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Science **290**, 2110 (2000);
DOI: 10.1126/science.290.5499.2110

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

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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.

19 October 2000; accepted 14 November 2000

Orchestrated Transcription of Key Pathways in *Arabidopsis* by the Circadian Clock

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Like most organisms, plants have endogenous biological clocks that coordinate internal events with the external environment. We used high-density oligonucleotide microarrays to examine gene expression in *Arabidopsis* and found that 6% of the more than 8000 genes on the array exhibited circadian changes in steady-state messenger RNA levels. Clusters of circadian-regulated genes were found in pathways involved in plant responses to light and other key metabolic pathways. Computational analysis of cycling genes allowed the identification of a highly conserved promoter motif that we found to be required for circadian control of gene expression. Our study presents a comprehensive view of the temporal compartmentalization of physiological pathways by the circadian clock in a eukaryote.

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 regulated 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.

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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 (protoporphyrin 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 striking coregulation, with most peaking around midday at CT4 (*9*). Two *LHC* genes, the reaction center gene *PSAD1*, 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-

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 ultraviolet (UV) range (*17*). *Arabidopsis* plants deficient in this pathway have an increased susceptibility to UV radiation (*18, 19*). The coordinate expression 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 very 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 drought (*23*) 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* confers cold resistance on *Arabidopsis* (*23*). 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 coordinately 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

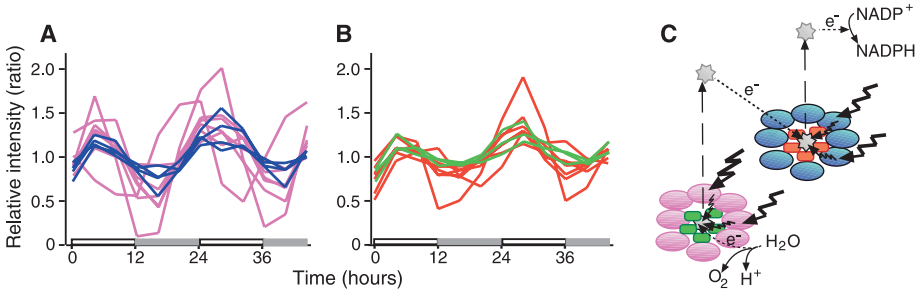

Fig. 1. Photosynthesis genes peak near the middle of the subjective day. Complementary RNA samples were prepared as described and hybridized to duplicate oligonucleotide-based microarrays (*7*). Data were normalized such that the median signal strength for each gene over all time points was 1. The average signal strength at each time point was then graphed as a ratio relative to the median signal strength of that gene. (**A**) *LHCA* genes are in blue; accession numbers (GenBank) are CAA39534, AAD28767, U32176, and P27521. *LHCB* genes are in pink; accession numbers are S23546, AF134122, AF134125, AAD28773, X71878, AAD28775, and AAD28777. (**B**) Photosystem I genes are in red; accession numbers are P49107, AJ245906, AJ245632, AJ245866, AJ245907, AJ245908, and AJ245909. Photosystem II genes are in green; accession numbers are X90769, CAA75629, and AJ223306. (**C**) Model for function of photosynthesis gene products in photosystems II (left) and I (right). Colors of proteins match colors of corresponding gene traces.

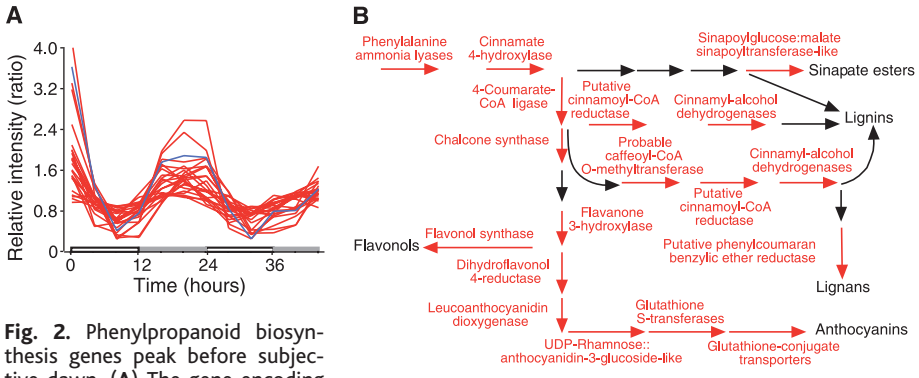

Fig. 2. Phenylpropanoid biosynthesis genes peak before subjective dawn. (**A**) The gene encoding the Myb transcription factor *PAP1* (accession number AAC83630) is in blue. The red traces represent phenylpropanoid biosynthesis genes. (**B**) Phenylpropanoid biosynthetic pathways. Genes encoding all enzymes indicated in red are clock-controlled. See Web table 2 (*8*) for gene names and accession numbers.

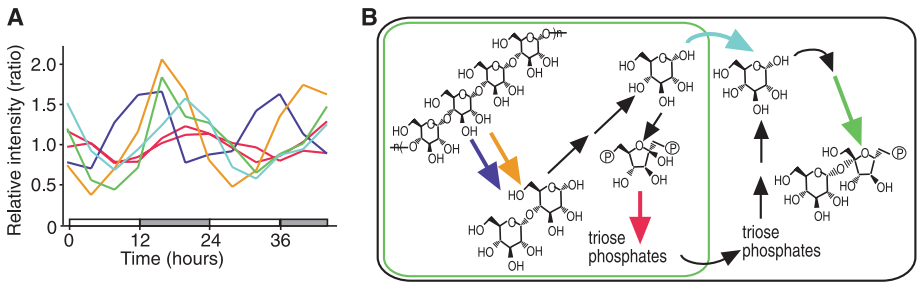

Fig. 3. Genes encoding starch-mobilizing enzymes peak during the subjective night. (**A**) Cycling genes encode a putative starch kinase (accession number AAD31337) that is related to potato R1 protein (*38*) (dark blue); a β-amylase (AJ250341) (gold); putative fructose-bisphosphate aldolase, plastidic form (AAD14543), and putative fructose-bisphosphate aldolase, predicted to be plastidic (AAD23681) (red); a putative sugar transporter (AAD03450) (light blue); and a sucrose-phosphate synthase homolog (T04062) (green). (**B**) Model for the enzymatic functions of these gene products in the mobilization of starch. Colored arrows indicate the function of the corresponding gene indicated in (A). The chloroplast is bounded by a green box and the cytoplasm by a black box.

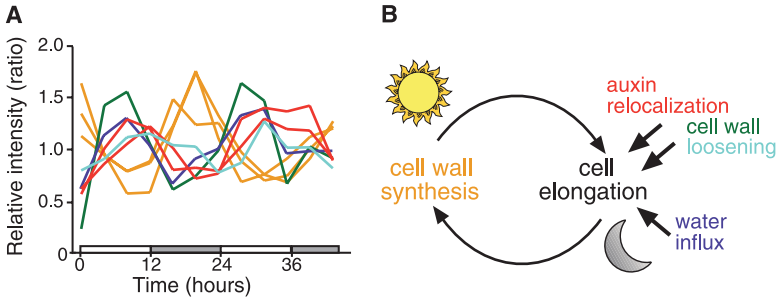

Fig. 4. Genes implicated in cell elongation are circadian-regulated. (**A**) Genes encoding the auxin efflux carriers *PIN3* (accession number AAD52695) and *PIN7* (AF087820) (red), a putative expansin (AAB87577) (green), a putative polygalacturonase (CAB81300) (light blue), and aquaporin δ-TIP (AAC49281) (dark blue) all peak toward the end of the subjective day. Three enzymes implicated in cell wall synthesis [cellulose synthase isolog (*AtCslG1*) (AF079178), cellulose synthase isolog (*AtCslG3*) (AAB63622), and similar to dTDP-D-glucose 4,6-dehydratase (AAD30579)], all in gold, peak toward the end of the subjective night. (**B**) Proposed mode of action of the products of these clock-controlled genes in cell wall remodeling.

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) (*8*). 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 at CT8. Asparagine has a higher nitrogen-to-carbon ratio than glutamine, and its production is favored under low-energy conditions (*27*). It therefore seems logical that the circadian clock would phase expression of *ASN1* toward the end of the day, a time when photosynthesis is near an end.

A cluster of five genes involved in sulfur assimilation was also under clock control, peaking between CT20 and CT0 (Web fig. 6, C and D) (*8*). These include genes encoding enzymes catalyzing the uptake and reduction of sulfate and the formation of *O*-acetyl-serine. Cysteine is formed from the condensation of *O*-acetyl-serine and sulfide, representing a link among the sulfur, carbon, and nitrogen assimilation pathways (Web fig. 6F) (*8, 9*). Regulatory interactions between nitrogen and sulfur assimilatory pathways have been previously reported (*28*).

Clock control of developmental processes. The circadian clock controls physiological processes such as the photoperiodic induction of flowering; indeed, a number of flowering-time genes have been previously reported to be under clock control (*2*). We found that five flowering-time genes were circadian-regulated, including the Myb factors *CCA1* and *LHY* (Web fig. 7A) (*8*). Circadian control of leaf movement has been recognized for even longer (*29*). In *Arabidopsis*, rhythms in cotyledon movement and hypocotyl elongation are underlain by a rhythm in cell elongation. Peak rates of cell elongation occur from late in the subjective day to early in the subjective night, with virtually no elongation from late in the subjective night to early in the subjective morning (*30*). The rigid plant cell wall normally prevents cell expansion, but a simultaneous loosening of cell wall components and reduction in cellular turgor and water potential allows cells to take up water and expand (*31*).

We found that a number of genes implicated in cell elongation were circadian-regulated and peaked coordinately between CT8 and CT12. These include the auxin efflux carriers *PIN3* and *PIN7* (Fig. 4A). Auxin promotes growth in plant stems and hypocotyls, and its relocalization plays an important role in the control of cell elongation (*32*). 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, mediated by aquaporins, into plant vacuolar compartments. We found that an aquaporin gene was under clock control and peaked at CT8 (Fig. 4A). This aquaporin, δ-tonoplast integral protein (δ-TIP), is localized to the vacuole and in young seedlings is primarily expressed in the hypocotyl and cotyledons (*34*). δ-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 hypocotyls 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 (*35*), 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-

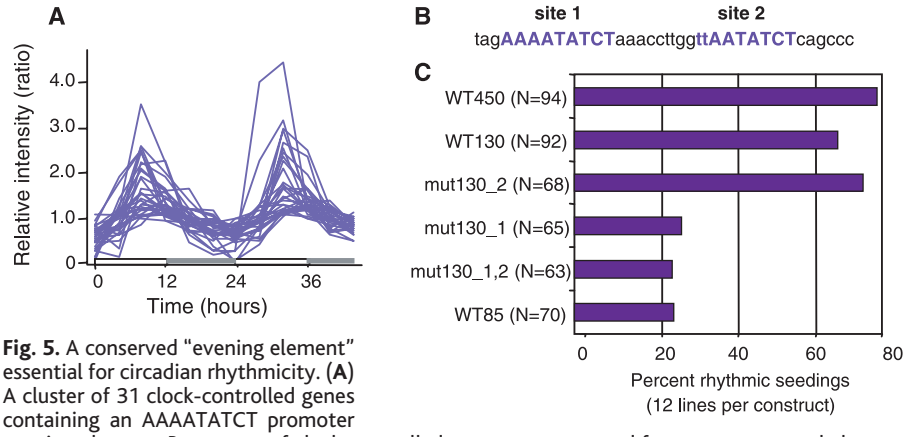

Fig. 5. A conserved "evening element" essential for circadian rhythmicity. (**A**) A cluster of 31 clock-controlled genes containing an AAAATATCT promoter evening element. Promoters of clock-controlled genes were scanned for overrepresented elements using AlignACE and ScanACE (*37*). The evening element was not overrepresented in any other circadian phase cluster. (**B**) Sequences of sites 1 and 2 are indicated in blue. (**C**) Mutation of the conserved AAAATATCT, but not a closely related motif, greatly reduced the ability of a promoter to confer circadian rhythmicity on a luciferase reporter gene in plants. Fusions to the firefly luciferase gene consisted of 450 bp (WT450), 130 bp (WT130, mut130_1, mut130_2, mut130_1,2), and 85 bp (WT85) of the *CCR2* promoter upstream of the putative transcriptional start site. Site 1 was replaced by gagcagctgc in constructs mut130_1 and mut130_1,2; site 2 was replaced by gagcagctgc 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.

tunity to identify novel promoter elements that confer circadian rhythmicity on gene expression. We surveyed genomic DNA regions upstream of cycling genes for overrepresented promoter elements. We found an absolutely conserved motif, the nine-nucleotide AAAATATCT "evening element," 46 times in the promoters of 31 cycling genes (Web table 2) (*8*), giving it a specificity score of 7.5×10^{-10} (*37*). These genes demonstrated impressive coregulation, with all but one peaking toward the end of the subjective day (Fig. 5A). We therefore performed mutagenic analysis on the *CCR2* promoter, which contains four evening elements, to determine whether this motif was in fact important for conferring circadian rhythmicity in plants.

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–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 as great a 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) (*8*). 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 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 clusters of clock outputs. Investigation of these factors could provide a link between the core clock components and the transcriptional responses they control.

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8. Web tables and figures can be viewed at *Science* Online (www.sciencemag.org/cgi/content/full/290/5499/2110/DC1).
9. See *Science* Online (www.sciencemag.org/cgi/content/full/290/5499/2110/DC1) for statistical methods, further discussions 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.
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40. Supported by the Novartis Agricultural Discovery Institute (S.A.K.), National Institute of General Medical Sciences grant 5F32GM20118-02 (S.L.H.), and the NSF Center for Biological Timing (NSF DIR-8920162) and NIH grant R01 DK51562 (M.S.). We thank C. Orozco and A. Schopke for excellent technical assistance; J. Borevitz, K. Palme, D. Somers, C. Strayer, and D. Weigel for communicating results before publication; and A. Weber, T. Richmond, D. Cosgrove, J. Maloof, and members of the Kay laboratory for helpful discussions and advice.

7 September 2000; accepted 26 October 2000