Orchestrated Transcription of Key Pathways in Arabidopsis by the Circadian Clock

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Circadian rhythms control processes ranging from human sleep-wake cycles to cyanobacterial cell division. This is made possible by the circadian clock, an internal biochemical oscillator. The circadian clock allows organisms to anticipate daily changes in the environment such as the onset of dawn and dusk, providing them with an adaptive advantage (1). Physiological processes coordinated by the clock in higher plants include photoperiodic induction of flowering (2) and rhythmic hypocotyl elongation, cotyledon movement, and stomatal opening (3). A small number of genes regulated by the clock have been found in an essentially serendipitous fashion (4, 5). However, a global examination of genes controlled by the clock in plants, or in any eukaryote, has been lacking.

The circadian clock regulates hundreds of genes. We have used highly reproducible oligonucleotide-based arrays (6) to determine steady-state mRNA levels in Arabidopsis at 4-hour intervals during the subjective day and night. We examined temporal patterns of gene expression in Arabidopsis plants under constant light conditions using GeneChip arrays representing about 8200 different genes. We hybridized duplicate microarrays with biotin-labeled probes derived from plant tissues harvested every 4 hours over 2 days (7). Reproducibility between arrays was excellent (Web fig. 1) (8). The mean hybridization signal strength and the standard error of the mean for each probe set at each time point were calculated from the duplicate hybridizations.

To objectively determine which genes exhibited a circadian pattern of expression, we empirically tested for statistically significant cross-correlation between the temporal expression profiles of each probe set and cosine waves of defined period and phase. Genes with a greater than 95% probable correlation with a cosine test wave with a period between 20 and 28 hours were scored as circadian-regulated (9). This analysis is independent of signal strength and imposes no minimal change in amplitude. According to this criterion, 494 probe sets, representing 453 genes or 6% of the genes on the chip, were classified as cycling (Web table 1) (8); 28% of these genes have not been characterized, and no conclusions can be drawn about their function. More than 20 of the known genes we found to be clock-regulated have been previously reported to be under circadian control (3, 10), validating our experimental methods.

We placed the cycling genes into phase clusters of peak expression time. All six possible phases (given our 4-hour time resolution) were well represented, although there were fewer genes peaking at CT16 (11) than in other phases [Web table 1 and Web fig. 2 (8)]. This is in contrast to cyanobacteria, in which 80% of circadian-regulated genes peak near subjective dusk (12). Many of the genes we found to cycle can be clustered into functional groups on the basis of their known and predicted physiological roles.

Clock-controlled anticipation of dawn and dusk. A large cluster of genes implicated in the light-harvesting reactions of photosynthesis were found to be under clock control. mRNAs encoding four LHCA and seven LHCB proteins, chlorophyll binding proteins that funnel light energy to the reaction centers of photosystems I and II, were cycling (Fig. 1A). Also, mRNA encoding an enzyme (protoalloxyan IX magnesium chelatase) involved in the synthesis of their ligand, chlorophyll, was cycling (Web table 1) (8). Seven photosystem I reaction center genes and three photosystem II reaction center genes were likewise cycling (Fig. 1B). These 22 photosynthesis genes exhibit strict coregulation, with most peaking around midday at CT4 (9). Two LHC genes, the reaction center gene PSAT1, and the magnesium chelatase gene have been previously reported to cycle (10, 13).

Light also regulates growth and development and resets the circadian clock. Genes encoding phytochrome B (PHyB), cryptochrome 1 (CRY1), cryptochrome 2 (CRY2), and phototropin (NPH1) (Web fig. 3A) (8) were clock-regulated. Homologs of the blue light photoreceptor genes CRY1 and CRY2 are also clock-controlled in animals (14). Downstream mediators of phototransduction pathways, SPA1 and RPT2, were also clock-

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below that threshold were inspected by eye. To be considered significantly similar, the two proteins had to show >50% identity over a region of at least 75% of the length of one of them.

56. InterPro (www.ebi.ac.uk/interpro/) is a database that integrates protein domain and motif sequence patterns from other databases, like PROSITE, Pfam, and PRINTS.
57. We acknowledge the work of all those who have participated in the Arabidopsis Genome Initiative (AGI), as well as the AGI policy of immediate release of sequence data, which made possible the analysis presented here. We thank all of our colleagues at Mendel Biotechnology for their input and work in our functional genomics research program and E. Meyerowitz and F. Ausubel for discussions and comments on the manuscript.

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regulated (Web fig. 3B) (8). Because phytochromes and cryptochromes mediate light input to the clock (15), they are both clock input and output components, creating outer feedback loops (Web fig. 3C) (8). The “gating” (16) of light signal transduction by the circadian clock may be due to this circadian control of light signaling components.

The circadian clock may also orchestrate the production of photoprotective pigments early in the day. Remarkably, 23 genes encoding enzymes in the phenylpropanoid biosynthetic pathway were coordinately regulated to peak before dawn at CT20 (Fig. 2A). The phenylpropanoid pathway produces many secondary metabolites (Fig. 2B), several of which act as “phenolic sunscreen” by absorbing light in the visible and near-ultraviolet (UV) range (17). Arabidopsis plants deficient in this pathway have an increased susceptibility to UV radiation (18, 19). The coregulation of these genes suggests that they may be controlled by one or more common regulatory factors. Myb family transcription factors, often acting in concert with basic helix-loop-helix proteins, activate the transcription of a number of flavonoid genes (20). A cycling gene homologous to the petunia Myb AN2 gene had a phase and waveform similar to that of the phenylpropanoid biosynthesis genes (Fig. 2A). This gene encodes PAP1, whose overexpression causes the overproduction of anthocyanins and lignins. In addition, PAP1-overexpressing plants up-regulate several key flavonoid enzymes (21), which are all under synchronous clock control (Fig. 2A). The striking coregulation of PAP1 with the phenylpropanoid biosynthesis genes suggests that PAP1 acts as a master regulator of clock-controlled transcription of these genes.

Chilling resistance is an important trait in plants. We found that a number of enzymes involved in lipid modification, including two desaturases, were under clock regulation and peaked near subjective dusk (Web fig. 4A) (8). This is consistent with previously observed rhythms in membrane lipid desaturation levels that correlate with increased resistance to cold treatments during the subjective night (22). Also, a number of genes previously shown to be up-regulated by cold or darkness were cycling and peaked around CT8. One of these, COR15B, is closely related to COR15a, whose overexpression confers freezing resistance on plant protoplasts (24). A gene encoding the transcription factor DREB1a/CBF3, which binds to the promoter elements of a number of these cold-induced cycling genes, was itself clock-controlled and peaked around midday (Web fig. 4A) (8). Overexpression of DREB1a/CBF3 conferred cold resistance on Arabidopsis (25). We suggest that the circadian rhythm in cold resistance seen in cold-sensitive plants may be mediated in part by the circadian regulation of DREB1a/CBF3 expression (Web fig. 4B) (8).

Clock-mediated coordination of carbon, nitrogen, and sulfur pathways. The daily action of photosynthesis results in the production of sugars that can be consumed by the cell in which they are produced, transported to nonphotosynthetic sink tissues, or stored for later use. Genes implicated in all of these processes were under clock control and peaked near the end of the subjective day.

These included six genes involved in the glycolytic and oxidative pentose phosphate pathways, two routes for the conversion of glucose into metabolites or its oxidation to produce adenosine triphosphate (Web fig. 5A) (8). Also peaking at this time were genes encoding enzymes that synthesize the sugar alcohol galactinol, which can be stored transiently in the plant vacuole or transported to other tissues via the phloem (Web fig. 5B) (8). Four genes encoding predicted hexose transporters also peaked at CT8 (Web fig. 5C) (8). These transporters may move sugars either from source to sink tissues or between organelles within cells. Transcripts for three isozymes of trehalose 6-phosphate synthase, which may play a role in the regulation of sugar metabolism in higher plants (25), were also clock-controlled and peaked coordinatey at CT8 (Web fig. 5D) (8). The up-regulation of these diverse genes near the end of the subjective day suggests that the clock may play an important role in allocating assimilated sugars to different pathways (Web fig. 5E) (8).

Another possible fate for sugar is its storage as starch in the chloroplast for use during the night, when the plant cannot photosynthesize. A cluster of genes encoding enzymes implicated in starch mobilization was under clock control, peaking during the subjective night between CT16 and CT20 (Fig. 3A). A model for how these proteins might work together to convert starch into sucrose, the predominant form in which sugars are translocated in plants, is presented in Fig. 3B. Our results establish that the circadian clock plays a role in the allocation of fixed carbon to its...
three principal fates (metabolic usage, transport, and storage) and suggest that circadian regulation of sugar metabolism may provide a mechanism for carbon homeostasis (26).

Assimilation of mineral nutrients such as nitrogen and sulfur involves a complex series of biochemical transformations that are among the most energy-intensive reactions in biology. We found nine genes implicated in nitrogen regulation to be under clock control (Web fig. 6, A and B) (6). Genes involved in early steps of nitrate assimilation are expressed at peak levels early in the subjective day, whereas asparagine synthase (ASN1), which converts aspartate to asparagine, peaks early in the subjective night, with virtually no expression almost 12 hours later, toward the end of the subjective night (31). Consistent with PIN3 playing a role in cell elongation in the hypocotyl, it is found on the lateral sides of hypocotyl epidermal cells (33). Auxin may activate expansins (enzymes that catalyze extension of cell walls), one of which was under clock control (Fig. 4A). Expansin activity is substantially enhanced by pretreatment of cell walls with hydrolases such as pectinases or cellulases (31), one of which peaked at CT8 (Fig. 4A). Cell expansion is also dependent on water influx, meditated by aquaporins, into plant vacuolar compartments. We found that an aquaporin gene was under clock control and peaked at CT8 (Fig. 4A). This aquaporin, 6-tonoplast integral protein (6-TIP), is localized to the vacuole and in young seedlings is primarily expressed in the hypocotyl and cotyledons (34). 6-TIP may work in concert with the PINs, the expansin, and the cell wall hydrolases to effect cell elongation in young plants (Fig. 4B). Peak expression of these genes occurs at the time of most rapid cell elongation in the hypocotyl of young Arabidopsis seedlings. After cell wall relaxation and expansion, new cell wall material must be laid down to reinforce the enlarged cell. Plant cell walls consist of a complex mix of polysaccharides, including celluloses, hemicelluloses, and pectins (31). We found that two cellulose synthase-like genes, both in the AtCslG gene family (33), peaked at CT20 (Fig. 4A). These genes probably synthesize polysaccharide backbones that are incorporated into the cell wall (36). Another gene implicated in cell wall biosynthesis, a dTDP-D-glucose 4,6-dehydratase homolog, likewise peaked at CT20 (Fig. 4A). This enzyme is predicted to act in a pathway leading to the synthesis of L-rhamnose, another component of plant cell walls (31). These three genes peak late in the subjective night, a time when no hypocotyl elongation is seen in young Arabidopsis seedlings. Therefore, the circadian clock temporally coordinates genes implicated in cell elongation, so that cell expansion genes are expressed together at the end of the subjective day and cell wall—synthesizing genes are expressed almost 12 hours later, toward the end of the subjective night.

A novel clock-controlled promoter element. The identification of more than 450 circadian-regulated genes offered the oppor...
tunity to identify novel promoter elements that confer circadian rhythmicity on gene expression. We surveyed genomic DNA regions upstream of cycling genes for overrepresented elements using AlignACE and ScanACE (37). The evening element was not overrepresented in any other regions of the genome, including the 130-bp region upstream of the putative transcriptional start site. Site 1 was replaced by gacgagctgc in constructs mut130_1 and mut130_1,2; site 2 was replaced by gacgagctgc in constructs mut130_2 and mut130_1,2. Constructs were introduced in Arabidopsis plants via Agrobacterium-mediated gene transfer (39). Luciferase assays were conducted and analyzed as described (5). Twelve T2 lines were examined for each construct.

We fused the CCR2 promoter to a luciferase reporter gene and introduced this construct into Arabidopsis. Surveying 12 transgenic lines for each construct, we found that the 130-bp base pair (bp) region upstream of the transcriptional start site was sufficient to confer rhythmic luciferase expression (Fig. 5C). These 130 nucleotides contained one evening element (site 1) and a related motif containing seven of the nine evening element residues (site 2) (Fig. 5B). Mutation of the partial evening element in the context of the 130-bp promoter fragment caused no reduction in seedling rhythmicity. However, mutation of the full-length evening element caused a great decrease in rhythmicity as did deletion of this region (Fig. 5C). These data demonstrate that the evening element motif, identified solely by computational means, plays an important role in conferring rhythmic gene expression in Arabidopsis. Most of the genes discussed in this article are involved in metabolism, primarily because these processes are the best understood in plants (9). However, numerous genes with probable regulatory roles, such as kinases and phosphatases, were also found to be clock-regulated (Web table 1) (4). In addition, more than 25% of the circadian-regulated genes found in this experiment are totally uncharacterized. These clock-regulated genes doubtless play important roles in metabolic pathways not discussed here and will provide fertile ground for future experimentation. Furthermore, the promoter element identified using bioinformatic approaches and confirmed by experimentation exemplifies how the characterization of output genes may point toward transcription factors that mediate phased expression of clock outputs. Investigation of these factors could provide a link between the core clock components and the transcriptional responses they control.

References and Notes
7. Wild-type Arabidopsis seeds of the Col-1 ecotype were sown on MS agar plates containing 3% sucrose. Seeds were stratified at 4° C for 2 days and then placed in growth chambers held at 22°C. Plants were grown in alternating light (60 μEinsteins m⁻² s⁻¹) and dark cycles of 12 hours each for 7 days and then released into constant light (60 μEinsteins m⁻² s⁻¹). Starting at subjective dawn on day 9 (CT0), plants were harvested every 4 hours over a 48-hour period.

Total RNA was then prepared from each sample, labeled, and hybridized to GeneChips (Affymetrix), and hybridization intensities were determined. See (9) for hybridization details.
8. Web tables and figures can be viewed at Science Online (www.sciencemag.org/cgi/content/full/290/5499/2110/DC1) for statistical methods, further discussion of functional interdependence between photosynthesis gene products, the possibility that dawn-phasing of uptake of minerals may facilitate their reduction, and a perspective on implications of this work.
10. By circadian convention, subjective dawn occurs at CT0 and subjective dusk occurs at CT12.
33. In addition, under some conditions, hypocotyl cells of plants deficient in PIN3 are 30% shorter than wild-type cells (K. Palme, personal communication).
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