Regulated gene expression is an important mechanism for controlling cell cycle progression in yeast and mammals, and genes involved in cell division-related processes often show transcriptional regulation dependent on cell cycle position. Analysis of cell cycle processes in plants has been hampered by the lack of synchronizable cell suspensions for Arabidopsis, and few cell cycle-regulated genes are known. Using a recently described synchrony system, we have analyzed RNA from sequential samples of Arabidopsis cells progressing through the cell cycle using Affymetrix Genearrays. We identify nearly 500 genes that robustly display significant fluctuation in expression, representing the first genomic analysis of cell cycle-regulated gene expression in any plant. In addition to the limited number of genes previously identified as cell cycle-regulated in plants, we also find specific patterns of regulation for genes known or suspected to be involved in signal transduction, transcriptional regulation, and hormonal regulation, including key genes of cytokinin response. Genes identified represent pathways that are cell cycle-regulated in other organisms and those involved in plant-specific processes. The range and number of cell cycle-regulated genes show the close integration of the plant cell cycle into a variety of cellular control and response pathways.

Cell division is a fundamental biological process and shares conserved features and controls in all eukaryotes (1–3). However, plants have a number of special features that give the control of cell division particular importance, including an indeterminate mode of development, the absence of cell migration, and responsiveness of growth rate and development to changes in environmental conditions. Cell division therefore plays a role both in the developmental processes that create plant architecture and in the modulation of plant growth rate in response to the environment (4, 5). It is therefore not unexpected that plant cell cycle control shows a novel number of aspects, together with conservation of the types of key regulators of cell cycle transitions such as cyclin-dependent kinases (CDKs), CDK inhibitor genes, cyclins, retinoblastoma (Rb) protein homologs, and E2F (6–16). However, important differences include the absence of direct CDC25 protein phosphatase homologs and the presence of cell cycle-regulated CDKs known as CDKB (17–22). As well as the presence of such novel regulators of the cell cycle, cell division control in plants might also show interactions with plant hormones and developmental regulators as well as with plant-specific processes such as cell wall metabolism.

Regulation of gene expression in different phases is proposed to be an important mechanism for control of progression through the cell cycle in yeast and mammalian cells, and around 800 genes have been identified using microarray analysis in both systems as potentially cell cycle-regulated (23–27). The wide scale analysis of cell cycle-regulated expression in plants has been hampered to date by the lack of a suitable system for the synchronization of cells from a sequenced species, and rather few genes are documented as cell cycle-regulated (28). Almost all of these genes are directly involved in cell cycle progression, thereby giving few clues as to mechanisms by which cell cycle control may intersect with other cellular processes (22, 29–35). Using a recently developed cell synchrony system for Arabidopsis cells (22), we have carried out an analysis of gene expression on high density Affymetrix microarrays (36).2 Cell cycle progression was reversibly blocked using the DNA polymerase inhibitor aphidicolin, and sequential RNA samples taken at two hourly intervals over a 19-h period were analyzed for gene expression. Expression of 4010 genes was detected and tested for statistically significant cell cycle regulation above the variation shown by a randomized data set, resulting in the identification of 463 candidate cell cycle-regulated genes, showing that cell cycle regulation of expression is found for a significant number of genes in plants. A close match was found for known regulated genes between the microarray expression analysis and RNA gel blots. Systematic analysis of their expression revealed common patterns of expression following release, suggesting coordinate regulation of a number of genes. Genes regulated in this experiment represent both processes known or suspected to be cell cycle-regulated in plants or other organisms and genes involved in a number of other cellular processes including hormone response, signal transduction, transcription control, and metabolic regulation (37–47). The insights provided by the first wide scale analysis and identification of cell cycle-modulated gene expression in plants reflect the central role of cell division in plant development and responses and forms an important foundation for future studies in plant cell biology.

*This work was supported by BBSRC Grant 8C15792 (to J. A. H. M.) and funding from the ETH (to W. G.). We also thank the Functional Genomics Center Zürich for technical and financial support. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Supported by the Deutsche Forschungsgemeinschaft.

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1 The abbreviations used are: CDK, cyclin-dependent kinase; PVE, plant cell cycle into a variety of cellular control and cycle-regulated genes show the close integration of the plant-specific processes. The range and number of cell regu-

2 Microsoft Excel spreadsheets with the full data set and the analyzed regulated genes will be made available on the World Wide Web at www.biot.cam.ac.uk/jahm/cellcycle.
Experimental Procedures

Arabidopsis Cell Suspension Culture—A suspension culture of the fast growing cell line MM2d was maintained as described (22). Briefly, cells were grown at 27°C in continuous darkness in a 300-ml flask, rotated at 130 rpm and were diluted by adding 3.5–100 ml of fresh MSS medium (Murashige and Skoog salt, 3% sucrose, 0.5 mg/liter 1-naphthaleneacetic acid, 0.05 mg/liter kinetin) every 7 days.

Synchronization by Aphidicolin Block/Release—MM2d cells were reversibly blocked in late G1/early S phase with aphidicolin as described (22). With the exception that 20 ml of early stationary phase cell suspension was transferred into 100 ml of fresh MSS medium. After incubation for 24 h under conditions as described above in the presence of 4 μg/ml aphidicolin (Sigma), cells were gently washed with 1 liter of MSS medium through a nylon net, followed by centrifugation, 357 × g for 1 min, no brake applied) to remove the drug. Cells were resuspended in a total volume of 120 ml of fresh MSS medium and incubated as above, and samples were taken hourly for analysis.

Cell Cycle Analysis—To determine cell cycle distribution, a sample of frozen cell pellet was treated to release nuclei and analyzed as described (22). On average, 10,000 particles were counted with a flow cytometer (PASII; Partec GmbH, Munster, Germany), and the cell cycle phases were analyzed using Multicycle for Windows (Phoenix Flow Systems, San Diego, CA). Cells actively replicating DNA were determined using bromodeoxyuridine (BrdUrd) labeling and subsequent immunocytochemical detection of the incorporated BrdUrd as described previously (22). Metaphase and anaphase cells were counted in the same samples by 4’,6-diamidino-2-phenylindole staining to determine the metaphase/anaphase index. Total RNA was extracted as described previously (33, 48). For Northern blot analysis, prezeEY markers genes were used for hybridization as described previously (22). Hybridized membranes were exposed to autoradiography film, scanned, and quantified using NIH Image 1.62 (available on the World Wide Web at rsb.info.nih.gov/nih-image/index.html). Equal loading was controlled by methylene blue staining of the membranes. The level of expression (in arbitrary units) was first corrected against the quantified loading control and then normalized by expressing each value as a proportion (percentage) of the maximum found expression.

High Density Oligonucleotide Array Expression Analysis—Preparation of cDNA and biotin-labeled cRNA were performed as recommended by Affymetrix (Santa Clara, CA). Hybridization to Arabidopsis Gene-Chips, detection of labeled cRNA using streptavidin-phycoerythrin, and reading of the arrays using a confocal scanner (Affymetrix) were performed according to the manufacturer's instructions. Raw data were processed with Affymetrix MicroarraySuite 5.0.

Bioinformatic Analysis—Based on results of the statistical algorithm of the MASuite 5.0 analysis software (Affymetrix), genes were selected for further analysis if they (i) were called at least once present (“present” call) in one of the 10 different experimental time points and (ii) were at least once changed among the samples (“difference” call) after comparative analysis of each experiment against the sample directly taken after release of the block (T0).

Numerical Characterization of Sinusoidal Expression—Analysis was performed as described by Shedden and Cooper (49). Briefly, data in the time series were compared with the first measurement to obtain relative expression values and were log-transformed. The measurements for each gene were centered across the chips. Suppose Yi(t) denotes the expression of gene i at time t. For all genes, the vector Yi(t) was fit with least squares to Yi(t) = a0 + b0C(t) + R(t), where S(t) = sin(2πT/T) and C(t) = cos(2πT/T) with T = 22 h being the time required for one entire cell cycle. Yi(t) can be decomposed into a periodic component Z(t) = a0 + b0C(t) with T = 22 h and a component R(t) that is aperiodic or has a period substantially different from 22 h. The proportion of variance explained by the Fourier basis (Fourier proportion of variance explained (PVE)) is the ratio m0 = var(Z(t))var(Yi(t)), which can range from 0 to 1. Values closer to 1 indicate greater sinusoidal expression with a period of 22 h, whereas values closer to 0 indicate a lack of periodicity or periodicity with a period that is substantially different. The fitted waveform Z(t) resembles a sine wave. For each gene, the phase of Z(t) (equal to time of maximal expression) was determined. Based on flow cytometric and cytological data, expression maxima between 0 and 5 h were considered as S phase, maxima between 5 and 10 h were considered G2, maxima between 10 and 17 h were considered M, and maxima between 17 and 22 were considered G1 phase. Because randomly distributed data will also show a certain tendency for periodicity, a random data set was constructed from the experimental data in which the variance for all time series Y(t) was conserved but not the order of the measurements. Data resampling was performed by allowing permutations of measurements for each gene. Subsequently, PVE values m0 were calculated for all vectors Yi(t) in the artificial control data set.

Cluster Analysis of Cell Cycle Expression Patterns—Expression patterns of genes defined as having a statistically significantly (p < 0.05) greater periodic expression in the experiment than the randomized data set were imported into GeneMaths (version 1.50; Applied Maths). Of the 4010 genes identified that passed the variation filter, a total of 493 gene expression profiles met the periodic fluctuation conditions (p < 0.05). All of the processed values for signal log ratios after comparative analysis against the sample directly taken after release block (T0) were subjected to principal component analysis (PCA) and self-organizing map (SOM) algorithms using GeneMaths 1.50 (50). Prior PCA and SOM analysis genes were labeled (GeneMaths) according to the annotated peak of expression found after statistical analysis for each phase as

Fig. 1. Aphidicolin block/release of Arabidopsis cell line MM2d. A, flow cytometry analysis of MM2d cells after release of aphidicolin block in late G1/early S phase, showing the coherent population of cells progressing through S phase. Each histogram represents a sample taken at 1-h intervals from T0 (time of release) to T19 (19 h later). B, DNA histogram of flow analysis results in A. C, LI determination of S phase (●) and metaphase/anaphase index determination of metaphase and anaphase cells (□). D, comparative mRNA analysis of gene expression by Northern blot and microarray analysis. Expression was normalized for microarray analysis by dividing the absolute detected signal through the maximum of expression (●). Signals after Northern blot analysis were quantified using NIH Image 1.62. The level of expression (in arbitrary units) was normalized by correcting against a loading control and expressing as a proportion of the maximum signal (□).
follows: S phase peak (blue), G2 (yellow), M phase (red), and G1 (green). Both the expression values of different genes (493 values) and different experiments (nine) were used as variables to calculate the PCA. Data were normalized across genes and experiments. SOM analysis was performed choosing as map (or matrix) the dimension $4 \times 4$. A dendrogram was created after the absolute expression pattern of each gene was normalized across the experiment by dividing the absolute signal at each time point by the maximum value for the same gene independently of whether it was called present or absent by MASuite. The hierarchical clustering analysis was performed by using as clustering algorithm the unweighted pair group method using arithmetic averages (large Nip) (51).

Data Base Search to Identify Regulatory Elements within the Promoter Region—The data base tool patmatch (available on the World Wide Web at www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) was used to search the promoter region 1 kb upstream of each open reading frame of the selected 493 genes. The following consensus sequences were used to search for regulatory motifs: E2F (TTTTYCGGY), mitotic-specific activation (YCYAACGGYY), Oct (CGCGGATC), and Hox (CCACGTCA).

RESULTS

Cell Cycle Progression after Aphidicolin-induced Synchrony—Analysis of gene expression during the cell cycle is predicated on effective synchronization and analysis of cell cycle progression. In many plant systems, the fungal toxin aphidicolin has been found to be an effective method of reversibly blocking cell cycle progression (22, 53). It inhibits both DNA polymerase $\alpha$ and $\delta$ (54) and therefore blocks cell cycle progression in early S phase. Removal of the inhibitor by washing leads to release of the block and the synchronous resumption of S phase and progression through the cell cycle. However, Arabidopsis cell culture systems have proven remarkably recalcitrant to efficient synchronization using this or other methods (29, 55, 56). We recently developed techniques for aphidicolin synchronization of the Arabidopsis Landsberg erecta cell line MM2d (22), which was used for the synchronization experiments reported here.

After treatment of MM2d cells with aphidicolin for 24 h and subsequent washing to remove the block, cell cycle progression was followed by flow cytometry over a 19-h period (Fig. 1A, and B). The majority of cells are arrested in G1/early S phase (G1/S, G1 = 1N) directly after release of the block. Within 1 h of removal of aphidicolin, a peak corresponding to a progressive increase in DNA content of S phase cells indicated that the majority of cells proceed synchronous through S phase (Fig. 1A). The DNA content of this S phase peak constantly increased in size before reaching the G2 phase DNA content (2N) after 5 h. Peak analysis of the flow cytometry data shows that 77% of the cell population is in S phase 1 h after block release, and a maximum of more than 90% is found in G2 after 7–8 h (Fig. 1B). At 8 h, a rapid increase in the number of metaphase cells is observed.

Synchrony was also monitored by pulse labeling with BrdUrd and detection of newly synthesized BrdUrd-containing DNA using immunocytochemistry and indirect immunofluorescence to identify S phase cells actively synthesizing DNA (22). The proportion of BrdUrd-positive cells observed is defined as the labeling index. Independent labeling index determination of the same samples confirmed the level of S phase synchrony measured by flow cytometry showing a labeling index peak of 76% observed 2 h after release (Fig. 1C). The metaphase/ anaphase index of cells in metaphase/anaphase reaches a peak value of around 11%, 11–12 h after release of the block. It should be noted that only cells in metaphase and anaphase were scored for the metaphase/anaphase index, which represents only around 35–40% of the total duration of mitosis, since it is difficult to score routinely other mitotic phases due to the small genome size and late condensation of Arabidopsis chromosomes in prophase (57).

Differential Analysis of Gene Expression—RNA was prepared from samples taken immediately after washing to remove aphidicolin (0 h) and at two hourly intervals until 16 h, followed by a final sample at 19 h. RNA was labeled and hybridized to high density Affymetrix GeneChip DNA arrays that contain ~8250 gene sequences and expressed sequence tags according to the manufacturer's instructions (Affymetrix). The hybridized chips were then analyzed, and genes were filtered as described under “Experimental Procedures.” Of the ~8250 genes and expressed sequence tags represented on the chip, 4010 passed the biological variation filter, indicating that they were both reliably detected on at least one chip (“present” call), and showing a change from the expression level at time 0 (“difference” call).

New analysis of microarray data has found that random variation can produce apparently systematic patterns of expression (49), throwing doubt on earlier identification of cell cycle-regulated genes in human fibroblasts (24). We therefore confirmed the existence of periodicity in our data set by creating a control set of randomized data (see “Experimental Procedures”) for the 4010 genes passing the first filter. Fourier-PVE values, indicating the degree of cyclicity in the data and phase of expression were determined. Fig. 2A shows a plot of the PVE values of the 1000 strongest expressed genes (highest mean expression; mean selection, left) or of the 1000 genes with the largest standard variation in expression (S.D. selection, right) against the similarly ranked genes from the random set. In such a plot, points close to or above the diagonal show that there is similar or even less
periodicity in the experimental data than in the random data. In contrast, points below the diagonal indicate periodic expression. Fig. 2A demonstrates considerable periodicity in the experimental data. Thus, the successful synchronization of the cell culture was confirmed and that indeed many genes are expressed in a cell cycle-dependent manner. Similar to observations of Shedden and Cooper (49), we observe stronger periodic expression among the genes with the highest S.D. than among the genes with highest mean expression (Fig. 2A, compare left and right). Similar observations were made with a plot of PVE values of the entire set of 4010 selected genes (data not shown).

Subsequently, we compared the distribution of phases between experimental and randomized data. Data in Fig. 2B show that the timing of maximal expression of the randomized data is relatively evenly distributed throughout the period of the experiment, as expected for effectively randomized data. In contrast, the distribution of phases in the experimental data strongly deviates from that of the random data. In particular, very few genes had expression maxima in G2 phase (Fig. 2C).

We then used the distribution of PVE values of the control data set to select those genes showing statistically significant higher periodic expression in the experiment than expected from the random data ($p < 0.05$), resulting in the definition of 493 (12%) gene signals of the total expressed (4010) as having a high probability of exhibiting significant regulation during the duration of the experiment. These included 213 gene signals with a peak of expression during S phase, nine genes peaking in G1, 135 in mitosis, and 136 in G2. The distribution of phases for the 493 selected signals was similar to that of the entire set of 4010 genes (data not shown). Although these genes were characterized by a low probability that their cyclical behavior was due to chance fluctuations, Fig. 2, B and C, demonstrates that a proportion of other genes among the set of 4010 is also likely to be expressed in a cell cycle-dependent manner. A number of the 493 gene signals identified as significantly regulated are represented by independent oligonucleotide sets on the array. 26 genes have duplicated oligonucleotide sets, and two have triplicated sets, resulting in a total of 463 different genes identified.

To confirm the reliability and sensitivity of the results obtained from the microarray analysis, the expression patterns of several genes known to be cell cycle-regulated (22) were determined by RNA gel (Northern) blot and compared with the normalized expression data from the microarray analysis (Fig. 1D). Cell cycle regulation of histone H4, CYCD2.1, CYCD3.1, CDKA, CDKB1, and CDKB2 could be readily detected both by Northern blot and among the 4010 expressed genes on the microarray. These comparative analyses clearly demonstrate that the expression profiles obtained by both methods show strikingly similar timing of their peak values and overall pattern, although small variations may be seen for individual time points. Striking is the clear difference between the timing of expression of CDKB1 and CDKB2 detected by both methods.

**Principle Component Analysis**—PCA was performed to analyze the extent to which the variation in expression seen among the 493 gene signals can be attributed to a limited number of variable components (58). Briefly, PCA can simplify the analysis and visualization of multidimensional data sets by determining key variables that explain the differences in the observation. The matrix to be analyzed using our data set has 493 rows of genes and nine columns of conditions corresponding to each of the measured time points. Fig. 3A is a plot of the observed variances in all nine principal components. The first two principal components account for 72% of the total variability observed in our data. Plotting all 493 genes onto the first and second principal component showed that all labeled genes fall into distinct quadrants (Fig. 3B). In addition, the position of known cell cycle-regulated genes, such as histones, mitotic
cyclins, and CDKs was identified. This result clearly demonstrates that annotated S phase and M phase genes have strikingly different locations in space after PCA. Thus, PCA confirms that the simple assignment of phase specificity by peak value is indicative of co-regulated genes and that the majority of variation observed can be explained by two principle variables.

Cluster Analysis of Gene Expression—Since PCA analysis demonstrated considerable structure in the expression data, clustering tools based on hierarchical neighbor joining and self-organizing maps were used to identify groups of co-regulated genes.

The relatedness of expression patterns of the 493 gene signals identified as differentially expressed was assessed by creating a dendrogram based on normalized expression levels of the absolute detected signal. This hierarchical cluster analysis clearly shows that different groups of genes show peaks of expression at specific time points throughout the time course (Fig. 4A, dark red). Abridged branch analysis resulted in the creation of sub-branches or nodes (Fig. 4A, A–G), which reflect differences in expression pattern and timing.

The hierarchical cluster analysis is based on consecutive pairwise comparisons, and although it is useful for grouping genes based on similarity of expression timing, it may not reflect the diversity of different regulatory patterns. The data set was therefore also clustered using SOMs, a neural network useful for clustering large data sets by classifying entries in a two-dimensional space or map. For this data set, SOM analysis using a 4 × 4 matrix resulted in the optimal classification of duplicated and triplicated genes between different cell cycle phases (S, G2, M, G1), based on peak expression time, between different nodes on the hierarchical cluster and different SOM clusters. Of the 28 replicated genes, only five pairs were not assigned to the same phase by peak expression, seven pairs were not assigned to the same branch node by hierarchical clustering, and, using the most stringent clustering assessment, 19 gene pairs were assigned to the same cluster group. Of the remaining nine pairs, all but one pair were assigned to groups showing very similar trends (groups 15 and 16, 2 and 3, 8–12, 8–16, and 1–6). Taken together with the comparison of signals with Northern data above, the analysis of duplicates shows highly reproducible detection and assignment of expression patterns. We also conclude that the SOM cluster analysis reliably assigns duplicate signals for the same gene to the same expression pattern in >65% of cases and to very similar patterns in >96% of cases observed.

Known Cell Cycle-regulated Genes—Although rather few genes are known to be cell cycle-regulated in Arabidopsis, there is direct evidence for regulation of histones (53, 59), mitotic cyclins (19, 60–63), and B-type CDKs (20, 22), proliferating cell nuclear antigen, and the CDC6 protein involved in initiation of DNA replication (16, 64). We examined the extent to which two classes of likely co-regulated genes were identified as cell cycle-regulated and whether known co-regulated genes were assigned to the same or similar clusters. The majority of histone genes are expressed primarily in S phase. 14 histone genes are represented on the Affymetrix array, of which 14 were detected as expressed and 10 different genes were identified within the set of cell cycle-regulated genes. All 10 regulated histones fall into the very similar clusters 12 (two genes), 15 (one duplicated gene signal whose pair is in 16) and 16 (eight gene signals), indicating high frequency of identification of histones and ro-
| Gene locus | Description | Tree | SOM | E2F | MSA |
|------------|-------------|------|-----|-----|-----|
| At4g12560  | Putative protein | C    | c16 |     |     |
| At1g14900  | Linker histone protein, putative | C    | c16 | 1   |     |
| At4g30860  | Putative protein | B    | c8  |     |     |
| At2g43590  | Putative endochitinase | B    | c16 |     |     |
| At3g54640  | Tryptophan synthase α chain | C    | c12 |     |     |
| At1g02920  | Glutathione S-transferase, putative | C    | c16 |     |     |
| At3g23340  | Putative casein kinase | C    | c8  |     |     |
| At2g43510  | Putative trypsin inhibitor | C    | c12 | 2   |     |
| At4g22690  | Cytochrome P450-like protein | B    | c16 |     |     |
| At1g14900  | Linker histone protein, putative | C    | c15 | 1   |     |
| At2g43570  | Endochitinase isolog | B    | c12 |     |     |
| At4g36990  | Heat shock transcription factor HSF4 | B    | c8  |     |     |
| At4g25900  | Possible apospory-associated-like | B    | c12 |     |     |
| At3g54560  | Histone H2A | A    | c12 | 1   |     |
| At1g21000  | Unknown protein | B    | c16 | 1   |     |
| At2g45300  | (EPSP) synthase | C    | c12 |     |     |
| At1g07640  | Metallothionein-like protein | B    | c4  | 1   |     |
| At2g47730  | Glutathione S-transferase (GST6) | B    | c8  |     |     |
| At5g61790  | Calnexin-like protein | C    | c7  |     |     |
| At2g21480  | Putative cytochrome P450 | B    | c12 |     |     |
| At2g44890  | Putative cytochrome P450 | B    | c8  |     |     |
| At2g02930  | Putative glutathione S-transferase | B    | c16 |     |     |
| At2g30620  | Histone H1 | C    | c16 | 1   |     |
| At1g78830  | Hypothetical protein | B    | c16 |     |     |
| At4g35450  | Monoxygenase 2 (MO2) | B    | c16 |     |     |
| At2g51640  | Putative PHD-type zinc finger | C    | c12 |     |     |
| At3g14940  | Phosphoepinephrulic acid carboxylase | B    | c4  | 1   |     |
| At4g32400  | Adenylate translocator-like | C    | c7  |     |     |
| At5g54160  | O-Methyltransferase | C    | c11 |     |     |
| At3g02520  | Putative 4-3-3 protein | C    | c8  |     |     |
| At5g22880  | Histone H2B like (emb CA69025.1) | B    | c16 |     |     |
| At1g89850  | Nitrate transporter (NTL1) | C    | c15 |     |     |
| At2g28760  | Putative nucleotide-sugar dehydratase | B    | c8  |     |     |
| At4g29520  | Putative protein | B    | c8  |     |     |
| At1g05460  | Hypothetical protein, 5’ partial | B    | c7  |     |     |
| At2g39420  | Putative phospholipase | B    | c7  |     |     |
| At5g41660  | DNA (cytosine-5)-methyltransferase | A    | c12 |     |     |
| At2g27730  | Glutathione S-transferase (GST6) | C    | c8  |     |     |
| At3g31570  | Putative glutathione peroxidase | C    | c8  |     |     |
| At3g54660  | Protein disulfide-isomerase-like | C    | c7  |     |     |
| At4g23460  | Putative potassium transport protein (TRH1) | C    | c11 | 1   |     |
| At5g06730  | Peroxidase | B    | c8  |     |     |
| At4g01020  | Pome.PID:1439562 | B    | c8  |     |     |
| At4g19420  | Putative pectinacetylesterase | B    | c7  | 1   |     |
| At5g47220  | EREBP 2 | B    | c12 | 1   |     |
| At3g45980  | Histone H2B | B    | c16 |     |     |
| At3g51030  | Thioredoxin | A    | c12 |     |     |
| At4g36640  | Putative protein | C    | c8  | 1   |     |
| At2g45300  | (EPSP) synthase | C    | c8  |     |     |
| At4g34230  | Cinnamyl alcohol dehydrogenase-like | B    | c7  |     |     |
| At3g16530  | Putative lectin | B    | c16 |     |     |
| At1g20690  | HMG1, putative | B    | c16 |     |     |
| At4g34550  | Caffeoyl-CoA O-methyltransferase-like | C    | c12 | 1   |     |
| At1g51950  | Auxin-regulated protein IAA18, putative | B    | c12 |     |     |
| At4g17500  | EREBP1 | B    | c8  |     |     |
| At3g05840  | ASK-GAMMA | B    | c16 |     |     |
| At1g78830  | Hypothetical protein | B    | c16 |     |     |
| At2g48990  | Putative cytochrome P450 | C    | c12 | 1   |     |
| At4g02110  | Unknown protein | B    | c16 | 1   |     |
| At4g39540  | Shikimate kinase-like protein | B    | c8  | 1   |     |
| At2g22250  | Putative asparagine aminotransferase | C    | c12 | 1   |     |
| At1g09430  | Unknown protein | B    | c8  |     |     |
| At2g46350  | Hypothetical protein | C    | c11 | 1   |     |
| At2g44160  | Putative methylenetetrahydrofolate reductase | C    | c7  | 1   |     |
| At2g29420  | Putative glutathione S-transferase | C    | c11 |     |     |
| At5g46640  | Heat shock protein 17.6-II | C    | c12 |     |     |
| At4g24020  | Putative protein | B    | c16 |     |     |
| At4g22770  | Putative DNA-binding protein | C    | c16 |     |     |
| At1g79450  | Hypothetical protein | B    | c4  |     |     |
| At1g21750  | Putative protein-disulfide isomerase precursor | C    | c11 |     |     |
| At1g05250  | S-Adenosylmethionine synthetase | C    | c6  | 1   |     |
| At2g24240  | Putative peroxidase | C    | c11 |     |     |
| At2g24990  | Putative replication protein A1 | A    | c8  |     |     |
| At2g33630  | Putative steroid dehydrogenase | B    | c8  |     |     |
| At4g26910  | Putative dihydrolylipoamide succinyltransferase | C    | c8  |     |     |
| At2g20980  | Hypothetical protein | C    | c7  |     |     |
| At4g27220  | Histone H2A-like protein | B    | c16 |     |     |
| Gene locus     | Description                              | Tree | SOM | E2F | MSA |
|---------------|------------------------------------------|------|-----|-----|-----|
| At1g78380     | Glutathione transferase, putative         | A    | c11 |     |     |
| At2g30490     | Cinnamate-4-hydroxylase                   | C    | e8  |     |     |
| At3g09010     | Putative receptor set                     | B    | c8  |     |     |
| At3g13790     | β-Fructofuranosidase 1                    | C    | c8  |     | 1   |
| At4g21810     | Putative protein                          | D    | c16 |     |     |
| At2g42790     | Putative citrate synthase                 | C    | c15 |     |     |
| At1g21760     | Unknown protein                           | A    | c16 |     | 1   |
| At1g47710     | Serpin, putative                          | B    | c4  |     |     |
| At1g08780     | Histone H1, putative                      | B    | c16 |     |     |
| At1g05470     | Hypothetical protein                      | B    | c4  |     |     |
| At1g30720     | Putative reticuline oxidase-like          | B    | c4  |     |     |
| At3g12500     | Basic chitinase                           | A    | c15 |     |     |
| At4g35520     | Putative protein                          | A    | c7  |     | 1   |
| At2g29570     | PCNA                                      | A    | c11 |     |     |
| At2g35610     | Unknown protein                           | B    | c4  |     |     |
| At2g43020     | Putative amine oxidase                    | B    | c4  |     |     |
| At2g28740     | Histone H4                                | C    | c16 |     |     |
| At4g22140     | Putative protein                          | B    | c4  |     |     |
| At2g42750     | Unknown protein                           | D    | c8  |     |     |
| At4g21810     | Putative protein                          | A    | c8  |     |     |
| At5g26340     | Hexose transporter-like                   | B    | c16 |     |     |
| At2g30490     | Cinnamate-4-hydroxylase                   | C    | c8  |     |     |
| At4g24520     | ATRI                                      | D    | c7  |     |     |
| At4g01700     | Putative chitinase                        | B    | c8  |     |     |
| At2g01680     | Unknown protein                           | C    | c12 |     |     |
| At3g59970     | MTHFRI                                    | C    | c11 |     |     |
| At4g21910     | Putative protein                          | B    | c8  |     |     |
| At2g38860     | Unknown protein                           | B    | c8  |     |     |
| At4g16370     | Isp4 like protein                         | C    | c12 |     |     |
| At1g59740     | Oligopeptide transporter, putative        | C    | c16 |     |     |
| At3g13790     | β-Fructofuranosidase 1                    | C    | c8  |     | 1   |
| At2g38810     | Histone H2A                               | C    | c12 |     |     |
| At2g45330     | Unknown protein                           | A    | c12 |     |     |
| At2g36320     | Unknown protein                           | C    | c15 |     |     |
| At2g45500     | Hypothetical protein                      | B    | c4  |     |     |
| At6g07380     | Putative amidase                          | C    | c11 |     |     |
| At4g03920     | Putative WD-repeat protein                | B    | c11 |     |     |
| At2g22430     | ATRIB                                    | C    | c15 |     |     |
| At2g21710     | Putative chromosome assoc. protein        | C    | c11 |     |     |
| At1g75750     | Unknown protein                           | C    | c15 |     |     |
| At4g17890     | Putative protein                          | B    | c16 |     |     |
| At1g23560     | Hypothetical protein                      | B    | c4  |     |     |
| At5g51440     | IAA-aminio acid hydroxase homolog ILL3    | C    | c16 |     | 1   |
| At2g22420     | Putative peroxidase                       | C    | c11 |     |     |
| At4g35220     | Putative protein                          | C    | c12 |     |     |
| At5g08910     | DnaJ homologue (gb AAB91418.1 )           | A    | c8  |     |     |
| At3g52060     | Putative protein                          | C    | c15 |     |     |
| At2g22480     | Putative pyrophosphate-fructose-6-phantase| A    | c12 |     |     |
| At4g18710     | Shaggy-like protein kinase etha           | C    | c12 |     |     |
| At4g34100     | Putative protein                          | C    | c12 |     |     |
| At4g21070     | Putative protein (fragment)               | B    | c16 |     |     |
| At5g05730     | Anthranilate synthase component I-1      | B    | c4  |     |     |
| At4g17900     | Putative protein                          | B    | c4  |     | 1   |
| At2g24470     | Unknown protein                           | A    | c8  |     |     |
| At2g37110     | Unknown protein                           | C    | c12 |     |     |
| At2g02390     | Putative glutathione S-transferase        | B    | c4  |     |     |
| At4g14630     | Germin precursor oxalate oxidase          | B    | c8  |     |     |
| At2g27190     | Purple acid phosphatase precursor         | C    | c15 |     |     |
| At1g53540     | 17.6-kDa heat shock protein (AA 1–156)   | C    | c8  |     |     |
| At3g29200     | Chorismate mutase                         | B    | c4  |     |     |
| At2g29500     | Putative small heat shock protein         | A    | c12 |     |     |
| At4g21070     | Putative protein (fragment)               | D    | c16 |     |     |
| At1g60420     | Unknown protein                           | B    | c4  |     |     |
| At5g19530     | Spermine synthase (ACL5)                  | A    | c4  |     |     |
| At5g47040     | Mitochondrial Lon protease homolog 1      | B    | c7  |     | 1   |
| At2g33700     | Putative protein phosphatase 2C           | B    | c4  |     |     |
| At4g00300     | Awaiting functional assignment            | B    | c4  |     |     |
| At4g15070     | Hypothetical protein                      | C    | c15 |     |     |
| At2g48340     | Putative photomorphogenesis repressor     | D    | c15 |     | 1   |
| At2g13870     | Root hair-defective 3 (RHD3)              | B    | c8  |     |     |
| At4g32160     | Putative protein                          | D    | c4  |     | 1   |
| At1g55530     | Putative protein                          | B    | c4  |     |     |
| At3g52850     | Spot 3 protein and vacular sorting        | A    | c15 |     | 1   |
| At2g22860     | Unknown protein                           | B    | c4  |     |     |
| At2g39550     | Putative geranylgeranyl transferase type  | B    | c4  |     |     |
| At1g54110     | Unknown protein                           | B    | c4  |     |     |
| At3g50780     | Putative protein                          | C    | c11 |     |     |
| At2g27650     | Putative SCARECROW gene regulator         | A    | c7  |     | 1   |
| At2g41790     | Putative zinc protease                    | B    | c4  |     |     |
bust assignment to clusters. All histone signals are within branches A–C of the hierarchical tree. Interestingly, the only two H2A genes are both assigned to cluster 12, suggesting differential regulation compared with other histones. In contrast, CDC6, also previously reported as S phase-regulated (16, 64), shows clearly different expression in branch D and cluster 7, indicating that it is down-regulated in mitosis and up-regulated during G1 of the second cycle.

Mitotic cyclins of both A and B classes are primarily expressed during G2 and M in Arabidopsis and in other plants (31, 32, 65). Nine mitotic cyclins are present on the chip, of which all nine are detected as expressed, and eight gene signals (representing seven distinct genes) are defined as cell cycle-regulated in this experiment. All fall into branch E and the very similar cluster 9, 13, or 14. Expression of CDKB1 and CDKB2 also peak in mitosis (Fig. 1D) (22), with CDKB1 showing earlier expression (branch D, cluster 15) than CDKB2 (branch E, cluster 13), which is thus co-regulated with mitotic cyclins. The robust identification of mitosis-specific genes validates the synchrony of the culture used, despite the relatively low metaphase/anaphase index recorded for the reasons discussed above.

Novel Regulated Genes—The genes identified as regulated fall into a wide range of cellular processes as defined by MIPS based on collapsed automatically derived functional categories (available on the World Wide Web at mips.gsf.de/proj/thal/db/ tables/tables_func_frame.html; Fig. 5). Genes that are highest expressed at the time of aphidicolin removal (t/H11005) are grouped in sub-branch A (31 genes). The application of aphidicolin for 24 h and the treatment of cells with fresh medium during washing, are likely to induce stress responses. It is therefore not surprising that we identify potential stress-associated genes within this cluster including chitinases, peroxidase, glutathione transferase, proteolysis (F-box protein, serine carboxypeptidase), and heat shock-related proteins (see Tables I–IV for details). Nevertheless, we also observe expression of genes likely to be involved in S phase, such as histone H2A.F/Z already known to be cell cycle-regulated at the G1/S boundary.

| Gene locus   | Description                           | Tree | SOM | E2F | MSA |
|--------------|---------------------------------------|------|-----|-----|-----|
| At5g39950    | Thioredoxin                           | C    | c12 |     |     |
| At2g32720    | Putative cytochrome b$_{5}$           | C    | c12 |     |     |
| At1g23020    | Putative NADPH oxidase                | C    | c12 |     |     |
| At1g59820    | Chromaffin granule ATPase II homolog  | C    | c11 |     |     |
| At2g71630    | Putative SET-domain transcript regulator | B   | c4  |     |     |
| At1g10410    | Unknown protein                       | B    | c4  |     |     |
| At1g20690    | High mobility group protein, putative | B    | c16 |     |     |
| At2g91310    | Unknown protein                       | C    | c8  | 2   |     |
| At1g05530    | Unknown protein                       | C    | e8  |     |     |
| At2g47130    | Putative alcohol dehydrogenase        | B    | e8  |     |     |
| At1g09560    | Germin-like protein                   | B    | e8  | 1   |     |
| At4g16660    | HSP-like protein                      | C    | c7  |     |     |
| At2g31570    | Putative glutathione peroxidase       | C    | c12 |     |     |
| At5g40780    | Glucose-6-phosphate dehydrogenase     | C    | c8  |     |     |
| At1g67480    | Unknown protein                       | D    | c15 |     |     |
| At2g17720    | Putative prolly 4-hydroxylase, α subunit | B   | e8  |     |     |
| At1g55920    | Serine acetyltransferase              | A    | c12 |     |     |
| At1g20690    | High mobility group protein, putative | B    | c8  |     |     |
| At4g02050    | Putative hexose transporter           | B    | c16 |     |     |
| At5g44790    | ATP-dependent copper transporter      | C    | c16 |     |     |
| At2g44790    | Phytocyanin                           | B    | c4  | 1   |     |
| At5g17980    | Anthranilate phosphoribosyltransferase| B    | c13 |     |     |
| At2g30140    | Putative glucosyltransferase          | B    | c12 |     |     |
| At3g49120    | Peroxidase                            | B    | c8  | 2   |     |
| At4g38540    | Monoxygenase 2 (MO2)                  | B    | c16 |     |     |
| At3g51030    | Thioredoxin h                         | A    | c12 |     |     |
| At4g38140    | Putative disease resistance protein   | B    | c4  |     |     |
| At1g09200    | Histone H3                            | C    | c16 |     |     |
| At4g35110    | Putative protein                      | B    | e8  |     |     |
| At2g27350    | Unknown protein                       | C    | c16 | 1   |     |
| At5g63570    | Glutamate-1-semialdehyde2,1-aminomut, 1| B   | c4  |     |     |
| At1g70250    | Receptor serine                       | B    | c4  |     |     |
| At4g11600    | Phospholipid hydroperoxide glutathione peroxide | A | c16 |     |     |
| At2g45290    | Putative transketolase precursor      | C    | c12 | 1   |     |
| At2g36310    | Hypothetical protein                  | A    | c11 |     |     |
| At2g37520    | Unknown protein                       | B    | c7  | 1   |     |
| At2g34970    | Putative translation initiation factor cIF-2B | C | c11 |     |     |
| At2g33530    | Putative serine carboxypeptidase II   | A    | c16 |     |     |
| At4g39270    | Receptor protein kinase-like protein  | B    | c4  |     |     |
| At1g77220    | Unknown protein                       | A    | c11 |     |     |
| At2g27200    | Putative nucleotide-binding protein   | D    | e4  |     |     |
| At4g02390    | NAD+ ADP-riboosyltransferase          | C    | c16 |     |     |
| At5g39950    | Thioredoxin                           | C    | c12 |     |     |
| At2g37480    | Unknown protein                       | A    | c4  |     |     |
| At4g16850    | Growth regulator-like protein         | C    | c11 |     |     |
| At1g74310    | Heat shock protein 101                | B    | c8  |     |     |
| At3g08760    | Putative protein kinase               | D    | c4  |     |     |
| At4g01870    | Predicted protein of unknown function | B    | c4  |     |     |
| At3g12500    | Basic chitinase                       | A    | c15 |     |     |
| At2g43260    | Hypothetical protein                  | C    | c11 |     |     |
| At2g34400    | Hypothetical protein                  | B    | c12 |     |     |
| At4g39230    | NAD(P)H oxidoreduct isoflavonereduct-like | B | c4  |     |     |
| At5g10180    | Sulfate transporter                   | C    | c15 |     |     |
| At4g27910    | Putative protein                      | B    | c12 | 1   |     |
in Arabidopsis suspension cultures (29), proliferating cell nuclear antigen (cluster 11), and a DNA cytosine methyltransferase (cluster 12), which these results suggest are regulated genes. In sub-branch B, a large group of genes (147 genes) is found showing a peak of expression at 2 h after the block is released, corresponding to early to mid-S phase, including several genes involved in DNA metabolism and replication such as histones, a CDC50 homologue, and FAS1, which shows strong periodic regulation in cluster 4 (67). Also in node B/cluster 4 are found the mitogen-activated protein kinase AtMPK6, which is known to be involved in signaling of abiotic stress (68), as well as the mitogen-activated protein kinase kinase AtMPK2. In addition, a large group of genes are annotated as oxidative stress-responsive genes, such as peroxidases (3), chitinases (4), glutathione transferases (six total, all in nodes B/C), superoxide dismutase, and ethylene-responsive element-binding factors (2).

Within the next node (C), 91 genes are clustered, which are highly expressed over multiple time points mainly in S phase including further histone genes. A few genes in sub-branches C and D show high expression in S phase (2–6 h) and then decrease, with higher expression again seen in the last experiment after 19 h. This expression profile is found, for example, for the CDC6 gene, which is specifically expressed in G1 and S phases (16, 64), and the mitogen-activated protein kinase kinase kinase kinase ATN1. ATN1 is related to mammalian transforming protein kinases, but no biological role is known in plants (68). Two casein kinase I genes are also found in clusters B and C.

In node E, 102 genes are grouped together, which are assigned as M phase-specific, belonging to clusters 9, 10, 13, and 14. In addition to mitotic cyclins and CDKs (see above), the Arabidopsis homolog of budding yeast CDC20 is within this node and cluster 13 and is one of the most highly regulated genes detected. CDC20 is one of two proteins required to activate the anaphase-promoting complex. Other genes with clear mitotic associations are three genes for kinesin heavy chain, two kinesin-like potential spindle proteins (At2g28620, At4g14330), a homolog of an extragenic suppressor of bin1D61 involved in chromosome structure and segregation (69), a homolog of human TOG that targets CDK activity to microtubules in mitosis (70), a fimbrial involved in F-actin filament cross-linking (71), and two helicases. Putative regulatory proteins include a protein phosphatase 2C (At2g30020), MYB70, an AP2 domain protein (At3g16280), and an FCA-like protein (At2g47310).

Node F includes 71 gene signals whose expression peaks at the M/G1 boundary, and node G includes a further 27 genes expressed during G1 phase. Some genes in these nodes are also expressed in the early stages of the experiment and are hence in clusters 1 and 2. Notable in node F are the very highly regulated histidine kinases (HKs) encoding the cytokinin receptor CRE1 (AtHK4) and the osmosensor AthK1 as well as the response regulators ARR4 and ARR7. Two MCM proteins required for prereplication complex assembly, CDC21 (72) and MCM5, which has an E2F site in its promoter as does human MCM5 (73), are both expressed at this time in cluster 5, as is the DNA mismatch repair protein MSH2, which associates with p53 in S phase in mammalian cells (74). In node G, the SKP1 homolog At2g03160 is found in cluster 3, indicating a G1/S expression pattern in both cycles. SKP1 functions as part of the SCF complex and regulates the destruction of G1 cell cycle regulators at the onset of S phase. The D-type cyclin CYCD4;1 (75) not previously known to be expressed or regulated in cell suspension cultures is also found in this node.

**Links to Other Cellular Processes**—In addition to processes likely to be cell cycle-regulated based on studies in other organisms, the data hold clues to novel plant-specific processes that may be integrated with cell division control.

Plants coordinate nuclear division with mitochondrial and plastid duplication and segregation. Two genes related to yeast ABF2, a high mobility group protein involved in mitochondrial DNA segregation (76), both have very strong regulation in the mitosis peak node E/cluster 13. Since both ABF2 homologs (At4g23800, At4g11080) are predicted to be plastid-targeted (data not shown), this could provide a link between nuclear and plastid division. Moreover, the only gene in Arabidopsis for organelle methionyl-tRNA synthetase that provides both mitochondrial and chloroplastic activity (77) is also regulated in node E/cluster 5, suggesting a link to organelle protein synthesis.

The data set includes a number of genes suggesting links to hormone perception, biosynthesis, and response, including cytokinin, brassinosteroids, auxin, ethylene, and jasmonate. One of the most highly regulated genes detected is the cytokinin receptor AtHK4 (CRE1, WOL1), which shows strong periodic expression in G1 (node F, cluster 1). It is thus expressed in early time points, decreases, and then increases in later time points. Interestingly, ATHK4 is co-regulated in the cell cycle with its downstream transcriptional targets ARR4 and ARR7, negative regulators of the cytokinin response presumably involved in a feedback mechanism (78, 79). Since ARR4 is also linked to phytochrome and light signaling, it could provide a link between cytokinin, other signals, and cell cycle (79, 80). The coordinate response of AtHK4, ARR4, and ARR7 cytokinin regulatory genes is consistent with the requirement for cytokinins for the G1/S transition through the regulation of CYCD3;1 expression (81) and suggests that roles for cytokinin at the G2/M transition (82) may be regulated by different genes.

**Dwarf1** encodes the enzyme that converts 24-methylenecholesterol to campesterol in brassinosteroid biosynthesis and is found in node F, cluster 5 with a G1 maximum. Brassinosteroid controls both cell growth and cell division (83) and regulates expression of the D-type cyclin CYCD3;1 (84, 85), and the up-regulation of DWARF1 during G1 phase suggests a mechanism by which this may be mediated.

Auxin is essential for cell division and cell cycle progression (86–88), although rather little is known of its precise molecular interaction with the cell cycle. The auxin-induced transcript-
### TABLE III

Potential cell cycle-regulated genes showing significant fluctuation and a peak in expression in M phase

| Gene locus  | Description                                      | Tree | SOM | E2F | MSA |
|------------|--------------------------------------------------|------|-----|-----|-----|
| At5g38410  | Ribulose bisphosphate carboxylase small           | E    | c9  |     |     |
| At3g23890  | Topoisomerase II                                   | E    | c13 | 1   |     |
| At5g08150  | Mitosis-specific cyclin 1b                        | E    | c13 | 2   |     |
| At1g18250  | Pathogenesis-related group 5 protein, putative    | E    | c13 |     |     |
| At2g26760  | Putative cyclin                                   | E    | c13 | 2   |     |
| At1g03780  | Hypothetical protein                              | E    | c9  | 1   |     |
| At4g32380  | WD-repeat protein-like protein                     | E    | c13 | 1   |     |
| At4g38230  | Putative protein                                  | E    | c13 |     |     |
| At3g48230  | Cytochrome P450-like protein                      | F    | c5  |     |     |
| At4g15570  | SEN1-like protein                                 | E    | c13 |     |     |
| At2g26180  | Putative SF16 protein (Helianthus annuus)         | E    | c14 | 1   |     |
| At4g03100  | Putative rac GTPase-activating protein             | E    | c13 |     |     |
| At4g23800  | 98b-like protein                                  | E    | c13 |     |     |
| At4g35620  | Cyclin 2b protein                                 | E    | c9  | 1   |     |
| At1g04250  | Putative auxin-induced protein, IAA17             | F    | c5  |     |     |
| At1g23790  | Hypothetical protein                              | E    | c13 |     |     |
| At1g20620  | Hypothetical protein                              | E    | c9  |     |     |
| At4g18140  | Putative protein                                  | E    | c13 |     |     |
| At2g25060  | Similar to early nodulins                         | E    | c13 | 1   | 2   |
| At1g77390  | Mitotic cyclin a2-type, putative                  | E    | c13 | 2   |     |
| At2g25060  | Similar to early nodulins                         | E    | c13 | 1   | 2   |
| At5g51280  | MS5-like protein                                  | E    | c13 |     |     |
| At1g01620  | Plasma membrane intrinsic protein 1c, putative    | E    | c13 | 1   |     |
| At4g35730  | Putative protein                                  | E    | c13 |     |     |
| At2g20590  | Hypothetical protein                              | E    | c14 | 1   |     |
| At1g09680  | Unknown protein                                   | E    | c9  | 1   |     |
| At1g03470  | Hypothetical protein                              | E    | c14 |     |     |
| At1g04680  | Putative pectate lyase A11                        | F    | c5  | 1   |     |
| At2g42960  | Putative protein kinase                           | G    | c9  |     |     |
| At2g46890  | Putative auxin-regulated protein                  | E    | c14 | 1   |     |
| At2g02250  | Lectin-like protein                               | F    | c1  |     |     |
| At5g01600  | Ferritin 1 precursor                              | E    | c13 | 1   |     |
| At4g17980  | NAM (no apical meristem)-like protein             | F    | c9  |     |     |
| At4g37570  | Phosphoenolpyruvate carboxykinase (ATP)-like      | E    | c9  |     |     |
| At4g36360  | β-Galactosidase-like protein                      | F    | c5  |     |     |
| At1g15540  | Unknown protein                                   | E    | c9  | 1   |     |
| At3g09390  | Metallothionein-like protein                      | E    | c14 |     |     |
| At1g77490  | Thylakoid-bound ascorbate peroxidase              | E    | c10 |     |     |
| At4g18560  | Hypothetical protein                              | E    | c14 |     |     |
| At4g27280  | Putative protein                                  | F    | c10 |     |     |
| At3g47840  | Putative protein                                  | E    | c14 |     |     |
| At4g01730  | Hypothetical protein                              | E    | c14 |     |     |
| At2g21330  | Putative fructose bisphosphate aldolase           | F    | c9  |     |     |
| At4g39090  | Drought-inducible cysteine proteinase RD19A        | F    | c1 |     |     |
| At4g29940  | Pathogenesis related homodomain protein (PRHA)    | F    | c9  |     |     |
| At2g35550  | Hypothetical protein                              | F    | c5  |     |     |
| At3g46090  | Zinc finger protein ZAT7                          | G    | c9  |     |     |
| At2g25350  | Unknown protein                                   | F    | c9  |     |     |
| At4g12800  | Probable photosystem I chain XI precursor         | E    | c10 |     |     |
| At4g24780  | Putative pectate lyase                            | F    | c5  |     |     |
| At2g39220  | Similar to latex allergen from Hevea brasiliensis | F    | c5  |     |     |
| At5g66570  | 33-kDa polypeptide of oxygen-evolving complex     | E    | c9  |     |     |
| At4g00430  | Probable plasma membrane intrinsic protein 1c     | E    | c14 |     |     |
| At3g49120  | Peroxidase                                        | E    | c10 | 2   |     |
| At5g01530  | Chlorophyll a                                     | E    | c9  | 1   |     |
| At2g21300  | Putative kinesin heavy chain                      | E    | c9  |     |     |
| At4g11080  | 98b-like protein                                  | E    | c13 |     |     |
| At2g35740  | Putative sugar transporter                        | E    | c10 |     |     |
| At2g41800  | Unknown protein                                   | F    | c5  | 1   |     |
| At2g28620  | Putative kinesin-like spindle protein             | E    | c13 |     |     |
| At4g14330  | Kinesin like protein                              | E    | c14 |     |     |
| At5g04590  | Sulfitreductase                                    | F    | c5  | 1   |     |
| At4g33120  | Putative protein                                  | E    | c13 |     |     |
| At4g22860  | Hypothetical protein                              | E    | c13 |     |     |
| At2g03090  | Putative expansin                                 | F    | c5  |     |     |
| At2g25880  | Putative protein kinase                           | E    | c13 |     |     |
| At3g48280  | Cytochrome P450-like protein                      | F    | c5  |     |     |
| At4g13460  | Putative protein                                  | E    | c13 |     |     |
| At4g22120  | Putative protein                                  | E    | c13 | 1   |     |
| At2g18520  | Unknown protein                                   | E    | c13 |     |     |
| At4g37330  | Cytochrome P450-like protein                      | E    | c13 |     |     |
| At4g02800  | Hypothetical protein                              | E    | c13 | 1   |     |
| At1g70210  | Cyclin, putative                                  | E    | c14 |     |     |
| At1g04030  | Unknown protein                                   | E    | c13 | 1   |     |
| At2g47500  | Putative kinesin heavy chain                      | E    | c13 | 1   |     |
| At2g40300  | Putative ferritin                                 | F    | c5  | 1   |     |
| At3g48280  | Cytochrome P450-like protein                      | E    | c5  |     |     |
| At3g48280  | Putative AP2 domain transcription factor           | E    | c13 | 1   |     |
regulator AXR3 (IAA17) (89) is strongly regulated (node F, cluster 5), showing continuously up-regulated expression, whereas IAA18 shows an S phase peak (B/12). A further putative auxin-regulated gene shows a mitotic peak, suggesting that differential regulation of auxin response genes could explain some of auxin’s multiple effects on all stages of the cycle.

Allene-oxide synthase is critical for the biosynthesis of all biologically active jasmonates, which is involved in wound and other pathogen responses and blocks cell cycle progression during G1 (90). Duplicated gene signals for allene-oxide synthase show both in node G and the similar clusters 1 and 6, suggesting possible roles in G1 control.

A unique aspect of cell division in plants is close coordination required between cell wall synthesis and the cell cycle. It is therefore interesting to note at least 23 genes identified that have known or putative links to cell wall or biosynthesis of cell wall components that are found in several clusters. For example, expansins are a group of extracellular proteins that directly modify the mechanical properties of plant cell walls, leading to turgor-driven cell extension (91). Three expansin genes are detected as regulated, of which two are expressed in G1 (F, 5), whereas the third is expressed in both S and G1 phases (B, 2). Extensins are abundant proteins presumed to determine physical characteristics of the plant cell wall, and expression of one is found with a G1 peak (G, 3).

**TABLE III—continued**

| Gene locus | Description | Tree | SOM | E2F | MSA |
|-----------|-------------|------|-----|-----|-----|
| At2g40230 | Putative anthranilate N-hydroxycinnamoyl | E    | c13 |     |     |
| At1g31970 | p68 RNA helicase, putative | E    | c10 |     |     |
| At1g16330 | Putative mitotic cyclin | E    | c9  |     |     |
| At1g55490 | Rubisco subunit binding-protein θ subunit | E    | c9  | 1   |     |
| At2g17620 | Putative cyclin 2 | E    | c13 | 1   | 1   |
| At2g33110 | Putative protein | E    | c13 |     |     |
| At2g22610 | Putative kinesin heavy chain | E    | c13 |     |     |
| At1g52110 | Putative protein | E    | c4  |     |     |
| At2g26570 | Phosphate transporter, putative | E    | c14 | 1   |     |
| At2g07690 | Putative DNA replication licensing factor, Mem5 | F    | c5  | 1   |     |
| At4g33400 | Dem-like protein | E    | c14 | 2   |     |
| At4g16020 | Probable ribosomal protein | E    | c5  |     |     |
| At2g24990 | Similar to extragenic suppressor of binD6 mutation | E    | c10 |     |     |
| At2g53880 | Hypothetical protein | E    | c13 |     |     |
| At2g16440 | Putative CDC21 protein | F    | c5  |     |     |
| At2g17620 | Putative cyclin 2 | E    | c13 | 1   | 1   |
| At2g35630 | Similar to ch-TOG protein from Homo sapiens | E    | c14 |     |     |
| At1g73550 | Hypothetical protein | E    | c14 |     |     |
| At2g48420 | Unknown protein | E    | c14 |     |     |
| At5g02500 | DnaK-type molecular chaperone hsc70.1 | F    | c1  |     |     |
| At1g47500 | DNA binding protein, putative | E    | c10 | 1   |     |
| At2g44540 | Putative ethylene response element-binding protein | E    | c9  |     |     |
| At4g37110 | Putative protein | E    | c13 |     |     |
| At2g11910 | Unknown protein | E    | c9  |     |     |
| At1g77050 | RNA helicase, putative | F    | c10 |     |     |
| At3g47860 | Putative peptide transporter | F    | c1  |     |     |
| At2g01290 | Putative ribose 5-phosphate isomerase | E    | c10 | 1   |     |
| At3g54890 | Chlorophyll A | E    | c9  |     |     |
| At2g38750 | Putative annexin | F    | c1  |     |     |
| At5g48460 | Fimbrin 2 (gb AAB97844.1) | F    | c14 |     |     |
| At1g26800 | Hypothetical protein | D    | c1  |     |     |
| At1g20930 | Putative cell division control protein, Cdc2 kinase | E    | c13 | 1   |     |
| At2g40330 | Unknown protein | D    | c14 |     |     |
| At4g39530 | Putative protein | E    | c13 | 1   |     |
| At2g47310 | Putative PCA-related protein | E    | c14 |     |     |
| At4g06620 | Putative tetrahydrofolate synthase | E    | c10 | 1   |     |
| At1g74470 | Geranylgeranyl reductase | E    | c14 | 1   |     |
| At1g15820 | Chlorophyll-binding protein, putative | E    | c10 | 1   |     |
| At2g20800 | Putative NADH-ubiquinone oxireductase | F    | c5  |     |     |
| At3g50060 | R2R3-MYB transcription factor | F    | c1  | 1   |     |
| At1g94210 | Unknown protein | E    | c14 |     |     |
| At4g12420 | Putative pollen-specific protein | F    | c9  |     |     |
| At2g19090 | Hypothetical protein | E    | c14 |     |     |
| At4g33570 | Putative protein | E    | c14 | 1   |     |
| At4g02150 | AtKAP α | E    | c13 |     |     |
| At5g23300 | Dihydroorotate dehydrogenase precursor | E    | c10 |     |     |
| At4g19710 | Aspartate kinase-homoserine dehydrogenase-like protein | E    | c9  |     |     |
| At4g31290 | Predicted protein | F    | c9  |     |     |
| At5g25380 | Cyclin 3a | E    | c14 |     |     |
| At2g02020 | Putative peptide | E    | c10 |     |     |
| At2g30020 | Putative protein phosphatase 2C | E    | c13 |     |     |
| At2g23290 | Putative MYB family transcription factor | E    | c13 |     |     |
| At3g56940 | Leucine zipper-containing protein AT103 | E    | c10 |     |     |
| At1g42970 | Glyceraldehyde-3-phosphate dehydrogenase | E    | c10 |     |     |
| At2g44630 | Hypothetical protein | E    | c10 |     |     |
| At4g25730 | Putative protein | E    | c10 |     |     |
| At4g38680 | Glycine-rich protein 2 (GRP2) | F    | c9  |     |     |
### TABLE IV
Potential cell cycle-regulated genes showing significant fluctuation and a peak in expression in G1 phase

| Gene locus   | Description                                      | Tree | SOM | E2F | MSA |
|-------------|--------------------------------------------------|------|-----|-----|-----|
| At2g01830   | Putative histidine kinase                        | F    | c1  |     |     |
| At2g04100   | Hypothetical protein                             | B    | c2  | 1   |     |
| At1g70710   | Endo-1,4-β-glucanase                             | F    | c1  |     |     |
| At2g17820   | Putative histidine kinase                        | F    | c1  |     |     |
| At2g02810   | Unknown protein                                  | B    | c3  |     |     |
| At2g25110   | Unknown protein                                  | C    | c7  |     |     |
| At4g38940   | Putative protein                                 | F    | c6  |     |     |
| At2g04100   | Hypothetical protein                             | B    | c3  |     |     |
| At2g17500   | Unknown protein                                  | B    | c2  |     |     |
| At2g17120   | Predicted GPI-anchored protein                   | B    | c3  |     |     |
| At1g14010   | Unknown protein                                  | B    | c7  | 1   |     |
| At4g39980   | 2-Dehydro-3-deoxyphosphoheptonate aldolase       | B    | c3  |     |     |
| At1g01530   | MADS-box transcription factor, putative          | F    | c6  |     |     |
| At2g40300   | Putative ferritin                                | F    | c5  |     |     |
| At2g30810   | Putative gibberellin-regulated protein           | F    | c1  |     |     |
| At2g44160   | Putative methylenetetrahydrofolate reductase     | C    | c7  |     |     |
| At1g67110   | Cytochrome P450, putative                        | F    | c1  |     |     |
| At1g05520   | Transport protein, putative                      | F    | c3  |     |     |
| At2g44460   | Putative β-glucosidase                           | B    | c2  |     |     |
| At2g47000   | Putative ABC transporter                         | G    | c2  |     |     |
| At1g71695   | Peroxidase ATP4a                                  | B    | c3  |     |     |
| At4g16150   | Transcription factor-like protein                | F    | c2  |     |     |
| At1g18890   | Calcium-dependent protein kinase, putative       | B    | c3  |     |     |
| At2g29120   | Putative ligand-gated ion channel protein        | B    | c2  |     |     |
| At5g42650   | Allene oxide synthase (emb CAA73184.1)           | G    | c1  |     |     |
| At2g31410   | Unknown protein                                  | E    | c5  |     |     |
| At1g54580   | Acyl-carrier protein (ACP), putative             | F    | c5  |     |     |
| At1g70410   | Carbonic anhydrase, putative                     | B    | c3  |     |     |
| At4g28540   | Protein kinase ADK1-like protein                 | B    | c3  |     |     |
| At2g28830   | Hypothetical protein                             | B    | c1  |     |     |
| At2g20370   | Unknown protein                                  | D    | c1  |     |     |
| At1g08830   | Superoxide dismutase                             | B    | c2  |     |     |
| At1g23800   | Putative aldehyde dehydrogenase                  | A    | c7  |     |     |
| At4g20840   | Reticuline oxidase-like protein                  | G    | c7  |     |     |
| At3g01540   | RNA helicase, DRH1                                | D    | c1  |     |     |
| At1g01470   | Hypothetical protein                             | B    | c3  | 1   |     |
| At2g39700   | Putative expansin                                | B    | c2  |     |     |
| At1g65530   | Hypothetical protein                             | G    | c2  |     |     |
| At4g22470   | Extensin-like protein                            | G    | c3  |     |     |
| At3g48340   | Cysteine endopeptidase-like                      | G    | c1  |     |     |
| At4g36210   | Putative protein                                 | F    | c2  | 1   |     |
| At1g01480   | 1-Aminocyclopropane-1-carboxylate synthase (ACC2)| B    | c2  |     |     |
| At4g21120   | Amino acid transport protein AAT1                | B    | c3  |     |     |
| At2g39710   | Unknown protein                                  | F    | c5  |     |     |
| At2g25970   | Unknown protein                                  | G    | c6  |     |     |
| At4g17490   | Ethylene responsive element binding-like         | F    | c1  |     |     |
| At2g17130   | Putative NAD⁺ dependent isocitrate dehydrogenase| D    | c8  |     |     |
| At1g78780   | Hypothetical protein                             | G    | c6  | 1   |     |
| At2g25670   | Unknown protein                                  | D    | c2  | 3   |     |
| At4g39980   | 2-Dehydro-3-deoxyphosphoheptonate aldolase       | B    | c2  |     |     |
| At1g03020   | Putative glutaredoxin                            | G    | c7  |     |     |
| At2g43790   | MAP kinase (ATMPK6)                              | B    | c4  |     |     |
| At4g27110   | DNA-directed RNA polymerase (EC 2.7.7.6) II      | F    | c6  |     |     |
| At1g01480   | 1-Aminocyclopropane-1-carboxylate synthase (ACC2)| B    | c2  |     |     |
| At1g01070   | Putative response regulator 3                    | F    | c2  | 1   |     |
| At4g30100   | Putative protein                                 | F    | c1  |     |     |
| At2g45740   | Unknown protein                                  | G    | c7  |     |     |
| At4g16830   | Nuclear antigen homolog                          | C    | c7  |     |     |
| At1g07480   | Transcription factor HA large subunit            | B    | c3  | 2   |     |
| At4g13710   | Putative pectate lyase A11                       | F    | c5  |     |     |
| At2g28950   | Expansion AtEx6                                  | F    | c5  |     |     |
| At5g65420   | D-type cyclin (emb CAB41347.1)                   | G    | c6  |     |     |
| At4g27070   | Tryptophan synthase β-subunit (TSB2)             | F    | c6  |     |     |
| At4g25690   | Hypothetical protein                             | D    | c3  |     |     |
| At2g26920   | Unknown protein                                  | B    | c3  |     |     |
| At1g30750   | Unknown protein                                  | B    | c3  |     |     |
| At2g33600   | Putative cinnamoyl-CoA reductase                 | F    | c2  | 1   |     |
| At1g21450   | Scarecrow-like 1                                 | B    | c3  | 1   |     |
| At4g19050   | Putative protein                                 | B    | c7  |     |     |
| At4g31820   | Putative protein                                 | F    | c2  |     |     |
| At1g30040   | Unknown protein                                  | B    | c2  |     |     |
| At3g50930   | BCS1 protein-like protein                        | B    | c2  |     |     |
| At3g46920   | Protein kinase-like protein                      | D    | c1  | 1   |     |
| At4g02330   | Awaiting functional assignment                    | F    | c5  |     |     |
| At3g19820   | Cell elongation protein, Dwarf1                  | F    | c5  | 1   |     |
| At2g29060   | Putative SCARECROW gene regulator               | B    | c2  |     |     |
| At4g32250   | Putative protein kinase                          | B    | c3  |     |     |
| At2g03180   | Putative kinetochore (Skplp-like) protein        | G    | c3  |     |     |
(93), both present on the array, the latter being represented by duplicate oligonucleotide sets. Both MTHFR genes are found in node C and in the closely related periodic clusters 7 and 11, which show a sharp decrease in mitosis. Both genes also carry E2F sites in their upstream regions. These results suggest that MTHFR transcription is S phase-regulated in Arabidopsis. Since lignin biosynthesis is a major utilizer of methionine via S-adenosyl methionine (94), this may be linked to cell wall synthesis or alternatively reflect the control of methionine pools for protein synthesis.

A number of genes identified provide clues to possible links with developmental and differentiation processes through genes previously identified as having developmental phenotypes when mutated. These include genes for an Argonaute (AGO1)-like protein (F, 5) possibly involved in RNA turnover processes (95), a homolog of tomato DEM1 (defective embryo and meristem) (96), which is mitosis-regulated and FCA-related (E, 14), and a NAM (no apical meristem)-like protein (F, 9) (97) as well as four scarecrow-like transcription factors (98), three of which are in nodes A or B.

**Cell Cycle-regulated Promoter Elements—** Three main groups of regulatory elements have been described in plants that control cell cycle expression. E2F binding sites regulate expression by binding to activating or inhibitory E2F factors, which are themselves regulated by the recruitment of hypophosphorylated Rb to E2F sites, which inactivates expression (99). Phosphorylation of Rb in late G1 results in activation of E2F-regulated genes including ribonucleotide reductase (100) and CDC6 (16, 64). Expression of mitotic cyclins has been shown to depend on specific elements conferring mitotic-specific activation.
in tobacco and Arabidopsis (61), whereas histone gene expression depends on octamer (Oct) and hexamer (Hex) elements (59).

We searched the regions 1 kb upstream of each open reading frame within the regulated gene set for the E2F (TTTYYCGYY), mitotic-specific activation (YCYAACGGYY), Oct (CGCGGATC), and Hex (CCACGTCA) consensus sequences. Oct and Hex sequences were found in only a few genes, mostly histones. The relatively loose E2F and mitotic-specific activation consensus sequences identified a relatively large number of genes, not all of which are likely to be regulated by these factors. However, when we examined the distribution of detected sites between different clusters (Table V), we found that clusters contained either relatively few or relatively frequent sites. In all cases except cluster 13, the clusters with frequent E2F sites are distinct from those with larger numbers of genes with mitotic-specific activation sites, suggesting that these generally confer regulation at different times in the cell cycle. Cluster 13 represents mitotic peaking genes and may suggest a role for E2F in regulating expression of genes peaking in G2/mitosis as previously found for mammalian E2F-regulated genes (39).

**DISCUSSION**

Here we present for the first time the results of a wide scale analysis of regulated gene expression in a plant cell cycle synchronized culture. The results demonstrate that a large number of plant genes are likely to show cell cycle-dependent regulation of their expression. The identified genes are involved in a wide range of cellular processes including cell cycle control, cytoskeleton, transcription, proteolysis, phosphorylation, signal transduction, biosynthesis, carbon and amino acid metabolism, hormone response, and organelle function (Fig. 5 and Tables I–IV).

Shedden and Cooper (49) have shown that microarray analysis is prone to random fluctuations, which can be interpreted as consistent regulation. We show that the data from this experiment show significantly greater regulation than a control randomized data set. We have applied statistical analysis to identify 463 genes among 4010 passing initial filters with a high probability of showing significant regulation (p < 0.05), and over 200 of these are significant (p < 0.01).

Shedden and Cooper (49, 66) have also criticized analysis of cell cycle expression because of the perturbations caused by synchronization methods. It is clear that the synchronization carried out here does indeed cause induction of some stress-related genes. However, the procedure was developed to minimize stress, and indeed Arabidopsis cells readily arrest division. Moreover, we have identified almost all Arabidopsis genes previously known to be cell cycle-regulated, including genes whose cell cycle regulation has been demonstrated in situ hybridization and are therefore independent of synchronization procedures (30). A large number of genes also fall into clusters not consistent with a simple stress response due to their periodic response to cell cycle position, and in particular clusters indicative of roles in G2/M or G1/S processes.

It is interesting to note that, compared with the analysis in mammalian cells, we identify large numbers of genes regulated during G1 phase (136 genes). This may reflect a greater role for transcriptional control in G1 in plants. It is also likely that G1 control in plants must integrate a larger number of potential
signals due to the multiple developmental and environmental influences on commitment to cell division.

We conclude that the analysis has not only successfully identified known cell-cycle-regulated genes but also identified as cell-cycle-regulated a number of other genes involved in cell cycle progression, DNA replication, and its control, and cytokinetic processes that might be suspected to be regulated but have not previously existed. In addition, a number of novel controlling genes including kinases, phosphatases, and transcription factors have been identified, as well as links to genes previously known for their role in differentiation or developmental processes.

Acknowledgments—J. A. H. M. and M. M. are very grateful to Klaus Herbermann and Bart den Boer for assistance and discussions regarding bioinformatic analysis. We also thank the Functional Genomics Center Zürich for technical support.

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