates the enzyme. These results suggest an additional mechanism of circadian-regulated control of stomatal pore size via phase-specific phosphorylation-dependent enzyme activity.

The clock regulates nitrate reductase (NR) activity in many plant species and circadian control may be at the transcript or protein level, depending on the species (75–80). In higher plants, NR inactivation is mediated by the phosphorylation of a conserved serine residue (S534) (supplemental Table S3) and binding of 14–3-3 proteins in the presence of divalent cations or polyamines (81). Mutation of this phosphorylation site in Arabidopsis to aspartate (82) results in the complete abolition of activation/inactivation in response to light/dark transitions or other treatments known to regulate the activation state of NR. In agreement with this finding, we observed a strong morning-phased oscillation in the phosphorylation state of this regulatory residue (S534) in nitrate reductase 2 (NIA2) that is antiphasic to the maximum of NR activity (80). These results now implicate clock-controlled phosphorylation of NR as a primary regulator of the activity of this enzyme.

Interestingly, mRNA expression of nitrate transporter 1.7 (*NRT1.7*) is diurnally regulated and temporally opposite to that of *NIA2*, with *NRT1.7* expression highest at dusk and *NIA2* highest at dawn (83). The circadian expression patterns for phosphorylated peptides of both proteins are similarly antiphasic (supplemental Table S3). The opposite diurnal patterns of NRT1.7 and NIA2 suggest that when nitrate reductase activity is down regulated during the night, excess nitrate in the leaf can be exported out by NRT1.7, which is localized to the phloem (83). Using different time periods for assimilation and export could ensure that the older leaves still get sufficient nitrate for assimilation to meet their own nitrogen demand, and only excess nitrate is transported out.

We also identified a third element of nitrate regulation that undergoes circadian phospho-oscillations. Anion channels/transporters contribute to a wide range of physiological functions such as control of stomatal movements, plant-pathogen interactions, root xylem loading, compartmentation of metabolites and coupling with proton gradients (84). In our data set one or both members of the tonoplast-localized chloride channel (CLC) protein family, CLCa and CLCb, undergo phospho-oscillations with the same late evening phase of maximum phosphopeptide accumulation followed by a rapid decrease during subjective day (supplemental Table S3) (85, 86). AtCLCa facilities nitrate accumulation in the vacuole, acting as a NO_3^-/H^+ exchanger (87) and it is likely that CLCb performs a similar role.

As some of these components involved in nitrogen regulation and homeostasis do not exhibit robust, sustained circadian oscillations in mRNA levels (*e.g.* NRT1.7 and CLCa), our findings implicate a significant role for the circadian system in the post-translational control of nitrate regulation via phosphorylation.

Phosphorylation of Aquaporins is Clock-regulated—Water channel proteins (aquaporins) are important in the control of water permeability in plant cells. Diurnal and circadian changes in water permeability result, in part, from changes in aquaporin expression levels. For example, gene expression of both tonoplast (δ-TIP) and plasma membrane aquaporins (AtPIP1;2 and AtPIP2;1) are clock-regulated (27, 88). Other more recent work has identified post-translational processes as significant control points in aquaporin function (89–91).

We identified sites for two aquaporins, AtPIP2;2 and AtPIP2;4, that exhibit circadian oscillations in their phosphorylation state but not mRNA levels (supplemental Table S3). Diphosphorylation of the related aquaporin, AtPIP2;1, at S280 and S283 in Arabidopsis leaf mesophyll increases hydraulic conductivity (K_{ros}), with K_{ros} (and Ser phosphorylation) higher in the dark than in light (89). These diphosphorylations lie within the C-terminal sequence GSFRS, which is closely conserved in AtPIP2;2 and AtPIP2;4 and correspond to the residues undergoing rhythmic oscillations in LL (AtPIP2;2 S-278; S-281 and AtPIP2;4 S283; S286; supplemental Table S3). Additionally, the dusk/early evening phasing of maximum phosphorylation of the PIP2;4 residues (LL 37–41) corresponds well with the time of maximum hypocotyl expansion in constant light (92). Taken together, these findings suggest that circadian oscillations in the phosphorylation state of key regulatory serines in aquaporins may contribute to the circadian regulation of water uptake and drive hypocotyl and leaf movement rhythms.

Components of the Translational Machinery Undergo Phase-specific Changes in Phosphorylation—Eukaryotic protein translation is mainly controlled at the level of initiation, which involves multiple events of protein phosphorylation (93). In higher plants changes in the phosphorylation status of ribosomal protein S6 (RPS6) have been found responsible for rapid adjustments in growth patterns under environmental change (94). RPS6 phosphorylation in plants leads to the

selective recruitment of ribosomal mRNAs to polysomes and thus regulates the switch of translational capacity between growth promoting and stress conditions (95). Previous work has established a positive correlation between phosphorylation of the S6 protein and translation, particularly of ribosomal proteins and a subset of proteins related to protein synthesis (94–98).

Several phosphorylation sites on the ribosomal protein S6 from Arabidopsis and maize have been mapped, and ties to diurnal and hormonal factors have been reported (64, 96, 99, 100). In Arabidopsis, Turkina *et al.* (2011) found a two- to fourfold increase in the day/night phosphorylation ratio at S231, S237 and S240 sites in the ribosomal proteins S6–1 and S6–2, suggesting that their differential phosphorylation may contribute to modulation of diurnal protein synthesis (100). We find that the phosphorylation status of some of these same residues (S237, S240, and S241; supplemental Table S3) on these ribosomal proteins oscillate in constant light, confirming these earlier reports but now implicating circadian control of phosphorylation as a significant factor in the control of translation. The higher phosphorylation state of S6 during the day could be at least partially responsible for the elevated level of general protein synthesis during the photoperiod (101).

In addition to RPS6, the acidic P-proteins, which form a lateral stalk structure in the active site of the 60S ribosomal subunit of ribosomes, undergo N-terminal phosphorylation, which may play a role in translational responses to external stimuli (102, 103). We have identified an early morning-phased Ser-105 phosphorylation of 60S acidic ribosomal protein P2–2 (supplemental Table S3). The phasing of this modification is similar to the RPS6 phosphorylations, which, together with the absence of mRNA cycling, may suggest a coordinated effect of translational control by the circadian system. Interestingly, the phosphorylation states of two plastid ribosomal proteins (RPL4 and PSRP4) oscillate together but out of phase with the cytosolic ribosomal proteins (supplemental Table S3).

Photosynthesis—Numerous proteins of the thylakoid membrane system undergo changes in phosphorylation state in response to a host of environmental conditions and stresses (104, 105). These include diurnally-driven phospho-oscillations of some chloroplast proteins in response to light/dark cycles, and circadian control of the phosphorylation state of the D1 photosystem II reaction center protein (106–108). We have identified circadian control of the phosphorylation state of three thylakoid membrane proteins: Two light harvesting complex proteins of Photosystem I (PSI) LHCA2 and LHCA4, and CURVATURE THYLAKOID1B (CURT1B)/TMP14 protein, a modifier of thylakoid membrane architecture (109). Previous studies identified light/dark dependent phosphorylation of CURT1B/TMP14 at T65 (110, 111), which we confirm and also show to be phased early in the subjective day (LL29) (supplemental Table S3). For LHCA2 we identified a novel evening-phased site (LL37–41) at S164 and a morning-phased (LL29) S38 phosphorylation on LHCA4 (supplemental Table S3).

Phosphorylation and Clock Dependent Gating in ABA Signaling—Abscisic acid (ABA) is a key regulatory factor in various developmental and physiological processes in plants, including seed maturation, dormancy, seedling development, and stomatal behavior. ABA signaling is in part modulated by the circadian system at the transcriptional level (112, 113). We have identified phase-dependent phosphorylation of several ABA response pathway proteins. ABF2, SNF1-related kinase 2s (SNRK 2.2 and SNRK 2.3), and low temperature induced 78Da protein 78A (COR 78A/RD29A) share very similar phosphorylation profiles that tend to peak in the early to middle subjective night (supplemental Table S3). Transcript abundance of the four genes shows circadian oscillations with an earlier peak, from between the middle and end of the subjective day. SNRK2.2 and SNRK2.3 are phosphorylated at T178 and T177 respectively, whereas S45 and S691 are phosphorylated in ABF2 and COR78 respectively.

Active phosphorylated SNRK2 phosphorylates the transcription factor ABF2(AREB1), which drives the expression of numerous ABA-responsive genes (114–116). SNRK2.2 and SNRK2.3 both interact with the protein phosphatase 2C ABA INSENSITIVE1 (ABI1), which is a negative regulator of ABA responses. In the absence of ABA, protein phosphatase 2Cs (PP2Cs) dephosphorylate and consequently inactivate the SNF1-related kinases (117). SNRK2.3 also interacts with PYRABACTIN RESISTANCE1 (PYR1), the ABA-binding inhibitor of ABI1 that forms the core of an ABA receptor system (118, 119). Taken together, transcript oscillations of these four genes suggest that clock-dependent gating in ABA signaling occurs through protein oscillations and/or phase-dependent phosphorylation of these SNRKs.

The Ca^{2+}-dependent protein kinase CPK6 showed a strong early morning phasing of phosphorylation at residues S536 and S540 (supplemental Table S3). CPK6 acts during ABA-induced stomatal closure as a positive regulator of ABA control of S-type anion channels (SLCA) in guard cells (120). Additionally, CPK6 strongly activates SLAC1 channels and CPKs can replace SnRK2 kinases in ABA regulation of SLAC1 channels, suggesting that both of these protein kinase families function in parallel *in vivo* (121). Hence, phase-dependent CPK phosphorylation is another likely point of ABA-mediated signaling that intersects with control by the circadian system.

A significant subset of ABA-regulated genes is regulated by the Ca^{2+} mobilizing signaling molecule cyclic adenosine diphosphate ribose (cADPR) (122). Given that the concentration of cADPR and cytosolic free Ca^{2+} are under circadian oscillations (29), it seems that circadian timing information encoded within cADPR and Ca^{2+} signals likely becomes integrated with environmental information via pathways involving CPK6 and SNRK2 phosphorylation events.

Circadian Clock Related Proteins—Many transcripts and proteins involved in maintaining the circadian oscillator are

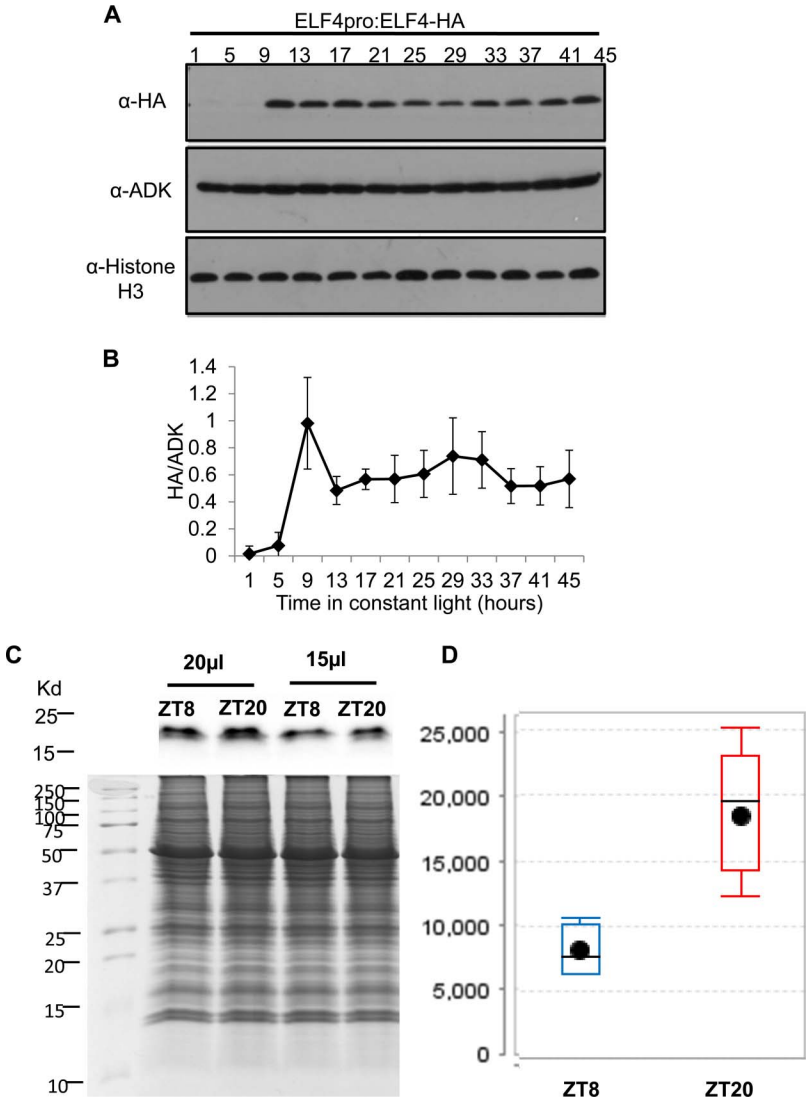


FIG. 6. **ELF4 phosphorylation levels do not follow protein abundance.** (*A*) and (*B*) ELF4-HA levels do not oscillate in constant light. (*C*) and (*D*) ELF4 levels in 12 h light/12 h dark conditions at 8 h after lights on (ZT8) and 8 h after lights off (ZT20) show twofold changes in phosphorylation state (*D*) despite no changes in ELF4 levels (*C*). Adenosine kinase (ADK) and histone H3 shown as loading controls for cytosolic and nuclear compartments, respectively. Coomassie stained total protein shown as loading controls in (C*). y* Axis in (*D*) is mean ion peak intensity. Figs. representative of at least three trials. Bars show S.E. in (*B*).

themselves clock controlled. We identified two clock-related peptides that undergo circadian oscillations in phosphorylation. PRR3 (Pseudo Response Regulator 3) is one of a five member gene family that oscillates with strong rhythms in mRNA and protein levels (16). A deficiency in PRR3 slightly shortens circadian period and PRR3 overexpression alters flowering time (123–125). PRR3 interacts with TOC1 and helps to stabilize TOC1 levels through sequestration from degradation by ZTL (21, 124). N-terminal and C-terminal regions of these proteins share strong amino acid sequence similarities, denoted as pseudoreceiver (PR) and CCT motifs, respectively (126, 127). The peptide identified here lies within the PRR3-specific region and the phosphorylation state oscillates in the opposite phase of maximum PRR3 mRNA and protein abundance (supplemental Table S3).

ELF4 is a component of a tripartite transcriptional "evening complex" (EC) that oscillates in abundance with maximum expression in the late evening (128). The EC is responsible for the evening repression of a number of clock components that are expressed early in the circadian cycle. We detected a very strong oscillation in the phosphorylation of S45, which is in phase with the mRNA and protein rhythms of ELF4 early in LD and early LL (supplemental Table S3) (128). However, by the second day in constant light (LL 25–45), both ELF4 message and protein levels show very little oscillation whereas rhythms in the S45 phosphorylation state remain very robust (supplemental Table S3; Fig. 6*A*, 6*B*). We also assayed the ELF4 phosphorylation state under LD at two time points when the protein abundance was very similar (ZT 8 and ZT 20; Fig. 6*C*, 6*D*). S45 phosphopeptide levels at ZT20 were more than double those found at ZT8, very similar to those found under constant light. These results show that phospho-oscillation at S45 is independent of protein levels and light/dark cycles, and suggest that ELF4 phosphorylation is clock-regulated, possibly through clock-gated kinase or phosphatase activity.

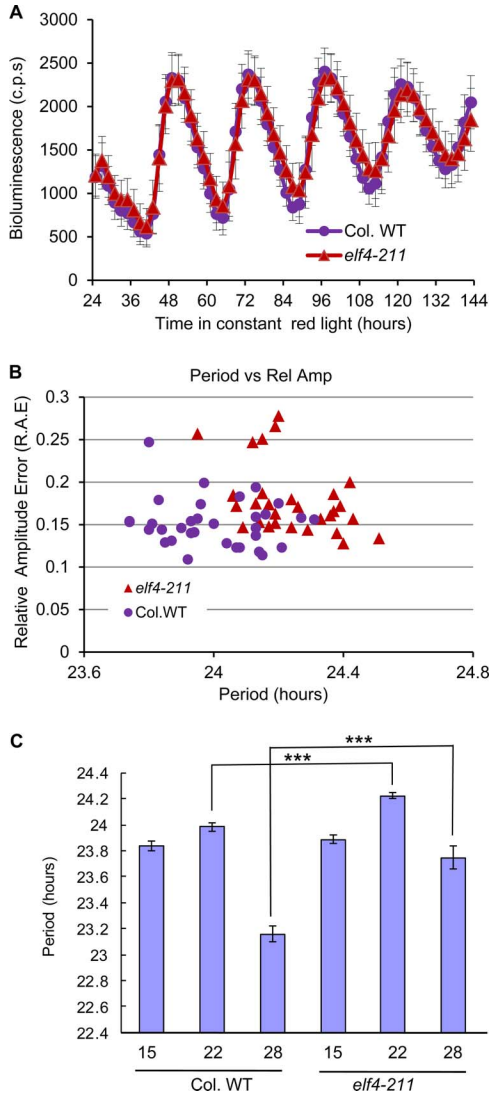


FIG. 7. **Circadian period of *elf4–211*.** (*A*) and (*B*) Oscillations in *CCA1-luciferase* expression in wild-type (Col. WT) and *elf4–211* under constant red light at 22 C. Relative amplitude error (RAE) indicates robustness of circadian amplitude, with levels closer to 0 indicating high amplitude oscillations (147). *C*, Circadian period of WT and *elf4–211* at different temperatures. Bars show S.E. *** indicates $p < 0.001$; $n = 25–55$ from two to three biological trials.

To understand the significance of phosphorylation of S45 we took advantage of the *elf4–211* allele, which bears an S45L transition (129). *elf4* null mutants are very poorly rhythmic, flower early in short days and have long hypocotyls (130–132). We first assayed circadian period and these developmental phenotypes associated with *elf4–211*. Free-running period in *elf4–211* was as robust as WT and slightly, but reproducibly, longer than WT (Fig. 7*A*, 7*B*). *elf4–211* flowering time in long and short days was as for WT, and hypocotyl lengths in short days and dim red light were also unchanged from WT (supplemental Fig. S5). We next tested known molecular interactions and control points that are affected by ELF4. In the context of the EC, ELF4 binds ELF3 (128, 133). We performed transient *in planta* immunoprecipitations between ELF3 and ELF4 (WT and S45L). In quantitating the ratio of IPed ELF3-TAP to coIPed ELF4-HA we found that the S45L allele reduced the ELF4-ELF3 interaction to ca. 64% of WT (Fig. 8). Similar tests of the ELF4-GI interaction (44) showed no effect (supplemental Fig. S6).

Allelic differences in ELF4 could affect the transcriptional repression activities of the EC complex. We next tested the expression pattern of *PIF4*, *PIF5*, *PRR7,* and *PRR9*, four genes that are direct targets of the EC complex (128, 134, 135). After 1 day in constant light, *PRR7* and *PRR9* mRNA levels oscillated robustly with early morning phases in the WT. The *PRR9* message levels in the *elf4–209* (null) mutant were low and arrhythmic, and *PRR7* mRNA showed a much delayed phase with low amplitude cycling (Fig. 9*A*, 9*B*). Both transcripts in the *elf4–211* background showed similar phasing and amplitude as the WT, suggesting that misregulation of these genes is not the cause of the slight period lengthening. Over the same time course, *PIF4* and *PIF5* expression levels were slightly phase-delayed relative to WT but not as strongly damped as in *elf4–209* (Fig. 9*C*, 9*D*).

At higher temperatures (28°C) period was affected more strongly, with a ~0.5 h lengthening in the *elf4–211*line (supplemental Fig. S7). We also tested the elf4–211 at 15°C but found very little effect on period, relative to WT (supplemental Fig. S8). When taken together (Fig. 7*C*) it is apparent that the greater difference between WT and *elf4–211* at higher temperatures results from the greater shortening effect of higher temperatures on WT period.

Current models depict a tripartite ELF4-ELF3-LUX complex as a central evening-phased repressor (Evening Complex; EC) in the circadian clock (128, 136). Based on genetic and physical interaction data ELF4 may be considered as an amplifier of the EC amount/function given the ability of overexpressed ELF3 to rescue the arrhythmicity of the *elf4–1* mutant (133). Part of this function may arise from the ability of ELF4 to facilitate nuclear localization of ELF3, resulting in an increase in functional (nuclear) ELF3 levels in the context of the EC. The reduction in the ELF3-ELF4 interaction arising from the S45A allele (*elf3–211*) (Fig. 8) may be the cause of the slightly longer period, especially at higher temperatures (Fig. 9*C*). Although ELF4 also interacts with nuclear GI to sequester it from the *CONSTANS* promoter, affecting flowering time in Arabidopsis, it is not known whether ELF3 and LUX are part of this process (44). *elf3*, *elf4* and *lux* mutants show similar phenotypes of early flowering, long hypocotyls and reduced amplitude or near arrhythmicity (128, 132, 137). A recent comparison of gene expression differences among these three mutants showed that *elf3–1* affects HY5 expression differently in response to UV stress (138). Hence, ELF4 may likewise participate in processes independent of its currently known partners.

Strong phosphorylation rhythms of ELF4 in the absence of protein cycling and in both constant light (Fig. 6*A*, 6*B*) and

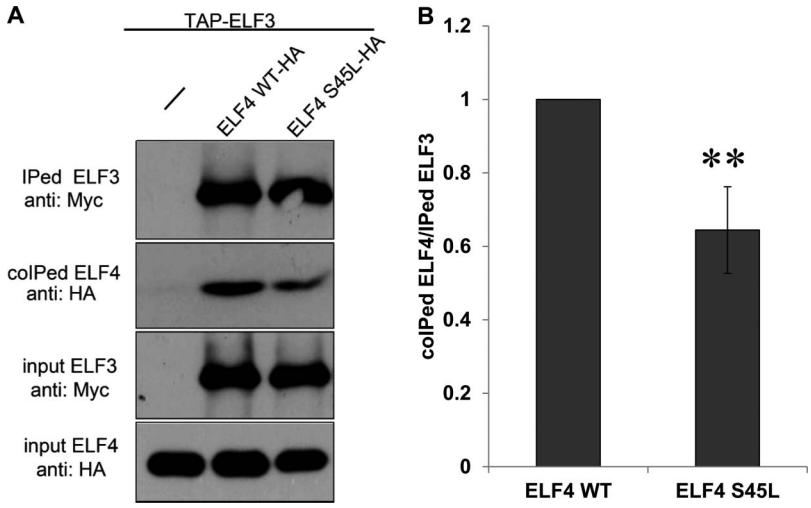


FIG. 8. **ELF4 S45L diminishes interaction with ELF3.** *A*, Immunoprecipitates of TAP-ELF3 co-expressed transiently in *N. benthamiana* with either ELF4-HA or ELF4 S45L-HA were probed with either anti-MYC (ELF3) or anti-HA. *B*, Quantitation of ELF4/ELF3 ratio. Mean of 3 biological replicates. ** denotes $p < 0.01$. Bars show S.E.

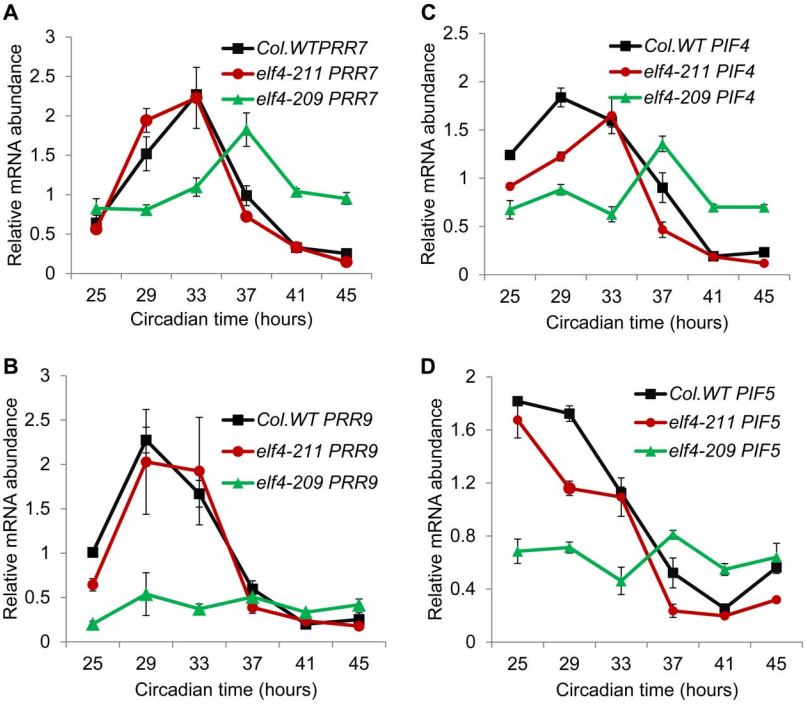


FIG. 9. **Expression of four genes regulated by ELF4 in *elf4–211* and *elf4–209* (null) relative to WT.** Quantitation by qPCR of (*A*) *PRR7*, (*B*) *PRR9*, (*C*) *PIF4,* and (*D*) *PIF5* mRNA levels after 24 h in constant white light. Mean of 3 biological replicates. Values relative to time course-averaged WT. Bars show S.E.

light/dark cycles (Fig. 6*C*) indicate other factors are important to sustain this phospho-oscillation. It is possible that other EC components (ELF3 or LUX) may oscillate more strongly in LL, and the formation/dissociation of the complex may be rhythmically dependent on their oscillations. If so, this larger tripartite complex may provide a docking platform for kinases which could explain the ELF4 phospho-oscillations.

CONCLUSIONS

Circadian control of gene expression in Arabidopsis has been largely based on studies of mRNA abundance, demonstrating that a large percentage of the transcriptome oscillates daily (24–26). Very often rhythms in transcript levels result in concomitant oscillations in protein levels, usually with a phase delay of 2–3 h or more (16, 139, 140). However, some studies in other circadian systems have shown that up to 50% of the cycling proteome is not reflected in mRNA rhythms, indicating a significant degree of clock-mediated post-transcriptional control (34, 36, 139).

Proteomics methods now allow direct characterization of abundance changes of essential proteins and phosphoproteins during the circadian cycle. Although there have been limited reports on circadian oscillations of the phosphorylation state in certain tissues or proteins (34, 141), there has been no systematic phosphoproteome-wide circadian analysis in any species. In this first large scale quantitative circadian phosphoproteomic study, we identified circadian oscillations in phosphoprotein abundance and compared them to

their respective transcript rhythms. Of the more than 100 phosphopeptides which oscillate under constant light and temperature, nearly half were genes with nonoscillatory mRNAs, implying post-translational rhythms either in protein abundance or in their phosphorylation state or both (Fig. 3). These results clearly show that circadian clocks can coordinate Arabidopsis cellular processes by orchestrating cycles of phosphorylation separate from transcriptional control.

Our network analysis using the STRING database highlighted an important subset of processes that undergo circadian regulation of phosphoprotein abundance. We especially note that coordinated phosphorylation of a number of ribosomal proteins indicates that the clock plays a central role in controlling this fundamental cellular process (Fig. 5; supplemental Table S3). Consistent with this is a recent study showing that the circadian clock influences ribosome biogenesis in mice by regulating the expression and activation of essential translation factors (142). Similarly, our finding that >40% of the rhythmic phosphoproteins do not possess a corresponding rhythmic transcript, suggests that phosphorylation, translation and/or protein stability of these gene products are subject to circadian regulation. Taken together, our findings support the emerging notion (33, 139) that circadian regulation of protein stability may be as common of a regulatory mechanism as found for mRNA processing and stability (143–145).

Additionally, another important node in our interaction network is comprised of a number of chaperones showing phospho-oscillations with a dusk phase (Fig. 5; Supplemental Table S3). This may indicate a higher demand for protein folding or quality control near the light/dark transition. Interestingly, a similar cohort of chaperones (HSP70B) and putative co-chaperones (chloroplast-targeted DnaJ-like proteins) were identified in *Chlamydomonas* and linked to redox-regulated control of chloroplast protein clusters (146). Further work is necessary to determine if such dusk-phased oscillations are uniquely present in photosynthetic organisms, although late-night phased oscillations of chaperones and co-chaperones proteins in mouse liver have been reported (139).

More specific to plants, circadian phospho-oscillations in components of the abscisic acid (ABA) hormone signaling pathway, nitrogen metabolism and photosynthesis appear significant and stand out from other metabolic processes as particularly subject to post-translational clock control (Fig. 5; supplemental Table S3). These findings elucidate new interaction networks that confer previously uncharacterized rhythms onto metabolism and physiology. Thus, our data set provides several new starting points for further investigation of phosphoproteins and pathways involved in, or regulated by, the circadian system in Arabidopsis.

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S This article contains supplemental Figs. S1 to S8 and Tables S1 to S6.

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