Cytochrome P450 Monooxygenases as Reporters for Circadian-Regulated Pathways

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Cytochrome P450 monooxygenases (P450s) play important roles in the synthesis of diverse secondary compounds in Arabidopsis (Arabidopsis thaliana). Comparison of four data sets analyzing seedlings harvested over a 2-d period of constant conditions after growth with varying photoperiods and thermocycles recorded a total of 98 P450 loci as circadian regulated for at least one of the four conditions. Here, we further describe the circadian-regulated pathways using, as reporters, individual P450 loci that are likely to be rate limiting in secondary metabolic pathways. Reverse transcription-polymerase chain reaction gel blot analyses have confirmed circadian regulation of P450s in phenylpropanoid, carotenoid, oxylipin, glucosinolate, and brassinosteroid biosyntheses and have shown that both P450 and non-P450 genes in the many branches of the phenylpropanoid pathway have similar circadian patterns of expression. In silico analyses of the subsets of coregulated promoters have identified overrepresented promoter elements in various biosynthetic pathway genes, including MYB and MYB4 elements that are significantly more abundant in promoters for the core and lignin sections of phenylpropanoid metabolism. Interactions with these elements important for circadian regulation do not involve the MYB transcription factor PAPI, as previously proposed, since the expression patterns of circadian-regulated P450s are the same in pap1-D mutant seedlings as in wild-type seedlings. Further analysis of circadian-regulated promoters in other biochemical pathways provides us with the opportunity to identify novel promoter motifs that might be important in P450 circadian regulation.

The biological clock controls many processes in organisms as diverse as cyanobacteria and humans. In higher plants, circadian rhythms regulate physiological events including growth and development, photosynthesis, metabolic adaptation, protein synthesis, carbohydrate transport and storage, leaf and cotyledon movements, and hormone signaling responses (Harmer et al., 2000; Covington and Harmer, 2007; Michael et al., 2008). Microarray and enhancer-trapping experiments have estimated that between 15% and 36% of the Arabidopsis (Arabidopsis thaliana) genome is under circadian regulation at the transcriptional level (Michael and McClung, 2003; Edwards et al., 2006; Michael et al., 2008). Promoter analysis using these large microarray data sets has identified several promoter elements involved in phase-specific light and circadian regulation of expression, including the Morning Element (ME), CCA1-Binding Site (CBS), GATA, Evening Element (EE), and Midnight Module (PBX/TBX/SBX; Wang et al., 1997; Harmer et al., 2000; Hudson and Quail, 2003; Harmer and Kay, 2005; Michael et al., 2008). However, it remains unclear how specific pathways downstream of the core circadian clock are regulated at specific times of the day.

Cytchrome P450 monooxygenases (P450s) play critical roles in the synthesis of lignin, pigments, defense compounds, fatty acids, hormones, and signaling molecules in all plant species (Schuler, 1996; Werck-Reichhart et al., 2002; Nielsen and Moller, 2005). Because of their wide distribution in diverse metabolic processes, P450s can serve as downstream reporters for many biochemical pathways in Arabidopsis, where 246 P450 full-length genes and 26 P450 pseudogenes have been annotated (Paquette et al., 2000; Werck-Reichhart et al., 2002; Schuler et al., 2006). With many studies detailing the responses of this highly diverse
gene family to biotic and abiotic stresses, the extent to which its members are regulated by circadian cues is unclear at present.

Carotenoids are the pigments responsible for many fruit and flower colors and some components of the light-harvesting complexes in photosynthesis (Bartley and Sc Olson, 1995; DellaPenna and Pogson, 2006). Carotenoids also serve as precursors in the synthesis of abscisic acid. The carotenoid pathway contains two P450s in its downstream lutein branch mediating β-ring hydroxylations on α-carotenes (CYP97A3; LUT5) and subsequent ε-ring hydroxylations (CYP97C1; LUT1; Tian et al., 2004; Kim and DellaPenna, 2006).

Oxylipins are acyclic or cyclic oxidation products derived from polyunsaturated fatty acids that regulate many defense and developmental pathways in plants (Creelman and Mullet, 1997). The oxylipin pathway in Arabidopsis contains allene oxide synthase (CYP74A1; AOS), mediating the synthesis of 12-oxo-phytodienoic acid, jasmonate (JA), and methyl jasmonate (MeJ) from their linolenic acid precursor (Laudert et al., 1996), and hydroperoxide lyase (CYP74B2; HPL), mediating the breakdown of this precursor into C₆-volatiles and octadecanoic acid (Bate et al., 1998; Matsui et al., 1999).

Glucosinolates are a class of naturally occurring thioglucosides responsible for some of the unique tastes of many condiments. Many P450s exist in the pathways branching to the production of these compounds, with CYP79F1 and CYP79F2 mediating distinct functions in the conversion of short- and long-chain Met derivatives to oximes (Hansen et al., 2001; Reintanz et al., 2001; Chen et al., 2003) prior to their modification by CYP83A1 to produce aliphatic glucosinolates (Bak and Feyereisen, 2001; Hemm et al., 2003; Naur et al., 2003). In a parallel pathway, CYP79B2 and CYP79B3 mediate steps in the conversion of Trp derivatives to indole-3-acetyldoxime (Hull et al., 2000; Mikkelsen et al., 2000) prior to its oxidation by CYP83B1 to produce indole glucosinolates (Bak and Feyereisen, 2001; Bak et al., 2001; Naur et al., 2003).

Brassinosteroids are steroidal plant hormones essential for many plant processes, including cell expansion and elongation, xylem differentiation, and pollen tube growth (Müssig et al., 2002; Asami et al., 2005; Baiguz, 2007). They have also been reported to improve resistance to chilling and drought stress (Clouse and Sasse, 1998). The brassinosteroid synthetic pathway, whose central CPD component is circadian regulated (Bancos et al., 2006), consists of a complex grid of metabolic reactions that includes CYP90A1 (CPD), CYP90B1 (DFW4), CYP90C1 (ROT3), CYP90D1, CYP85A1, and CYP85A2 (Bishop and Koncz, 2002; Fujioaka and Yokota, 2003). Brassinosteroid degradation is mediated by P450s in two other families, CYP72C1 (SOB7) and CYP73A1 (BSA1; Neff et al., 1999; Turk et al., 2003, 2005; Nakamura et al., 2005; Takahashi et al., 2005).

Phenylpropanoid synthesis represents one of the best-characterized pathways because it generates a wide variety of products found in most plants, including flavonoids that act as signaling molecules, protectants against UV light damage and microorganisms, lignins that are structural components of cell walls, and anthocyanins that act as floral pigments and attractants to insect pollinators (Dixon and Paiva, 1995; Whetten and Sederoff, 1995; Winkel-Shirley, 2001). Harmer et al. (2000) first reported that the circadian clock regulated a large number of genes in this pathway, resulting in the daily cycling of its transcripts. Within this collection, four P450s mediate the hydroxylation of t-cinnamic acid (CYP73A5; C4H), p-coumaroylshikimic/quinic acids (CYP98A3; REF8), coniferaldehyde/ferulic acid (CYP84A1; FAH1), and narigenin/dihydrokaempferol (CYP75B1; T7T). These are widely distributed in the core pathway and the lignin and flavonoid/anthocyanin branches, which are postulated to provide scaffolds for the assembly of multienzyme channeling complexes (Meyer et al., 1996; Mizutani et al., 1997; Urban et al., 1997; Ruegger et al., 1999; Schoenbohm et al., 2000; Schoch et al., 2001; Winkel, 2004). From this work, it was also proposed that phenylpropanoid metabolism is regulated by PAP1, a MYB transcription factor (Harmer et al., 2000). More recent analyses have shown that PAP1 itself cycles and also that it regulates late flavonoid and anthocyanin gene expression (Borevitz et al., 2000; Tohge et al., 2005; Gonzalez et al., 2008). To date, there has been no evidence directly tying PAP1 to circadian regulation of the phenylpropanoid pathway.

The analyses presented here of P450 expression patterns in four data sets, varying with respect to the thermal and photoperiod cycles used for entrainment, indicate that different combinations of these P450s display coordinated in-phase expression in the different entrainment conditions. The characterization of P450 responses to circadian regulation has potential to identify nodes that globally coordinate transcript abundance of many pathways to specific times of the day. Until now, the activities of specific pathways have, for the most part, only been inferred from analysis of genome-wide patterns. To better understand the coordination of the downstream synthetic and catabolic pathways conferring time-of-day-specific activities, we have utilized P450s as reporters for different nodes in the network emerging from the central circadian clock.

RESULTS
Circadian Variations in P450 Transcripts

Given the importance of P450s in many metabolic pathways, it is clear that they can serve as global reporters for cellular responses to internal and external cues. To analyze the extent to which P450s might be regulated by the circadian clock, four previously published circadian time courses (Michael et al., 2008) were compared for their patterns in P450 expression. The four circadian conditions represent plants grown under different conditions of light and temperature (Table I).
They are named to reflect how they were sampled: DD_DDHC, LL_LLHC, LL_LDHC, and LL_LDHH. The abbreviation DD (continuous dark, 22°C) or LL (continuous light, 22°C), before the underscore, represents the continuous circadian conditions under which the plants were harvested. Abbreviations after the underscore indicate the conditions under which plants were grown (entrained before being released into circadian conditions; these include light/dark cycles (LD), continuous light (LL), continuous dark (DD), continuous temperature (HH, 22°C), and/or thermocycles (HC, 22°C/12°C) as detailed by Michael et al. (2008). Samples were harvested at 4-h intervals over a 2-d period and compared on Affymetrix ATH1 Genechips. Expression for all genes on the ATH1 Genechips across these circadian conditions as well as other diurnal conditions can be accessed at http://diurnal.cgrb.oregonstate.edu/. Identification of cycling genes, the time of their peak expression over the day (phase, in hours from subjective dawn), and statistical analysis of the entire data set have been described by Michael et al. (2008). Briefly, all four time courses were gcRMA (see “Materials and Methods”) normalized together, and cycling genes were called using the pattern-matching program HAYSTACK (http://haystack.cgrb.oregonstate.edu/) using a 5% false discovery rate (FDR; Michael et al., 2008). All of the cycling genes described are statistically significant using these criteria. Expression for all genes on the ATH1 Genechips across these circadian conditions as well as other diurnal conditions can be accessed at DIURNAL (http://diurnal.cgrb.oregonstate.edu/).

Across the four circadian conditions, 233 of the 246 full-length P450 genes and 26 P450 pseudogenes in Arabidopsis were detected on Affymetrix ATH1 arrays, with 11 of these array elements representing closely related P450 genes. Using a 0.8 correlation cutoff for predicting cycling loci (all four time courses analyzed together; \( P = 0.05, \) FDR = 5%; Michael et al., 2008), 98 P450 loci listed in Table II showed statistically significant circadian phasing for at least one of the four array conditions. Between 4% and 22% of the 250 P450 transcripts were circadian regulated under any one of the four conditions, with 39% of the gene list overlapping with one of the other three conditions (Fig. 1). Between 33% and 42% of the genes were specifically circadian regulated under only one condition. The fewest genes displaying circadian rhythms were detected under DD_DDHC conditions, consistent with the fact that generally fewer genes cycle under this condition (Michael et al., 2008). The greatest numbers of genes showing circadian rhythms were detected under LL_LDHH and LL_LDHC conditions rather than LL_LLHC conditions, suggesting that entrainment by light/dark cycles plays a role in P450 expression.

Reverse transcription (RT)-PCR gel blot analyses of samples from the LL_LLHC and LL_LDHC time courses performed with gene-specific primers and probes confirmed the cycling of P450 transcripts distributed in many different pathways, including CYP73A5, CYP75B1, CYP98A3, and CYP84A1 in phenylpropanoid synthesis (nos. 1–4 in Table II; highlighted in red in Fig. 2A online), CYP97CI in carotenoid synthesis (no. 7 in Table II), CYP74A1 in jasmonate synthesis (no. 9 in Table II), CYP79B3 in glucosinolate synthesis (no. 15 in Table II), and CYP90A1 in brassinosteroid synthesis (no. 17 in Table II). Comparison of the UBQ10-normalized RNA levels shown in the left panels of Figure 3 with the gcRMA-normalized ATH1 array data shown in the right panels of Figure 3 indicates strong correlations for most of the transcripts analyzed. Analysis using the model-based pattern-matching HAYSTACK algorithm (Fig. 3K) indicates that the four P450s in the phenylpropanoid pathway are phased with a maximum at 22 h under the LL_LLHC conditions. Complementary analysis using semiquantitative RT-PCR gel blots on these same RNA samples (Fig. 3, A and F) indicates that these four P450s are phased at around 24 h. Comparisons between these two analytic methods indicate that, in some instances, the RT-PCR gel blots show obvious cycling variations and the ATH1 array data show much lower levels of variation. This is the case for CYP75B1 and CYP84A1 in the LL_LLHC time course (Fig. 3, F and K) as well as for CYP98A3 in the LL_LLHC time course, where RT-PCR gel blot signals show as much as a 10-fold difference between the initial time point and each day’s minimum and microarray data show only a 2.5-fold difference. This is also the case for CYP90A1 in the LL_LDHC time course, where RT-PCR gel blot signals decrease more dramatically than the microarray data to an extreme minimum at 16 to 20 h (Fig. 3, I and N). Differences in the magnitude of these variations likely arise from the greater sensitivity of the RT-PCR gel blots. It is well known that microarray data, especially oligonucleotide microarray data, suffer from poor dynamic range.

| Table I. Plant growth and harvest conditions |
|---------------------------------------------|
| Name                  | Entrainment Conditions          | Harvest Conditions | Researcher/Laboratory |
|------------------------|--------------------------------|--------------------|------------------------|
| DD_DDHC                | DD (continuous dark)            | DD (continuous dark, 22°C) | Hazen, Kay             |
| LL_LLHC                | HC (thermocycles of 22°C/12°C)  | LL (continuous light, 22°C) | Michael, Chory         |
| LL_LDHC                | LL (continuous light)           | LL (continuous light, 22°C) | Michael, Chory         |
| LL_LDHH                | LD (12-h-light/12-h-dark cycles)| LL (continuous light, 22°C) | Edwards, Millar; Pan, Schuler |
|                        | HC (thermocycles of 22°C/12°C)  |                    |                        |
|                        | HH (continuous temperature of 22°C) |                  |                        |
Table II. P450s showing circadian regulation

The 98 P450 loci shown to have statistically significant circadian phasing for at least one of the four array conditions (DD_DDHC, LL_LDHH, LL_LDHC, and LL_LLHC) are listed, with the numbers in columns 5 to 8 indicating the phasing time relative to subjective dawn. The first 27 P450s that have designated functions are sorted by biochemical pathway; the remaining 71 P450s (nos. 28 to 98) currently have no biochemically defined function.

| No. | Affymetrix Identifier | Arabidopsis Genome Initiative No. | Locus | DD_DDHC | LL_LDHH | LL_LDHC | LL_LLHC | Pathway |
|-----|-----------------------|-----------------------------------|-------|---------|---------|---------|---------|---------|
| 1   | 267470_at             | At2g30490                         | CYP73A5 | 1       | 20      | 22      |         | Phenylpropanoid core |
| 2   | 250558_at             | At5g07990                         | CYP75B1 | 20      |         |         |         | Phenylpropanoid/flavonol |
| 3   | 253088_at             | At4g36220                         | CYP84A1 | 1       |         |         |         | Phenylpropanoid/lignin |
| 4   | 245101_at             | At2g40890                         | CYP98A3 | 21      | 23      | 22      |         | Phenylpropanoid/lignin |
| 5   | 267380_at             | At2g26170                         | CYP711A1| 20      | 23      | 22      |         | Flavonoid |
| 6   | 246268_at             | At1g31800                         | CYP97A3 | 14      |         | 19      |         | Carotenoid |
| 7   | 251969_at             | At3g33130                         | CYP97C1 | 13      |         | 20      |         | Carotenoid |
| 8   | 251979_at             | At4g15110                         | CYP97B3 | 16      |         |         |         | Carotenoid |
| 9   | 249208_at             | At5g42650                         | CYP74A1 | 19      |         | 22      |         | Oxylipin |
| 10  | 245253_at             | At4g15440                         | CYP74B2 | 15      |         | 17      |         | Oxylipin |
| 11  | 262717_s_at           | At1g16410                         | CYP79F1 | 1       |         |         |         | Aliphatic glucosinolate |
| 12  | 262717_s_at           | At1g16400                         | CYP79F2 | 1       |         |         |         | Aliphatic glucosinolate |
| 13  | 254687_at             | At4g37770                         | CYP83A1 | 22      |         |         |         | Aliphatic glucosinolate |
| 14  | 252827_at             | At2g39950                         | CYP79B2 | 23      |         |         |         | Indole glucosinolate |
| 15  | 264052_at             | At2g22330                         | CYP79B3 | 0       | 23      |         |         | Indole glucosinolate |
| 16  | 256598_at             | At3g30180                         | CYP85A2 | 1       |         |         |         | Brassinolide |
| 17  | 250752_at             | At5g05690                         | CYP90A1 | 5       | 8       | 9       |         | Brassinolide |
| 18  | 252184_at             | At3g50660                         | CYP90B1 | 12      |         | 8       |         | Brassinolide |
| 19  | 246216_at             | At4g36380                         | CYP90C1 | 3       |         | 6       |         | Brassinolide |
| 20  | 267614_at             | At2g26710                         | CYP73A4 | 5       |         | 6       |         | Degradation of brassinosteroids |
| 21  | 25703S_at             | At3g19270                         | CYP707A4| 7       |         |         |         | Degradation of abscisic acid |
| 22  | 246864_at             | At5g25900                         | CYP701A3| 3       |         | 7       |         | GAs |
| 23  | 260241_at             | At1g63710                         | CYP86A7 | 3       |         |         |         | Fatty acid |
| 24  | 258973_at             | At3g01900                         | CYP99B2 | 1       |         |         |         | Fatty acid |
| 25  | 262820_at             | At1g11680                         | CYP51G1 | 1       |         | 1       |         | Sterols/steroids |
| 26  | 264877_at             | At2g17330                         | CYP51G2 | 5       |         | 5       |         | Sterols/steroids |
| 27  | 266996_at             | At2g34490                         | CYP710A2| 20      |         | 19      | 23      | Sterols |
| 28  | 267562_at             | At4g30770                         | CYP71A13| 2       |         |         |         | Sterols/steroids |
| 29  | 254767_s_at           | At4g13310                         | CYP71A20| 3       |         |         |         | Sterols/steroids |
| 30  | 256598_at             | At2g30180                         | CYP71B2 | 22      |         | 0       | 3       |         |
| 31  | 256735_at             | At2g36280                         | CYP71B4 | 4       | 7       | 7       |         |         |
| 32  | 267293_at             | At1g13110                         | CYP71B7 | 8       |         |         |         |         |
| 33  | 246947_at             | At5g25120                         | CYP71B11| 19      |         |         |         |         |
| 34  | 246949_at             | At5g25140                         | CYP71B13| 4       |         |         |         |         |
| 35  | 257636_at             | At3g26200                         | CYP71B22| 3       |         |         |         |         |
| 36  | 257623_at             | At3g26210                         | CYP71B23| 13      |         |         |         |         |
| 37  | 257625_at             | At3g26230                         | CYP71B24| 21      |         | 4       |         |         |
| 38  | 257628_at             | At3g26290                         | CYP71B26| 5       | 7       | 9       |         |         |
| 39  | 251988_at             | At3g33300                         | CYP71B31| 23      |         |         |         |         |
| 40  | 256870_at             | At3g26300                         | CYP71B34| 1       |         |         |         |         |
| 41  | 256873_at             | At3g26310                         | CYP71B35| 7       |         |         |         |         |
| 42  | 252674_at             | At3g42500                         | CYP71B38| 2       |         | 1       |         |         |
| 43  | 258063_at             | At3g14620                         | CYP72A8 | 15      | 17      | 20      |         |         |
| 44  | 258112_at             | At3g14640                         | CYP72A10| 6       |         |         |         |         |
| 45  | 267505_at             | At2g45560                         | CYP76C1 | 14      | 6       | 9       | 14      |         |
| 46  | 267559_at             | At2g45570                         | CYP76C2 | 8       |         |         |         |         |
| 47  | 267560_at             | At2g45580                         | CYP76C3 | 15      |         |         |         |         |
| 48  | 250859_at             | At5g04660                         | CYP77A4 | 0       |         |         |         |         |
| 49  | 258962_at             | At3g10570                         | CYP77A6 | 19      |         |         |         |         |
| 50  | 250838_at             | At5g04630                         | CYP77A9 | 19      |         |         |         |         |
| 51  | 262819_at             | At1g11600                         | CYP77B1 | 13      |         |         |         |         |
| 52  | 266321_at             | At2g46660                         | CYP78A6 | 1       |         |         |         |         |
| 53  | 250509_at             | At5g09970                         | CYP78A7 | 4       |         |         |         |         |

(Table continues on following page.)
Although the results from these two methods differ in magnitude, both demonstrate circadian regulation of the P450s analyzed, and within each method, the circadian expression pattern of each gene appears to be highly reproducible.

Circadian Regulation of P450 Transcripts in Different Secondary Pathways

With P450s occurring at important nodes in many secondary pathways displaying circadian cycling, variations in their activities can impact an array of downstream synthetic and catabolic pathways and alter physiological functions over the course of a day. Comparison of the circadian phasing of the four P450 transcripts in the core and various branches of the phenylpropanoid pathway (CYP73A5, CYP75B1, CYP98A3, and CYP84A1; Fig. 2) indicates that the LL_LDHH arrays show similar phasing just before subjective dawn (20–21 h maxima) for three of these P450s, with the most profound increase for CYP75B1 in anthocyanin synthesis and slightly later phasing for CYP84A1 (1 h maximum; Table II). Two of these

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### Table II. (Continued from previous page.)

| No. | Affymetrix Identifier | Arabidopsis Genome Initiative No. | Locus | DD_DDHC | LL_LDHH | LL_LDHC | LL_LLHC | Pathway |
|-----|-----------------------|----------------------------------|-------|---------|---------|---------|---------|---------|
| 55  | 249673_at             | At5g35920                        | CYP79A4P | 2        | 2       | 2       | 2       |         |
| 56  | 246620_at             | At5g36220                        | CYP81D1 | 20       | 17      | 17      | 17      |         |
| 57  | 253096_at             | At4g37330                        | CYP81D4 | 20       | 17      | 17      | 17      |         |
| 58  | 253097_at             | At4g37320                        | CYP81D5 | 14       | 10      | 10      | 10      |         |
| 59  | 256386_at             | At1g66540                        | CYP81D10 | 3        | 2       | 2       | 2       |         |
| 60  | 256589_at             | At3g28740                        | CYP81D11 | 3        | 2       | 2       | 2       |         |
| 61  | 253100_at             | At4g37400                        | CYP81F3 | 22       | 22      | 22      | 22      |         |
| 62  | 253052_at             | At4g37310                        | CYP81H1 | 14       | 14      | 14      | 14      |         |
| 63  | 253503_at             | At4g31950                        | CYP82C3 | 4        | 4       | 4       | 4       |         |
| 64  | 253502_at             | At4g31940                        | CYP82C4 | 4        | 4       | 4       | 4       |         |
| 65  | 249881_at             | At5g23190                        | CYP86B1 | 4        | 4       | 4       | 4       |         |
| 66  | 250576_at             | At5g08250                        | CYP86B2 | 5        | 5       | 5       | 5       |         |
| 67  | 265020_at             | At1g24540                        | CYP86C1 | 4        | 4       | 4       | 4       |         |
| 68  | 262882_at             | At1g64900                        | CYP89A2 | 0        | 0       | 0       | 0       |         |
| 69  | 247579_at             | At5g61320                        | CYP89A3 | 0        | 0       | 0       | 0       |         |
| 70  | 266155_at             | At1g64950                        | CYP89A5 | 20       | 20      | 20      | 20      |         |
| 71  | 262866_at             | At1g64940                        | CYP89A6 | 4        | 4       | 4       | 4       |         |
| 72  | 256651_at             | At5g06900                        | CYP93D1 | 23       | 23      | 23      | 23      |         |
| 73  | 248353_at             | At5g23220                        | CYP96A4 | 16       | 17      | 17      | 17      |         |
| 74  | 263894_at             | At2g21910                        | CYP96A5 | 4        | 4       | 4       | 4       |         |
| 75  | 262435_at             | At1g47620                        | CYP96A8 | 2        | 2       | 2       | 2       |         |
| 76  | 252896_at             | At4g39480                        | CYP96A9 | 23       | 23      | 23      | 23      |         |
| 77  | 252911_at             | At4g39510                        | CYP96A12 | 0        | 0       | 0       | 0       |         |
| 78  | 245550_at             | At4g15330                        | CYP705A1 | 4        | 4       | 4       | 4       |         |
| 79  | 248727_at             | At5g47990                        | CYP705A5 | 16       | 16      | 16      | 16      |         |
| 80  | 266308_at             | At2g27010                        | CYP705A9 | 11       | 11      | 11      | 11      |         |
| 81  | 263276_at             | At2g14100                        | CYP705A13 | 12      | 12      | 12      | 12      |         |
| 82  | 257112_at             | At3g20120                        | CYP705A21 | 12      | 12      | 12      | 12      |         |
| 83  | 261499_at             | At1g28430                        | CYP705A24 | 4        | 4       | 4       | 4       |         |
| 84  | 256801_at             | At3g20940                        | CYP705A30 | 1        | 1       | 1       | 1       |         |
| 85  | 256802_at             | At3g20950                        | CYP705A32 | 0        | 0       | 0       | 0       |         |
| 86  | 256803_at             | At3g20960                        | CYP705A33 | 5        | 5       | 5       | 5       |         |
| 87  | 254331_s_at           | At4g22690                        | CYP706A1 | 6        | 6       | 6       | 6       |         |
| 88  | 254331_s_at           | At4g22710                        | CYP706A2 | 6        | 6       | 6       | 6       |         |
| 89  | 254335_s_at           | At4g12320                        | CYP706A6 | 18       | 18      | 18      | 18      |         |
| 90  | 254335_s_at           | At4g12310                        | CYP706A7 | 18       | 18      | 18      | 18      |         |
| 91  | 248728_at             | At5g48000                        | CYP708A2 | 17       | 17      | 17      | 17      |         |
| 92  | 266251_s_at           | At5g68300                        | CYP708A3 | 20       | 23      | 23      | 23      |         |
| 93  | 252629_at             | At3g44970                        | CYP708A4 | 20       | 23      | 23      | 23      |         |
| 94  | 246726_at             | At2g22500                        | CYP712A1 | 3        | 3       | 3       | 3       |         |
| 95  | 246728_at             | At5g24910                        | CYP714A1 | 5        | 5       | 5       | 5       |         |
| 96  | 249684_s_at           | At5g36110                        | CYP716A1 | 5        | 5       | 5       | 5       |         |
| 97  | 245728_at             | At1g73340                        | CYP720A1 | 23       | 23      | 23      | 23      |         |
| 98  | 261134_at             | At1g19630                        | CYP722A1 | 17       | 17      | 17      | 17      |         |
also cycle with similar normalized profiles in the LL_LDHC arrays with peaks before subjective dawn (Fig. 3A). In addition, CYP711A1 (MAX1; no. 5 in Table II), which is reported to be a positive regulator of the flavonoid pathway (Lazar and Goodman, 2006) and to act downstream of carotenoid-derived hormones (Booker et al., 2005), shows circadian phasing (20–23 h maxima in LL_LDHH, LL_LDHC, and LL_LLHC arrays) similar to three of the four phenylpropanoid P450s.

In carotenoid synthesis (Fig. 4A), CYP97A3 and CYP97C1 occur in the lutein branch. Both of these (nos. 6 and 7 in Table II) show circadian phasing in the LL_LLHC arrays (19–20 h maxima) and the LL_LDHH arrays (13–14 h maxima; Fig. 5A). CYP97B3 (no. 8 in Table II), whose protein shares 45% to 46% amino acid identity with CYP97A3 and CYP97C1, shows circadian phasing at 12 h in the LL_LDHH arrays (below the cutoff used for Table II) and at 16 h in the LL_LDHC arrays, which is similar to the phasing of CYP97A3 and CYP97C1 on the arrays. The similarity of this phasing suggests that CYP97B3 may be under the same transcriptional regulation as the other CYP97 family genes. CYP711A1, which was previously mentioned as acting downstream of carotenoid cleavage dioxygenases (Booker et al., 2005), shows circadian phasing at 20 to 22 h in three of the time courses (LL_LDHH, LL_LDHC, and LL_LLHC); this is significantly later than the phasing of the other P450s in this carotenoid pathway.

In oxylipin synthesis (Fig. 4B), CYP74A1 in JA synthesis and CYP74B2 in C6-volatile production are...
Figure 3. Circadian-regulated transcripts in known pathways. A to E, RNAs from the LL_LLHC and LL_LDHC conditions were analyzed on RT-PCR gel blots for the transcripts listed above each panel or set of panels. The RT-PCR products for P450s in each
circadian regulated, with slightly different phasings in the LL_LLHC arrays (22 and 17 h maxima; nos. 9 and 10 in Table II). With many other loci mediating steps in JA synthesis, at least one locus at each step in the pathway is circadian regulated in the LL_LLHC and LL_LDHC arrays, with phasings between 13 and 18 h, at times slightly prior to the phasings seen for CYP74A1 and CYP74B2; among multiple loci coding for the same enzyme, those that are circadian regulated are indicated with boxes in Figure 4B. The only loci with noticeably different phasing from others in the JA synthetic pathway are one lipoxygenase (LOX1; no. 59 in Table III) mediating the synthesis of 9-hydroperoxides and not the 13-hydroperoxides needed for jasmonate production (Royo et al., 1996; Blee, 2002), one undefined 12-oxophytodienoate reductase (OPR; no. 66 in Table III), and S-adenosyl-L-Met:jasmonic acid carboxyl methyltransferase (JMT; no. 68 in Table III) catalyzing the last step in MeJ synthesis. As both LOX and OPR have many isoforms in Arabidopsis, it is likely that the individual members of these families are under different modes of transcriptional regulation. In the case of JMT, its phasing 2 to 11 h later than transcripts for previous steps in this pathway suggests that the proportions of JA and MeJ vary throughout these cycling periods.

Figure 4. Maps of four circadian-regulated pathways. These maps adapted from AraCyc Pathway correspond to the carotenoid (A), oxylipin (B), glucosinolate (C), and brassinosteroid (D) pathways. Black boxes indicate genes that are circadian regulated, and underlines indicate P450 loci. [See online article for color version of this figure.]
II) are circadian regulated, with similar phasings under LL_LLHC (21–1 h maxima) as a number of other enzymes in this branched pathway (Fig. 5B, top). Not surprisingly, CYP79B2 and CYP79B3 in the indole glucosinolate branch (nos. 14 and 15 in Table II) are circadian regulated, with exactly the same phasings as most other enzymes in its branch (Fig. 5B, bottom). The glucosinolate pathway has not previously been reported to have circadian rhythms.

In brassinolide synthesis (Fig. 4D), four of the six synthetic P450s have different circadian phasings depending on the array conditions (nos. 16–19 in Table II). For example, the LL_LDHH arrays show similar phasing for CYP85A2 and CYP90C1 (ROT3; 1–3 h maxima) and a slightly later phasing for CYP90A1 (CPD; 5 h maximum), which is considered the initial
Table III. Elements overrepresented in different branches of biosynthetic pathways

The numbers in the columns for the four circadian array conditions (DD_DDHC, LL_LDHH, LL_LDHC, and LL_LLHC) are the phasing of corresponding genes. EE, CBS, and ME are given; the numbers indicate the positions (bp) where they present in the promoter of each gene. The statistical significance (e-value) and ratio of overrepresented element frequency shown in the branch and in the 27,457 promoters of the Arabidopsis genome are also given.

| Pathways and Subdivisions | Locus | Arabidopsis Genome Initiative No. | P450 DD_DDHC | LL_LDHH | LL_LDHC | LL_LLHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|--------------------------|-------|-----------------------------------|--------------|---------|---------|---------|-----------|------------------------------------------|
| **Phenylpropanoid pathway** |       |                                   |              |         |         |         |           |                                          |
| Core pathway             |       |                                   |              |         |         |         |           |                                          |
| 1 Phenylalanine-lyase     | PAL1  | At2g17040                          | 20           | 23      |         |         | CBS:363   | MYB4:2.44e-12(54/77609)                   |
| 2 Phenylalanine-lyase     | PAL2  | At3g53260                          | 21           | 0       | 0       |         |           | MYB:1.84e-11(12/25/20163)                |
| 3 4-Coumarate-CoA 4-hydroxylation | PAL3 | At5g04230                          | 4            |         |         |         |           | GL-TRP5:4.26e-05(11/8446)               |
| 4 4-Coumarate-CoA 4-hydroxylation | C4H | At2g10490 CYP73A5                   | 1            | 20      | 22      |         |           | WL:1.10e-04(53/61066) GL-MET:3.6.16e-04/19/33391 |
| 5 4-Coumarate-CoA 4-hydroxylation | 4CL1 | At1g51680                          | 19           | 22      |         | CBS:1444  | CAROT:2:1.36e-03(26/49795) |
| 6 4-Coumarate-CoA 4-hydroxylation | 4CL2 | At3g21240                          | 22           | 22      |         |           | GL-MET:1.64e-04(22/37935) GL-TRP2:9.71e-03/18/35624 |
| 7 4-Coumarate-CoA 4-hydroxylation | 4CL3 | At1g65060                          | 20           | 18      | 20      |         | CBS:557    |                                          |
| 8 4-Coumarate-CoA 3-hydroxylation | 4CL4 | At1g21230                          | 0            |         |         |         |           |                                          |
| 9 4-Coumarate-CoA 3-hydroxylation | 4CL5 | At5g63380                          | 0            | 1       | 2       | 3       |           | CBS:1497    |                                          |
| 10 4-Coumarate-CoA 3-hydroxylation | 4CL6 | At1g20490                          | 21           | 20      |         |         | CBS:356    |                                          |
| **Intermediate flavonoid branch** |       |                                   |              |         |         |         |           |                                          |
| 11 Naringenin-chalcone synthase | CHS  | At5g13930                          | 21           | 20      | 22      |         |           | SORLIP2:7.85e-04(12/22714) GL-TRP2:9.71e-03/18/35624 |
| 12 Chalcone isomerase     | TT5   | At3g55120                          | 21           | 20      | 22      |         | CBS:1619   | MOTIF8:2.86e-03(26/76487)     |
| 13 Chalcone isomerase     | CHI   | At5g05270                          | 20           | 23      |         |         |           | ABRE-like:3.80e-03(8/121223) GL-MET:1.64e-04(22/37935) |
| 14 Naringenin 3-dioxygenase | F3H  | At3g12240                          | 23           | 20      | 22      |         | CBS:544    | GL-TRP2:8.50e-03/17/44906 |
| 15 Flavanone 3-hydroxylation | F3H  | At1g54530                          | 1            | 3       |         |         | CBS:601    |                                          |
| 16 Flavanone 3-hydroxylation | F3H  | At3g19000                          | 19           |         |         |         | CBS:815    |                                          |
| 17 Flavanone 3-hydroxylation | F3H  | At4g16330                          | 7            | 12      |         |         |           |                                          |
| **Flavonol branch**       |       |                                   |              |         |         |         |           |                                          |
| 18 Flavonol 3’-monooxygenase | TT7  | At5g07990 CYP75B1                   | 20           |         |         |         |           | EE:6.15e-04(36/3608) GL-MET:2.85e-04/17/26923 SORLIP1:9.63e-04/24/42489 |
| 19 Flavonol synthase      | FS    | At3g51020                          | 9            | 13      | 10      |         | EE:1037    |                                          |
| 20 Flavonol synthase      | F3H   | At3g19010                          | 9            |         | 9       |         | EE:692     |                                          |
| 21 Flavonol synthase      | F3H   | At5g63950                          | 20           |         |         |         |           |                                          |
| 22 Flavonol synthase      | F3H   | At5g08640                          | 21           | 19      | 21      |         | EE:270     |                                          |
| 23 Flavonol 3-O-glucosyltransferase | F3OG1 | At1g01420                          | 23           |         |         |         | EE:934,34  |                                          |
| 24 Flavonol 3-O-glucosyltransferase | F3OG2 | At1g01850                          | 22           |         |         |         | EE:881     |                                          |
| 25 Flavonol 3-O-glucosyltransferase | F3OG3 | At4q01070                          | 17           | 18      | 21      |         |           |                                          |
| 26 Flavonol 3-O-glucosyltransferase | F3OG4 | At5g54060                          | 22           | 21      |         |         |           |                                          |
| 27 Anthocyanin branch      |       |                                   |              |         |         |         |           |                                          |
| 28 Flavonol 3’-monooxygenase | TT7  | At5g07990 CYP75B1                   | 20           |         |         |         | CBS:1847   | G-box([LRE]:5.00e-06/8(8723) G-BOX EXTENDED:2.44e-04/5/4921 ABRE-like:1.97e-03(6/12125) SORLIP4:2.99e-03/2/627 BS2:9.98e-03/6/16468 |
| 29 Dihydroflavonol 4-reductase | DFR | At5g42800                          | 21           | 21      |         |         | ME:1090,868,4 |                                          |
| 30 Leucoanthocyanidin dioxygenase | ANS | At4q22870                          | 20           | 21      |         |         | ME:1701    | EE:1069 |                                         |
| 31 Leucoanthocyanidin dioxygenase | ANS | At4q22870                          | 20           | 21      |         |         | ME:1447,876,326,101 |                                         |
| Lignin branch             |       |                                   |              |         |         |         |           |                                          |
| 32 Hydroxycinnamoyl-CoA:shikimate/quinine hydroxy-cinnamoyltransferase | CST/CQT | At5g48930                          | 20           | 23      |         | CBS:1225,364 | MYB:1.14e-10/28/20163 MYB4:2.95e-07/63/77609 WLE1:2.78e-04/75/110661 |

(Table continues on following page.)
Table III. (Continued from previous page.)

| Pathways and Subdivisions | Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|---------------------------|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 33 Coumarate-3- hydroxylase | REF8  | At2g40890  | CYP98A3 | 21 | 23 | 22 | ME:1311,221 | GL-MET3:5.23e-03(25/33391) |
| 34 Caffeoyl-CoA o-methyltransferase | At4g34050 | 18 | 21 | CBS:1912 | ME:234 |
| 35 Caffeoyl-CoA o-methyltransferase | At1g24735 | 0 | 0 | 4 | ME:903,452,61 |
| 36 UDP-Glc 4-epimerase | At5g58490 | 14 | 18 | 18 | CBS:592 |
| 37 UDP-Glc 4-epimerase | At2g33590 | 20 | 2 | ME:1528 |
| 38 UDP-Glc 4-epimerase | At2g20400 | 2 | ME:1727,1434, 772,294 |
| 39 Cinnamyl-CoA reductase | At5g14700 | 19 | 16 | 18 | CBS:226 |
| 40 Cinnamyl-CoA reductase | At4g30470 | 20 | 21 | 0 |
| 41 Cinnamyl-CoA reductase | At5g34230 | 1 | CBS:1058 |
| 42 Cinnamyl-CoA reductase | At3g19450 | 19 | 22 | 23 | CBS:1628 |
| 43 Cinnamyl-CoA reductase | At4g23910 | 19 | 18 | ME:903,452,61 |
| 44 Cinnamyl-CoA reductase | At1g15950 | 22 | 1 | 1 | CBS:1541 |
| 45 Ferulate 5-hydroxylase | At5g54160 | 0 | 0 |
| 46 Flavonol 3′-O- methyltransferase | At2g23910 | 19 | 16 | 18 | ME:1757 |

**Carotenoid pathway**

**Copathway**

| Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 47 Phytoene synthase | PSY | At3g17230 | 20 | 18 | 22 | 2 | CAROT-CO:7.78e-05(21/106974) |
| 48 Phytoene dehydrogenase | PDS | At4g14210 | 23 |
| 49 7-Carotene desaturase | ZDS | At3g04870 | 16 | 19 |

**Zeaxanthin branch**

| Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 50 Lycopene cyclase | LYC | At3g10230 | 20 | 0 | 1 | CBS:401 | CAROT-A1:1.13e-08(35/96444) |
| 51 β-Carotene hydroxylase | B2 | At5g25270 | 5 | 21 | 1 | 2 | CAROT-CO:7.78e-05(21/106974) |
| 52 β-Carotene hydroxylase | B2 | At4g25700 | 17 | 22 | 21 | CBS:692,348 | CAROT-A1:1.13e-08(35/96444) |
| 53 Zeaxanthin epoxidase | ABA1 | At5g67030 | 23 | 4 | 3 |

**Feedback of 7**

| Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 54 Violaxanthin deepoxidase precursor | NPQ1 | At1g08550 | 19 | 10 | 12 | 14 |

**Lutein branch**

| Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 55 Lutein-deficient 2 | LUT2 | At5g37030 | 14 | 22 | 22 | CAROT-B1:8.51e-08(25/106974) |
| 56 Lutein-deficient 2 | LUT2 | At3g10230 | 20 | 0 | 1 | CBS:401 | CAROT-A1:1.13e-08(35/96444) |
| 57 β-Ring hydroxylase on carotenes | LUT5 | At1g31800 | 14 | 19 |
| 58 ε-Ring hydroxylase on carotenes | LUT1 | At3g33130 | 13 | 20 |

**Oxylipin pathway**

**Jasmonate branch**

| Locus | Arabidopsis Genome Initiative No. | P450 | DD-DHC | LL-LLDHC | LL-LLHHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|-------|----------------------------------|------|---------|-----------|-----------|----------|-----------------------------------------------|
| 59 Lipoxigenase | LOX1 | At1g355020 | 22 |
| 60 Lipoxigenase | LOX2 | At3g15140 | 18 |
| 61 Lipoxigenase | LOX3 | At1g17420 | 16 | EE:263 |
| 62 Allene oxide synthase | AOS | At5g42630 | CYP74A1 | 19 | 22 | EE:904 |
| 63 Allene oxide cyclase 1 | AOC1 | At2g25760 | 13 |
| 64 Allene oxide cyclase 2 | AOC2 | At2g25770 | 13 | EE:1573 |
| 65 Allene oxide cyclase 4 | AOC4 | At1g13280 | 7 | 13 | AG/API BS IN SUP:4.08e-03/2/104 |

(Table continues on following page.)
rate-limiting enzyme in this pathway. The LL_LDHC arrays show significantly earlier phasing for CYP90A1 (8 h maximum) than for CYP90B1 (DWF4; 12 h maximum). The LL_LLHC arrays show slightly earlier phasing for CYP90C1 (6 h maximum) than for CYP90A1 and CYP90B1 (8–9 h maxima).

In the inactivation of plant hormones, the catabolism of brassinolide and other brassinosteroids is mediated by CYP734A1 (BAS1) and CYP72C1 (SBO1; Neff et al., 1999; Turk et al., 2003, 2005; Nakamura et al., 2005; Takahashi et al., 2005) and the catabolism of abscisic acid is mediated by four members of the CYP707A family.

### Table III. (Continued from previous page.)

| Pathways and Subdivisions | Locus | Arabidopsis Genome Initiative No. | P450 | DD_DDHC | LL_DDHC | LL_LDHC | LL_LLHC | EE/CBS/ME | e-Value and Overrepresented Ratio in Element |
|---------------------------|-------|----------------------------------|------|---------|---------|---------|---------|-----------|---------------------------------------------|
| **Hexenal branch**        | 69    | LOX1                             | At1g55020 | 22      | 18      | EE:263  | EE:904  | ME:1391, 1134, 1197, 390, 376 CBS:1573  |
| Lipoygenase               | 70    | LOX2                             | At3g45140 | 1         |         |         |         | JA:2:1.9e-05(JT4520)                      |
| Lipoygenase               | 71    | LOX3                             | At1g17420 | 16      | 19      | EE:1573 |         | MYB4:5.3e-03(29/77609)                    |
| Allene oxide synthase     | 72    | AOS                             | At5g42650 | 19      | 19      | EE:804  |         | MYB1:1.8e-03(10/20163)                    |
| Allene oxide cyclase 1    | 73    | AOC1                            | At3g25760 | 13      | 13      | EE:1573 |         | MYB4:1.42e-03(25/77609)                   |
| Allene oxide cyclase 2    | 74    | AOC2                            | At3g25770 | 13      | 13      | EE:1573 |         | MYB4:1.1e-03(10/20163)                    |
| Allene oxide cyclase 4    | 75    | AOC4                            | At1g3280  | 7       | 7       |         |         | MYB4:1.42e-03(25/77609)                   |
| Hydroperoxide lyase       | 76    | HPL1                            | At4g15440 | 20      | 20      | EE:1383 |         | JA:2:7.9e-08(011/5533)                    |
| **Glucosinolate pathway** | 77    | Monoxygenase                     | CYP79F1  | 1 CBS:445  |         |         |         | JA:2:1.9e-05(JT4520)                      |
| Aliphatic glucosinolate branch | 78    | Monoxygenase                     | CYP79F2  | 1 CBS:1331 |         |         |         | JA:2:1.9e-05(JT4520)                      |
| Monoxygenase              | 79    | Monoxygenase                     | CYP83A1  | 22      | 22      |         |         | GL-TRP4:5.99e-04(14/14468)               |
| Alkylthiolydroximate C-S lyase | 80    | SUR1                            | At2g20610 | 19      | 22      |         |         | MYB1:1.8e-03(10/20163)                    |
| UDP- glycosyltransferase  | 81    | UGT1                            | At1g24100 | 19      | 21      | CBS:1867, 728, 576 | GL-MET4.5e-00(14/37915) |
| Sulfotransferase          | 82    | ST2                             | At1g74090 | 17      |         |         |         | IBOX:7.0e-03(12/30201)                    |
| **Indole glucosinolate branch** | 83    | Monoxygenase                     | CYP79B2  | 23      | 23      | CBS:1829, 810 EE:183 | GL-TRP2:1.8e-09(21/35624) |
| Monoxygenase              | 84    | Monoxygenase                     | CYP79B3  | 0 CBS:917, 782 |         |         |         | GL-TRP5:1.0e-05(8/8464)                   |
| Allkylthiolydroximate C-S lyase | 85    | SUR1                            | At2g22330 | 19      | 22      |         |         | GL-TRP4:3.61e-05(10/14468)               |
| UDP- glycosyltransferase  | 86    | UGT1                            | At1g24100 | 19      | 21      | CBS:1867, 728, 576 | GL-TRP1:7.25e-05(11/19861) |
| Desulfoglucosinolate sulfotransferase | 87    | DST                             | At1g74100 | 22      |         |         |         | MOTIF3A:1.03e-03(5/5059) CBS:1.5e-03(7/12234) |
| **Brassinosteroid pathway** | 88    | C-24 reduction                   | CYP90B1  | 7 8      | EE:1577 |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| Brassinsteroid biosynthesis | 89    | DWF4                            | CYP90B1  | 12 8     | CBS:1085, 177 |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| 3-Oxo-5α-steroid-4-dehydrogenase | 90   | DWF4                            | CYP90B1  | 12 8     | CBS:1085, 177 |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| Steroid                   | 91    | DWF4                            | CYP90B1  | 12 8     | CBS:1085, 177 |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| 6-Deoxo-cathasterone 23α-hydroxylation | 92    | CYP90B1  | 8 9      |         |         |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| C-2α-Hydroxylase          | 93    | CYP90C1                          | 3 6      | CBS:280  |         |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| Brassinolide synthase     | 94    | CYP90B1                          | 8 9      |         |         |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| Degradation of brassinosteroid breakdown | 95    | CYP90B1  | 3 6      | CBS:1796, 819 |         |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |
| Glucosyltransferase       | 96    | CYP90B1                          | 8 9      |         |         |         |         | ATMYB2 BS IN RD22:8.27e-03(3/7337)        |

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Identification of Circadian-Relevant Elements

To gain perspective on circadian controls over different pathways, circadian-regulated promoters in each branch of a pathway as well as in each overall pathway were searched for known elements that were overrepresented compared with their frequency in the 27,457 promoters of the Arabidopsis genome (annotated in the AGRIS sequence motif database [http://Arabidopsis.med.ohio-state.edu]; Davuluri et al., 2003; Palaniswamy et al., 2006). Promoters were also searched for novel elements using the Gibbs sampler program AlignACE (http://atlas.med.harvard.edu/; Hughes et al., 2000). These searches identified a number of five- to nine-nucleotide elements listed in Table III that are significantly overrepresented in these different pathways at a cutoff of \( P < 10^{-3} \); the sequences for elements identified in these searches are outlined in Supplemental Table S1.

In promoters of genes for different branches of phenylpropanoid metabolism, many circadian-relevant elements are evident. The CBS (AAAAATCT) is overrepresented in the core pathway and the intermediate flavonoid branch. The ME (AACCAC) is frequent in the lignin and anthocyanin branches. The EE (AAAAATCT) is overrepresented and has been identified as a separate motif (Kushiro et al., 2004; Saito et al., 2004). At the resolution of the 4-h time points evaluated in these arrays, CYP734A1 (no. 20 in Table II) displays any distinct circadian regulation (7 h maximum). Of the three P450s in GA synthesis (CYP88A3, CYP88A4, and CYP701A3), only the second multifunctional CYP701A3 (no. 22 in Table II) in this pathway (Hellriegel et al., 1998, 1999) is circadian regulated (3 h maximum LL_LDHC array, 7 h maximum in LL_LLHC array).

In the lutein branch of the carotenoid pathway, DPBF1&2 is the only previously described element that appears to be overrepresented; in the carotenoid intermediate pathway, ABFS and T-BOX are overrepresented. With few known elements overrepresented in these carotenoid intermediate and branched pathways, AlignACE algorithms identified a number of novel overrepresented elements in the promoters of the zeaxanthin/abscisic acid branch (designated A), lutein branch (designated B), and core pathway (designated CO). These elements are identified with alphabetic and numerical designations and correspond to CAROT-A1 (AGAGA[AG][AG]), CAROT-A2 (CCA AAN[CA]A), CAROT-A3 (GAGA[AT]GA[AG]), CAR OT-B1 ([CT][TG][AG]AAG), CAROT-B2 ([GA][AG]A GAAGCT), CAROT-B3 (GAAAGCT), and CAROT-C0 (AGAAAGA). Of these, CAROT-C0 is overrepresented in all three parts of this pathway, and the others are more specific for promoters in branches of this path-
way. Spacings of these elements in the CYP97A3 and CYP97C1 promoters are shown in Figure 6D. The promoter of CYP97B3, which codes for a P450 closely related to CYP97A3 and CYP97C1 in the lutein branch and shows intermediate phasing, contains CAROT-B1, CAROT-B2, and CAROT-CO as well as GL-MET2, GL-TRP3, CAROT-A3, and T-BOX. Notably, no EE exist in any of the carotenoid pathway promoters, and CBS exist in only two promoters.

In the oxylipin pathway, circadian-regulated promoters in the AOS branch have overrepresented MYB4, MYB, RAV1-A, AG/AP1 BS, ATMYC2 in RD22, GL-TRP1, and MOTIF8 (ATTACAA), and five of the 10 promoters in this branch have EE. AlignACE analysis of this entire pathway identified three novel overrepresented elements, JA1 (ATGTGAAT), JA2 (AAGAA[G]ANG), and JA3 (T[TC]GG[AG]CAA), that we had previously identified as overrepresented in MeJ-inducible promoters. Of these, JA2 is represented seven times in the AOC4 promoter, with six of these elements being present in short tandem direct repeats, indicating that its abundance is not uniform across promoters in the AOS branch. The circadian-regulated CYP74B2 promoter contains multiple ME, GATA [LRE], DPBF1&2, GL-TRP1, and MOTIF8, one each of the G-BOX [LRE], JA3, and CBS, and no EE; none of these, except possibly the ME, can be recorded as overrepresented, since this is the only locus in the HPL branch of oxylipin metabolism.

In the glucosinolate pathway, circadian-regulated promoters in the aliphatic glucosinolate branch contain MYB, MYB4, and I-BOX and the novel GL-MET3 (ANACCAAA), GL-TRP2 (ANNTTGAAA), GL-TRP4 (GTTG[AT]G), and GL-TRP5 (ACCA[AG]CNA[AG]). Circadian-regulated promoters in the indole glucosinolate branch contain STRE (Marchler et al., 1993) and MOTIF3A (TNG[AT]N[AG][AT]GGAA[AG]) and the novel GL-TRP1 (ACATATT), GL-TRP1 (ANNTTGAAA), GL-TRP4 (GTTG[AT]G), GL-TRP5 (ACCA[AG]CNA[AG]), and GL-MET4. Of these, MOTIF3A was previously identified as overrepresented in Arabidopsis P450 promoters induced by MeJ or salicylic acid (SA). CBS exist in five of eight promoters in these branched pathways.

Overrepresented elements in the collection of circadian-regulated brassinosteroid synthesis loci include the GATA [LRE] ([AT]GATA[GA]), ATMYZB BS in RD22 (CTAACCA), MOTIF1 (CT[GTNGATGTC]A), MOTIF8,
and novel BS1 (AAC[ACGT]CTTT) and BS2 (TATNT-TAG). MOTIF1 was originally found to be overrepresented in the promoter sequences of Arabidopsis P450s induced by MeJ, SA, or BION and their combinations; MOTIF8 was originally found to be overrepresented in promoters of root-specific P450s. The only overrepresented element in the two circadian-regulated brassinosteroid degradation loci is ATMYB2 BS in RD22.

**Figure 7.** Phenylpropanoid subnetwork. Genes networked in their expression profiles are shown with nodes corresponding to CHS (chalcone synthase; At5g13930), TT5 (chalcone isomerase; At3g55120), CHI (chalcone isomerase; At5g05270), F3H (naringenin 3-dioxygenase; At3g51240), TT7 (flavonoid 3'-monooxygenase; CYP75B1; At5g07990), F3OG2 (flavonol 3-O-glucosyltransferases; At4g01070), F3OG3 (At5g54060), and DFR (dihydrodihydroflavone 4-reductase; At5g42800). FS (flavonol synthase; At5g50210) is not connected to these. A, Genes at nodes in this network that contain G-BOX [LRE] have thick red circles; associated genes that also contain G-BOX [LRE] have thin red circles. B, Phasing of genes at nodes in this network. Values on the x axis indicate hours after each time-course sampling was initiated. White and gray bars on the x axis indicate subjective day and subjective night during the time course; blank and grid bars indicate high (22°C) and low (12°C) temperature maintained during the time course.
are present once in the DWF1 promoter, twice in the CYP85A2 (BR6ox2) promoter, and twice in the CYP734A1 (BAS1) promoter. CBSs are present in the 3-oxo-5α-steroid 4-dehydrogenase, CYP90C1, and CYP734A1 promoters. CYP72C1, another P450 involved in brassinosteroid degradation, is not circadian regulated but its promoter contains SORLREP3 and ATMYB2 BS in RD22, which are overrepresented in the CYP734A1 and UGT73C5 loci involved in brassinosteroid degradation as well as one CBS.

Analysis of a Hypothetical Node Controlling Circadian Cycling

To better understand the relationships between these pathways and some of their predicted transcriptional regulators, we analyzed the expression patterns of PAP1, one MYB transcription factor that had been proposed to control the circadian regulation of the anthocyanin and lignin branches (Harmer et al., 2000), and examined the downstream effects of perturbing its expression. Analysis of PAP1 transcript abundance throughout these circadian cycles indicates that, consistent with previous hypotheses, the PAP1 locus is under circadian regulation (Fig. 8B). Its phasings on both LL_LDHH and LL_LDHC arrays (19 h maximum) are very similar to the four circadian-regulated P450s in the phenylpropanoid pathway (Fig. 8, C–E) and other associated circadian-regulated transcripts (Table III). To directly determine whether PAP1 has a role in circadian regulation of these branched pathways, the circadian regulation patterns of several phenylpropanoid transcripts were compared in wild-type and PAP1-overexpressing (pap1-D) seedlings over a 48-h period starting at 7 d of growth under the LL_LDHH conditions (top versus bottom panels of each blot in Fig. 8). Of the four P450 loci in the phenylpropanoid pathway, only CYP75B1 transcripts appear to accumulate at any higher level in pap1-D seedlings compared with wild-type seedlings. However, most importantly, CYP75B1 and three other P450 transcripts in this pathway maintain their normal circadian cycling despite the constant overexpression of the PAP1 protein (Fig. 8B).

DISCUSSION

Our profilings of the four P450 transcripts responsible for rate-limiting steps in phenylpropanoid metabolism emphasize the similar circadian phasing of all transcripts in this pathway, even those in diverse branches (intermediate flavonoid branch, lignin branch, flavonol branch, and pelargonidin and cyanidin branches). Nearly all, including those needed in flavonoid and anthocyanin production, are expressed at their maximal levels just before subjective dawn, at a time when there is little light. Under some of these circadian regimes (LL_LDHC), the cycling of the CYP75B1 transcript is especially prominent, suggesting that its normal circadian cycling pattern is enhanced by exposure to light at subjective dawn. In Arabidopsis, regulation of the flavonoid and anthocyanin pathway transcripts such as CYP75B1 is controlled by a MYB and TTG1 complex with basic helix-loop-helix proteins (Dubos et al., 2008; Gonzalez et al., 2008). The light-dependent regulation of these genes by complex sets of regulators as well as circadian cycles highlights the complex regulatory mechanisms modulating each of the individual branches in this pathway.

One element likely to be involved in light induction of this promoter and others in the flavonoid/anthocyanin branch is the G-BOX [LRE]. This has previously been identified as a light-responsive element (Menkens and Cashmore, 1994; Chattopadhyay et al., 1998; Michael et al., 2008), and in this study, it has been seen as overrepresented throughout the entire downstream flavonoid pathway, including the intermediate flavonoid, flavonol, and anthocyanin branches (Fig. 6B). In a scale-free network of genes coexpressed with the flavonoid pathway (Fig. 7A), eight out of the nine promoters in the intermediate flavonoid pathway, flavonol, and anthocyanin branches that contain G-BOX [LRE] (circled in red) occur at significant nodes. The expression patterns of seven of these genes are highly connected, with only the FS gene not connected to any of the other nodal genes and the F30G2 gene isolated in a subnetwork. Analysis of the phasings of these last two genes indicates that F30G2 is phased up to 4 h earlier than the other seven genes and FS is phased 8 to 12 h earlier. Expression of the F30G2 transcript is correlated only with that of CHI in the nine genes. The coincidence of phasings for these seven other genes is very significant (Fig. 7B), making it likely that the multiple G-BOX [LRE] play a controlling role in the regulation of these promoters. Further comparisons between these simple G-BOX [LRE] motifs have indicated that a longer G-BOX EXTENDED element [CAGCTG/(G/T)(A/C)] exists within 900 bp of each of these seven similarly phased promoters. These seven promoters also all contain a minimum of one ABRE-like, two DPBF1&2, and two MYB4, suggesting that additional overrepresented elements are also important for coordinated regulation of these promoters. In contrast, the differently phased FS promoter contains only one G-BOX EXTENDED relatively far from its translation start site (–1,583 bp) and no additional simple G-BOX [LRE], making it likely that FS expression is under the control of another transcription factor. The very apparent shift in the phasing of the FS transcripts indicates that the flavonol branched pathway feeding off from the rest of the phenylpropanoid pathway is differentially regulated from the core and lignin and flavonoid branches.

Our promoter analyses have also indicated that MYB and MYB4 are significantly overrepresented in the core and lignin branch of this phenylpropanoid pathway. One potential MYB transcription factor, PAP1, which was proposed to control circadian regulation of the anthocyanin and lignin branches, is
indeed circadian regulated, with the same phasing as these branched pathways. However, direct analyses of phenylpropanoid pathway loci potentially targeted by this transcription factor in overexpressing pap1-D seedlings have indicated that the circadian-regulated \textit{FAH1} (\textit{CYP84A1}) and \textit{REF8} (\textit{CYP98A3}) loci in lignin synthesis and \textit{C4H} (\textit{CYP73A5}) in the core pathway are not modulated by PAP1. In contrast, the \textit{TT7} (\textit{CYP75B1}) locus, which is directly involved in flavonoid and anthocyanin syntheses, shows some degree of overall enhanced accumulation in pap1-D seedlings, suggesting that PAP1 can modestly enhance expression of the flavonoid branch of this pathway. And, contrary to the suggestion that PAP1 regulates circadian phasing of phenylpropanoid transcripts, these increases in \textit{CYP75B1} transcripts fluctuate, with a circadian rhythm that is unaffected by the high PAP1 levels in this mutant, providing further evidence that PAP1 does not control circadian fluctuations of these loci and indicating that other transcription factors modulate circadian cycles in this pathway. How PAP1 expression and these branched pathways are controlled certainly requires further investigation. These results also dramatically demonstrate that the relationships between cycling genes and the cycling network cannot be inferred from time-of-day information and that additional experiments are required to dissect cascades of regulation.

Since it has been demonstrated that the coordination of daily activities confers fitness for specific environments (Michael et al., 2003; Dodd et al., 2005), understanding how these branched pathways are coordinately as well as separately controlled can provide important information for optimizing plant growth.

**Figure 8.** Circadian regulation of P450s in the phenylpropanoid pathway in wild-type and PAP1-overexpressing (pap1-D) mutant seedlings. RT-PCR gel blots for constitutive \textit{UBQ10} (A), PAP1 (B), \textit{CYP73A5} (C), \textit{CYP75B1} (D), \textit{CYP98A3} (E), and \textit{CYP84A1} (F) were compared in wild-type and pap1-D seedlings over a 48-h period starting at 7 d of growth under LL_LDHH conditions (collected and analyzed in the Schuler laboratory). \textit{UBQ10} expression levels (A) under LL_LDHH were used for normalization, and each normalized transcript level is shown above each lane. The gRMA-normalized microarray data for PAP1 and these four phenylpropanoid P450s on the LL_LDHH arrays performed with wild-type seedling RNAs (collected and analyzed by the Millar laboratory) are shown in G. Values on the x axis indicate hours after each time-course sampling was initiated. White and gray bars on the x axis indicate subjective day and subjective night during the time course at continuous 22°C. [See online article for color version of this figure.]
and health. Interestingly, all of the genes involved in carotenoid synthesis exhibit circadian regulation, but with shifted phasings in the different branches of this pathway (nos. 47–58 in Table III; Fig. 5A). The phasings of the carotenoid intermediate pathway is 19 h on average, and that of the zeaxanthin branch is approximately 20 h relative to subjective dawn, except for NPQ1 (the violaxanthin deepoxidase precursor on the feedback loop), whose circadian phasing is opposite all other loci analyzed in carotenoid synthesis. The lutein branch shows phasing at 14 h prior to subjective dawn except for Lyc, the last common component shared between the lutein and zeaxanthin branches. This phasing of the lutein branch is 5 to 6 h earlier (or 18–19 h later) than that of the zeaxanthin branch, potentially producing maximum expression of lutein derivatives such as α-carotene at the beginning of dark and maximum expression of zeaxanthin derivatives such as β-carotene in the middle of night. With it known that light-harvesting complex II (Kim and DellaPenna, 2006) and β-carotene-containing photosystems are produced almost exclusively under high light conditions and that α-carotene-containing photosystems are produced primarily in shade-grown leaves (Thayer and Björkman, 1990; Demmig-Adams and Adams, 1992; Dall’Osto et al., 2007), these results indicate that the circadian-regulated accumulation of transcripts for these branched pathways precedes accumulation of these carotenoid components by as much as 12 h.

Recent research has indicated that multiple hormone responses are intertwined with circadian cycling, including abscisic acid, l-aminocyclopropane-1-carboxylic acid, brassinolide, cytokinin, indole-3-acetic acid, MeJ, and SA (S.L. Harmer, unpublished data). Auxin synthesis has been reported to be gated by the circadian clock, allowing a plant to respond to auxin at restricted times of day (Covington and Harmer, 2007). The key regulatory nodes, transcription factor/binding site relationships, and how time-of-day activities are maintained accurately in complex biochemical pathways remain to be established. Additional studies are also needed to determine whether these transcriptional variations manifest themselves in enzymatic and metabolic variations throughout the day. Initiated as an analysis of the factors affecting P450s in an array of synthetic and catabolic pathways, this study has provided, to our knowledge, the first glance at the varied range of biochemical pathways that are targeted by the circadian clock.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) Columbia ecotype and pap1-D mutant seeds were sterilized in 70% ethanol for 30 s and 15% bleach for 15 min and then rinsed in distilled water two to three times. One hundred to 200 seeds per time point were sown on half-strength Murashige and Skoog agar plates containing 0.8% agar without Suc and were kept in the dark at 4°C for 3 d before transfer to a growth chamber. Seedlings were entrained with 12-h white-light/12-h-dark cycles at a continuous temperature (22°C) for 7 d prior to being released into continuous white light at 22°C (LL, LDHH conditions). After 1 d in continuous conditions, seedlings were harvested at subjective dawn and every 4 h over the course of the next 44 h.

Data Sources

The gene lists used for the analysis of different pathway promoters are derived from data available at The Arabidopsis Information Resource (http://www.arabidopsis.org). The circadian-regulated Arabidopsis P450 gene lists of four data sets are derived from analysis of Affymetrix Arabidopsis genome chips as described by Michael et al. (2008). Raw data and analyzed data for the entire gene set can be accessed at http://diurnal.cgrb.oregonstate.edu/. Growth conditions for DD_DDHC were constant dark and 22°C, those for LL_LDH were constant light and 22°C/12°C, and those for LL_LDH were 12 h of light/12 h of dark and 22°C/12°C. For LL_LHH, growth conditions were constant light and 22°C/12°C and samples were collected on the second and third days after switching to these conditions.

Microarray Data Analysis and Normalizations

All microarray experiments were described previously (Mockler et al., 2007; Michael et al., 2008). Briefly, all techniques were as described in the manufacturer-supplied protocols, with RNAs extracted from frozen tissues and labeled probes were prepared and hybridized to Affymetrix Arabidopsis ATH1 GeneChips. Array quality was checked using standard tools implemented in the Bioconductor packages simpleaffy and affyPLM. All microarrays described here were normalized together using gcRNA (robust multiarray average; Wu et al., 2003), and relative values are recorded as gcRNA/gcRNA in the microarray plots. Present/absent calls were made using the Affymetrix MAS5 program. The resulting gcRNA-normalized unlogged values were used to identify cycling genes with the HAYSTACK pattern-matching tool (http://haystack.cgrb.oregonstate.edu/). HAYSTACK, a model-based pattern-matching algorithm, compares a collection of diurnal/circadian models against microarray time-course data to identify cycling genes. HAYSTACK has been implemented in Perl and uses least-squares linear regression for each gene against all model cycling patterns with 24 possible phases. A series of statistical tests were used to identify the best-fit model and phase of expression and to estimate a P value and FDR for each gene. We selected cycling genes using a correlation cutoff of 0.8, which corresponds to a maximum FDR of 3.3% to 5.8% in different data sets. All microarray data can be accessed through the DIURNAL Web interface (http://diurnal.cgrb.oregonstate.edu/).

 Autoradiographs of the RT-PCR gel blots were scanned using an Epson Perfection 1250 scanner and quantified using ImageJ 1.41 software (http://rsbweb.nih.gov/ij/). RT-PCR signals for each sample were then background corrected and normalized against the RT-PCR signals for UBQ10 and reported relative to the RT-PCR signal for the first sample in each time course.

RT-PCR Verifications

Approximately 100 seedlings per time point were frozen under liquid nitrogen and powdered, and total RNA was extracted using a beadbeater (Biospec Products), a Plant RNeasy kit, and on-column Rnase-free DNase (Qagen) according to the manufacturer’s instructions. Quantitative RT-PCR gel-blot analysis of individual P450 transcripts was carried out by amplifying approximately 0.1 mg of total RNA from each sample in one-step RT-PCR containing 50 mKCl, 10 mTris-HCl (pH 8.4), 200 m each dNTP, 200 mg ml⁻¹ gelatin, 40 pmol of a 5′ gene-specific primer, 80 pmol of a 3′ oligo(dt) primer, 2 units of AMV reverse transcriptase (Promega), 8 units of RNAsin (Promega), and 1 unit of GoTaq polymerase (Promega). First-strand cDNAs were synthesized for 30 min at 42°C and subsequently PCR amplified for 18 to 26 cycles, with each cycle consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension step of 72°C for 10 min. The numbers of PCR cycles used for each transcript (18 for CYP90A1 and UBQ10, 19 for CYP79B3, 20 for CYP73A5 and CYP84A1, 23 for CYP74A1, and 26 for CYP57B1 and CYP9BA3) were determined to be within the linear PCR amplification range for each transcript. PCR products were fractionated on 1.5% agarose gels, transferred to nitrocellulose, and hybridized to biotin-labeled probes corresponding to approximately 150 nucleotides derived from the 5′ untranslated region of each P450 locus or the Arabidopsis UBQ10 cDNA. The gene-specific primers used in this analysis were as follows:
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73A5-5', 5'-TTGCAATCTTTAACCCTTCC-3'; 75B1-5', 5'-GACTCGAGGGT-
CCGTTAAAAT-3'; 84A1-5', 5'-GGGTTTGGTATGTTGAAA-3'; 98A3-5', 5'-TTGACCGCATTTAACCAGG-3'; 74A1-5', 5'-AGAAAGACCTCCTAC-
CATCAATGT-3'; 90A1-5', 5'-CAGTCTGTCGCTGCAAGGC-3'; 79B3-5', 5'-ACGTTCGAGCTTATGGA-3'; PAPI-5', 5'-GACAAAGCAAAAGGG-
GGACA-3'; algalD-5', 5'-CGAAATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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