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Identification of Proliferation-Induced Genes in *Arabidopsis thaliana*[1]

Characterization of a New Member of the Highly Evolutionarily Conserved Histone H2A.F/Z Variant Subfamily

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The changes in gene expression associated with the reinitiation of cell division and subsequent progression through the cell cycle in *Arabidopsis thaliana* cell-suspension cultures were investigated. Partial synchronization of cells was achieved by a technique combining phosphate starvation and a transient treatment with the DNA replication inhibitor aphidicolin. Six cDNAs corresponding to genes highly induced in proliferating cells and showing cell-cycle-regulated expression were obtained by the mRNA differential display technique. Full-length cDNA clones (cH2BAt and cH2AvAt) corresponding to two of the display products were subsequently isolated. The cH2BAt clone codes for a novel histone H2B protein, whereas the cH2AvAt cDNA corresponds to a gene encoding a new member of the highly conserved histone H2A.F/Z subfamily of chromosomal proteins. Further studies indicated that H2AvAt mRNA expression is tightly correlated with cell proliferation in cell-suspension cultures, and that closely related analogs of the encoded protein exist in Arabidopsis. The implications of the conservation of histone H2A.F/Z variants in plants are discussed.

New tissues and organs are generally initiated throughout the life of the plant from small, highly organized groups of cells called meristems (Medford, 1992). Occasionally, however, adventitious organ development occurs as a consequence of the reactivation of cell division in previously quiescent cells of differentiated tissues (Yang et al., 1994). A better understanding of how developing plants achieve such a tight control of proliferative activity in meristematic and differentiated tissues will come from the elucidation of the molecular events involved in the reentry and progression through the cell cycle in the plant cell (e.g. Murray, 1994; Hirt, 1996).

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In eukaryotes the molecular events that commit a cell to enter into a new round of division are considered to take place essentially in the G1 phase of the cell cycle, in which a major regulatory checkpoint has been defined (Hunter and Pines, 1994). To pass this checkpoint, the expression of specific genes is required (Koch and Nasmyth, 1994; Sanchez and Dynlacht, 1996). In higher plants partial inhibition of mRNA synthesis during the G1 phase was found to induce an arrest of cell division in cultured cells of *Catharanthus roseus* (Ohnishi et al., 1990), indicating that the progression through the G1 phase is also strictly dependent on de novo gene transcription.

The subsequent progression of cell division also involves the sequential and coordinate expression of specific genes during the different phases of the cell cycle. Studies performed in yeast and animals have indeed shown that numerous genes encoding functions essential to cell-cycle progression are transcriptionally regulated (McKinney and Heintz, 1991; Koch and Nasmyth, 1994; Muller, 1995).

A number of cell-cycle-related genes have been isolated from various plant species (Jacobs, 1995). Similar to their animal counterparts, most of these genes show a periodic pattern of expression during the cell cycle. For example, among mammalian cyclins, many plant A-like (cycA) and B-like (cycB) cyclin mRNAs accumulate preferentially at specific phases of the cell cycle (Setiady et al., 1995; Fuerst et al., 1996; Reichheld et al., 1996; Shaul et al., 1996). Genes coding for histones and for the PCNA, a DNA polymerase auxiliary protein, show S-phase-specific expression patterns (e.g. Kodama et al., 1991b; Reichheld et al., 1995). The expression of some plant CDK genes is also cell-cycle-regulated (e.g. Fobert et al., 1996; Segers et al., 1996; Magyar et al., 1997; Sauter, 1997).

Despite increasing data highlighting the intimate link between transcriptional regulation and the control of cell division, differential gene expression has been used only occasionally as a selection criterion for the identification of genes coding for products that could be functionally involved in cell division. Studies on synchronized cells of *C. roseus* revealed that specific and very limited qualitative changes in the protein and mRNA content of the cells occur

at the different phases of the cell cycle (Kodama et al., 1989). Several cDNA clones corresponding to genes that are preferentially expressed at the G1/S boundary of the cell cycle were isolated in this system (Kodama et al., 1991a). This work demonstrated that screening for differential gene expression represents an additional means of identifying specific, possibly novel, functions acting during plant cell division (Kodama et al., 1994).

In this paper modifications in the mRNA content associated with the resumption of cell division and synchronous progression through the cell cycle of *Arabidopsis thaliana* cells have been studied by means of the mRNA differential display technique (Liang and Pardee, 1992). The isolation of six differential display products corresponding to genes highly induced in proliferating cells and showing cell-cycle-regulated expression is reported. Two of these genes were further characterized. One codes for a novel histone, H2B. The second gene encodes a protein belonging to the highly evolutionarily conserved histone H2A.F/Z subfamily of proteins, the members of which were previously found to be preferentially expressed in proliferating cells in animals (Hatch and Bonner, 1990).

MATERIALS AND METHODS

Cell-Suspension Cultures and Synchronization

The new *Arabidopsis thaliana* At112 and At202 cell lines were selected by cloning cell microclusters isolated from the T87-C3 cell suspension by a modification of the method of Coutos-Thevenot et al. (1990). Clusters of 5 to 15 cells expected to be of clonal origin (Hauptman and Widholm, 1982) were plated at a density of 35 clusters mL^{-1} in 9-cm-diameter Petri dishes (14 mL of culture medium per plate) in Gamborg B5 culture medium (Flow Laboratories, McLean, VA; supplemented with 20 g L^{-1} Suc and 1 μM naphthalene acetic acid) containing 0.5% agarose (LSM, Litex, Denmark) and 20% (v/v) conditioned culture medium obtained from 3-d-old cell suspensions. After 30 d the obtained microcalli were subcultured in fresh agarose-containing medium. Subculturing was performed every 20 d until the calli reached a mean diameter of 0.5 cm. The calli were then dissociated in 5 mL of Gamborg B5 medium in six-well culture plates (Nunc, Roskilde, Denmark) maintained on a rotating shaker. The cell cultures thus obtained were successively transferred to Erlenmeyer flasks containing increasing volumes of culture medium. The cells were subsequently cultured at 21°C and 110 to 130 oscillations min^{-1} under continuous illumination in 1-L flasks containing 250 mL of Gamborg B5 medium. Every 7 d the cells were subcultured at a density of 2 g fresh cells L^{-1}.

For synchronization 7-d-old At112 cells were transferred to Pi-free reconstituted Gamborg B5 medium at a cell population density of 1.5×10^6 cells mL^{-1} and cultured for 32 h. KH_2PO_4 and aphidicolin (Sigma) were then added to the culture medium at a final concentration of 1.087 mM and 25 μg mL^{-1}, respectively. After an additional incubation of 16 h, the cells were washed extensively with fresh medium on nylon membranes of 20-μm pore size and resuspended into new Gamborg B5 medium containing 20% (v/v) conditioned culture medium.

Determination of Cell Number

Protoplasts were prepared by enzymatic maceration of the cell clusters in Gamborg B5 medium containing 25 mM Mes, 12% (w/v) sorbitol, 1% (w/v) caylase 345L (CAYLA, Toulouse, France), and 0.1% (w/v) pectolyase Y-23 (Seishin Seiyaku, Tokyo, Japan), and incubating at 30°C for 90 min with mild agitation. The cell number was estimated by counting the resulting protoplasts with a hemocytometer.

Thymidine Incorporation

The cell culture (0.5 mL) was incubated with 0.2 μCi of [2-^{14}C]thymidine (56.5 mCi $mmol^{-1}$; DuPont-NEN) for 30 min at 21°C at 110 to 130 oscillations min^{-1}. Measurements of thymidine incorporation into DNA were performed according to the method of Minocha et al. (1991).

Flow-Cytometric Analysis

Cells were fixed for 15 min on ice in a 4% (w/v) solution of paraformaldehyde in Tris buffer (10 mM Tris, 10 mM Na_2-EDTA, 100 mM NaCl, pH 7.4) containing 0.6% (w/v) Triton X-100 (Sigma) according to the method of Sgorbati et al. (1986). Nuclei were isolated from the fixed cells by crushing with a Teflon potter in ice-cold buffer solution and filtrating successively through nylon membranes of 50- and 20-μm pore size. The nuclei were stained on ice with 4′,6-diamidino-2-phenylindole at a final concentration of 2 μg mL^{-1}. Flow-cytometric analysis was carried out on an Coulter flow cytometer (Elite, Epic, New York, NY). DNA histograms were analyzed with the MULTICYCLE computer program (Phoenix Flow Systems, San Diego, CA).

Nucleic Acid Extraction and mRNA Differential Display

Total RNA was extracted from the cells with acid guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). Standard techniques of RNA manipulation were as described by Sambrook et al. (1989). For the mRNA differential display, genomic DNA contamination of total RNA samples was removed by treatment with RNase-free DNAse I (Boehringer Mannheim) according to the method of Liang et al. (1993). Differential display analyses were then performed as previously described (Callard et al., 1994), except that PCR amplification was done using *Taq* polymerase (GIBCO-BRL) in the presence of 2 μCi of [α-^{33}P]dATP (2000 Ci $mmol^{-1}$; DuPont-NEN).

The PCR products were separated on a 6% DNA sequencing gel, blotted onto Whatman 3MM paper, dried without fixing, and autoradiographed. The PCR bands of interest were recovered from gel slices using a razor blade, and the DNA was diffused out by boiling for 15 min in 100 μL of sterile water followed by ethanol precipitation in the presence of 4 μg of glycogen carrier (Boehringer Mannheim). The reamplified PCR fragments were inserted into a pUC 18 *Sma*I/BAP vector (Sure-Clone DNA-ligation kit,

Pharmacia) and cloned in *Escherichia coli* DH5α (GIBCO-BRL). The sequence heterogeneity of the inserts was determined using a restriction enzyme (*Hin*fI, *Hae*III, and *Rsa*I) fingerprinting approach (Shoham et al., 1996) on 10 independent plasmid clones in each case.

Transcript Analysis

For northern-blot analysis total RNA (20 μg) was denatured with glyoxal and DMSO and size-fractionated by electrophoresis on 1% (w/v) agarose gels. The RNA was then blotted onto nylon membranes (Hybond-N^+, Amersham), fixed by UV cross-linking, and hybridized to ^{32}P-labeled DNA probes according to a previously described procedure (Regad et al., 1993). The GCK7, GGN9a, GGN9b, GCK12, GGA8, and GGL17 cDNA inserts were recovered from the plasmid vector by *Xba*I/*Kpn*I digestion. The cDNA inserts of the cH2BAt and cH2AvAt clones were prepared by PCR amplification with the universal primers M13-reverse and M13(−20). The Arabidopsis histone H4 cDNA clone was kindly provided by Dr. Claude Gigot (Université Louis Pasteur, Strasbourg, France).

The histone H4 probe was a *Hin*dIII/*Eco*RI fragment (196 bp) located in the coding region of the Arabidopsis H4A748 gene (Chaboute et al., 1987). The actin partial (0.7 kb) cDNA clone TAT3A7 (accession no. Z17483) was obtained from a cDNA library from Arabidopsis cultured cells (Regad et al., 1993) constructed in λ ZAP II (Stratagene). The insert of the actin λ clone was amplified by PCR with the universal primers M13-reverse and M13(−20). The different cDNA fragments and PCR products were gel-purified using a kit (Quiaex, Qiagen, Chatsworth, CA) and labeled with [α-^{32}P]dCTP using the oligolabeling procedure.

Screening of the cDNA Library

The GGA8 and GCK7 cDNA probes were prepared as described above and hybridized in each case against 3×10^5 plaque-forming units of the Arabidopsis cDNA library (Regad et al., 1993). Phage plaques were plated onto nitrocellulose membranes (Hybond-C-Extra, Amersham) according to the manufacturer's instructions. Prehybridization, hybridization, and washing were performed according to the method of Sambrook et al. (1989).

DNA Sequencing and Database Homology Searches

In vivo excision of the ZAP clones was performed as described in the manufacturer's (Stratagene) protocol. Nucleotide sequencing of the cDNA clones was carried out on the resulting double-stranded plasmids by the method of Prober et al. (1987). Each sequence was determined on both strands using an automated DNA sequencer (model 373A, Applied Biosystems/Perkin-Elmer). Homology searches against databases were performed using the BLAST program (Altschul et al., 1990) of the National Center for Biotechnology Information. Multiple amino acid sequence alignments were made using the PileUp program of the Genetics Computer Group software package (Devereux et al., 1984) and the MULTALIN program (http://www.toulouse.inra.fr/multalin.html; Corpet, 1988).

RESULTS

Cell Line Isolation and Synchronization Procedure

Flow-cytometric analysis of cell ploidy revealed that the Arabidopsis T87-C3 cell line previously established in our laboratory (Axelos et al., 1992) was heterogeneous, containing at least two cell populations exhibiting different ploidy levels (data not shown). New cell lines were therefore generated from this cell suspension by in vitro cloning of cell microclusters. Thirty-six independent cell cultures of presumably clonal origin were obtained; 22 of these were analyzed by flow cytometry and all were found to have a homogeneous nDNA content. Two of these cell lines, At112 and At202, made up of well-dispersed cells, homogeneous in size and shape and possessing an average cell-doubling time of 30 h, were maintained in liquid culture. The At112 cell line, which possessed the lowest chloroplast content, was used subsequently in all synchronization experiments.

To obtain synchronous cell cultures, various auxin- or nutrient-limiting culture conditions were first established and the point of cell cycle arrest determined in each case by flow-cytometric analysis of the DNA content of nuclei. These studies revealed that cells deprived of auxins (naphthalene acetic acid) were arrested in both the G1 and G2 phases of the cell cycle, as previously observed in other systems (John et al., 1993). In contrast, cells in which growth was arrested as a consequence of Suc, NO_3^-, or Pi starvation were predominantly arrested in the G1 phase. In addition, Pi starvation did not affect cell viability during a 3-d blocking period, and readdition of Pi to the culture medium resulted in a very rapid resumption of cell proliferation, as estimated by DNA-synthesis measurements.

Flow-cytometric studies revealed that a moderate proportion (approximately 20%) of the cells were able to synchronously enter into the S phase after release from the Pi block. To obtain a higher degree of synchrony, a second block at the G1/S boundary was induced immediately after the readdition of Pi by transiently treating the cells with the DNA-polymerase inhibitor aphidicolin (Huberman, 1981) (Fig. 1A).

The degree of synchrony in the cell population was followed by monitoring the incorporation of radioactive thymidine into DNA, by determining cell number, and by analyzing the DNA content of nuclei by flow cytometry (Fig. 1, B–D). When Arabidopsis cells were transferred to Pi-free medium, the growth-arrested state was maintained and after 32 h most (approximately 77%) of the cells were in the G1 phase, according to flow-cytometric measurements (Fig. 1D, −Pi 32h). Similar profiles were observed 16 h after the readdition of Pi in the presence of aphidicolin (Fig. 1D, 0h), and DNA synthesis appeared to be completely inhibited under these conditions (Fig. 1B).

The rate of incorporation of thymidine into DNA increased abruptly after the removal of aphidicolin (Fig. 1B), indicating that washing out the drug allowed the immediate and efficient entry of cells into the S phase, as has been

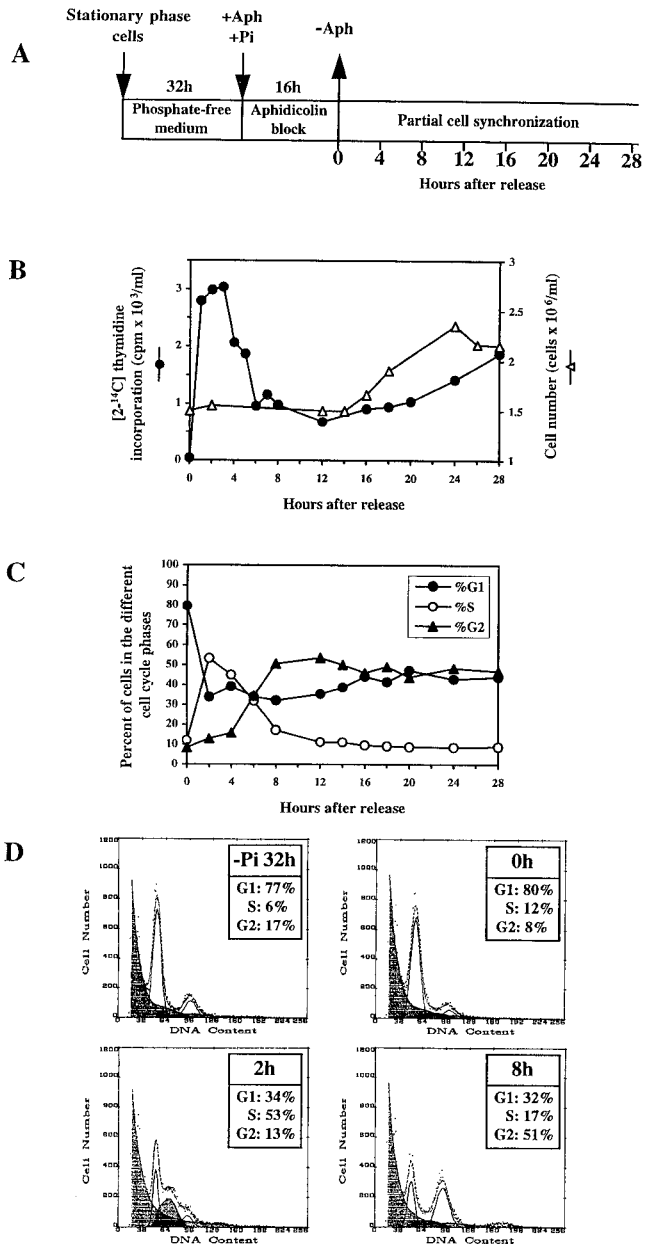

Figure 1. Synchronization of the At112 cell-suspension culture. A, Schematic representation of the synchronization procedure. At112 cells in the stationary phase were transferred to Pi-free medium. After 32 h, Pi (+Pi) and aphidicolin (+Aph) were added to the culture medium. After an additional 16 h of incubation, aphidicolin-arrested cells (0 h) were washed extensively (−Aph) and resumed growth in a synchronous manner. B, [2-^{14}C]Thymidine incorporation into DNA and cell number determination of the partially synchronized At112 cell culture after release from the aphidicolin block. C, Percentage of cells at the different phases of the cell cycle at the indicated time periods, calculated from flow-cytometric measurements. D, The original DNA histograms obtained from the cytometer were analyzed using the MULTICYCLE software program, which allows the relative proportions of cells in the various cell-cycle phases to be estimated. The dark-filled areas in the MULTICYCLE-calculated profiles represent background baseline due to cell and nuclear debris. The hatched areas correspond to cells in the S phase. −Pi 32h, Cells maintained for 32 h in Pi-free medium; 0h, aphidicolin-arrested cells; 2h and 8h, cells analyzed 2 and 8 h, respectively, after release from the aphidicolin block.

observed in other systems (e.g. Hirt et al., 1992; Nagata et al., 1992). Two hours after release from the block, 50 to 60% of the cells were found to be in the S phase (Fig. 1, C and D). This proportion then progressively declined and, after 8 h, 50% of the cells were found to be in the G2 phase. The first mitoses were observed after 16 h, at which time cell numbers started to increase, reaching approximately 50% after 24 h (Fig. 1B). No sharp variation in cell number was detected, however, indicating that synchrony was probably lost in our system during or before mitosis.

Identification, Cloning, and Sequencing of Differentially Amplified Display Products

The mRNA differential display technique (Liang and Pardee, 1992) was used to compare total RNA prepared from Pi-starved cells, cells arrested at the G1/S boundary by aphidicolin treatment, and cells collected at different times after release from the block. Using 2 of the 12 possible T_{11}XN-anchored primers, each in combination with 10 arbitrary decamer primers, we identified 6 differentially displayed bands corresponding to mRNA species that specifically accumulate in cycling cells in an apparently cell-cycle-regulated manner. The corresponding display profiles are presented in Figure 2. For each of the bands, highly reproducible display patterns were obtained when comparing RNA extracts prepared from two independent synchronization experiments (Fig. 2, patterns I and II). Three of the bands (GCK7, GGN9b, and GCK12) were stronger in profiles corresponding to aphidicolin-blocked cells and cells in the S phase. The GGA8 and GGL17 bands corresponded to genes that appeared to be induced in S-phase cells and were strongly down-regulated in the presence of the inhibitor. The GGN9a band was highly enhanced in cells in the late S and G2 phases.

The six labeled cDNAs corresponding to the display bands were recovered from gels, reamplified, and cloned into a plasmid vector. Tests for sequence heterogeneity of the inserts using restriction-enzyme fingerprinting (Shoham et al., 1996) indicated that each plasmid insert obtained from a single display band corresponded either to a unique or to a highly predominant cDNA species (data not shown).

The nucleotide sequence of the inserts was determined. Computer searches indicated that four of the six clones (GGN9a, GGN9b, GCK12, and GGL17) had no significant homology to any sequence present in the databases. The DNA-deduced peptide sequences of GGA8 and GCK7, however, presented similarities with H2B histones and histone H2A.F/Z variants, respectively.

Expression Analysis of the Selected Genes in Partially Synchronized Cells

To confirm that the differences in the display patterns were correlated to differences in the steady-state levels of

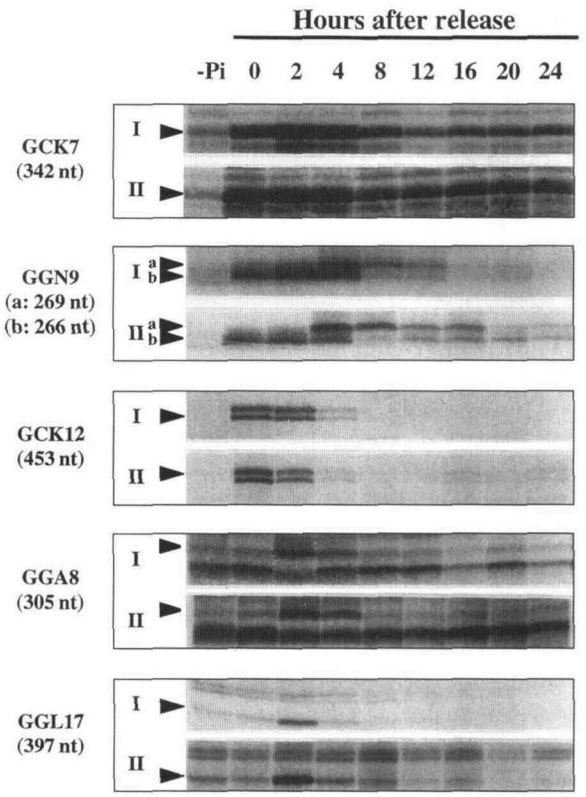

Figure 2. Differential display analysis of synchronized Arabidopsis cell cultures. Total RNA from Pi-starved cells (−Pi), aphidicolin-arrested cells (0 h), and cells collected 2 to 24 h after release from the aphidicolin block was reverse-transcribed and PCR-amplified with different primer sets: 5′T_{11}GC + OPK7 (5′-AGCGAGCAAG); 5′T_{11}GG + OPN9 (5′-TGCCGGCTTG); 5′T_{11}GC + OPK12 (5′-TGGCCCTCAC); 5′T_{11}GG + OPA8 (5′-GTGACGTAGG); and 5′T_{11}GG + OPL17 (5′-AGCCTGAGCC). The PCR products were resolved on a 6% (w/v) polyacrylamide sequencing gel and visualized by autoradiography. For each primer combination, two display profiles (I and II) obtained from independent cell synchronization experiments are presented. The arrowheads on the left indicate the differentially displayed bands. These bands were named GCK7, GGN9a, GGN9b, GCK12, GGA8, and GGL17 in relation to the primer sets used for their identification. The sizes (in nucleotides [nt]) of the PCR bands are indicated in parentheses.

the corresponding mRNAs, northern blots of total RNA isolated from synchronized cells were probed with the six cloned display products (Fig. 3). The hybridization profiles shown for GCK7 and GGA8 were obtained by using probes consisting of corresponding full-length cDNAs (cH2AvAt and cH2BAt, respectively), that were isolated by library screening (see below). These profiles were identical to those initially obtained with the cloned display products (data not shown).

No transcript corresponding to the GGN9a probe could be detected in these experiments. This may be attributable to a low representation of the corresponding mRNA in these Arabidopsis cells, below the detection limit of the technique. The other cDNA probes, however, hybridized in each case to a unique mRNA of variable abundance. Moreover, the deduced expression patterns of the corresponding genes were in close accordance with those observed by differential display analysis. The relative transcript level of the GCK7 gene was high in cells arrested at the G1/S transition and in cells in the S phase. It then decreased rapidly as the cells entered the G2 phase of the cell cycle. In addition, transcripts were not detected in growth-arrested cells. The GGN9b and GCK12 gene-expression profiles were similar to that of the GCK7 gene, although the steady-state levels of the corresponding transcripts were much lower.

Levels of GGA8 and GGL17 mRNAs were high in Pi-starved cells and low in aphidicolin-arrested cells, but strongly increased after release from the block. These results indicate that although the two genes are expressed at basal levels in conditions under which DNA synthesis is highly reduced, they also show strong up-regulation during the S phase.

As a control for S-phase-specific expression, we examined the mRNA accumulation pattern of the Arabidopsis histone H4 gene, the increased expression of which correlates with DNA replication (Reichheld et al., 1995). The histone H4 gene was highly expressed in cells in the S phase and gradually declined as the cells progressed into

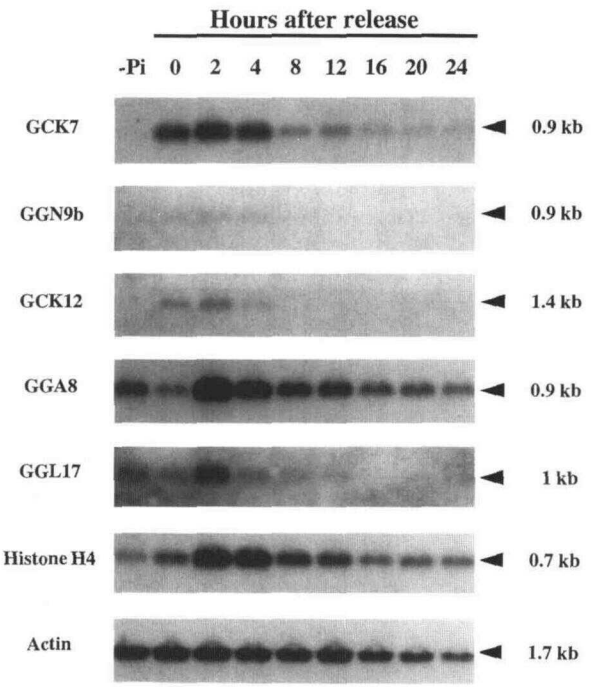

Figure 3. Northern-blot analysis of mRNAs differentially expressed in the synchronized At112 cells. Twenty micrograms of total RNA isolated from Pi-starved cells (−Pi), aphidicolin-treated cells (0 h), and cells collected 2 to 24 h after release from the aphidicolin block was subjected to denaturing agarose gel electrophoresis, blotted, and sequentially hybridized with radioactively labeled inserts of GGN9b, GCK12, and GGL17 clones and full-length cDNAs corresponding to the GGA8 and GCK7 products. The sizes (in kilobase pairs) of the identified mRNAs are indicated on the right. The RNA blot was also hybridized against two Arabidopsis probes corresponding to the histone H4-coding region and to an actin cDNA, which were used as controls for S-phase-specific and constitutive expression, respectively.

the G2 phase. The corresponding mRNA levels were strongly reduced in the presence of aphidicolin.

Isolation and Sequence Analysis of a cDNA Corresponding to a New Histone H2B Gene

A full-length cDNA corresponding to the GGA8 display product was isolated by cDNA library screening. Ten positive clones were analyzed and found to contain a cDNA insert of 700-bp maximum size. One of these clones was further isolated and the cDNA insert sequenced. The sequence shared 100% identity with that of the cloned display product used as a probe for the screening. A homology search through the databases identified the putative 150-amino acid-long translation product of GGA8 as a new member of the histone H2B family. This gene was named *H2BAt* and the corresponding cDNA clone is subsequently referred as cH2BAt.

The deduced amino acid sequence of H2BAt exhibits 86 and 92% identity with the two previously identified full-length (accession no. Y07745) and partial (accession no. Z18202) histone H2B sequences of Arabidopsis, respectively. Nucleotide sequence identities in the coding region are 79 and 83%, respectively. These results indicate that at least three closely related variants of the histone H2B protein exist in Arabidopsis.

Characterization of a New Member of the Histone H2A.F/Z Variant Subfamily

Ten positive clones detected after an initial screening of the Arabidopsis cDNA library with the cloned GCK7 display product were analyzed and found to contain a cDNA insert of 700-bp maximum size. Two of these clones were chosen for further characterization and one of them was sequenced. The sequence of the clone was identical to that of the cloned display product used for the screening. Computer searches against databases showed that the predicted protein sequence of GCK7 shared striking similarity with variant histone H2A proteins of the F/Z type. The selected cDNA encoding the new H2A.F/Z histone variant was named cH2AvAt and the corresponding gene is referred to as *H2AvAt*.

An optimal alignment of the H2AvAt predicted protein sequence with several members of the histone H2A.F/Z subfamily is shown in Figure 4. An Arabidopsis expressed sequence tag corresponding to a cDNA most probably coding for a second H2A.F/Z variant was also identified in the database and the corresponding polypeptide was included in the alignment (Fig. 4; *est*H2AvAt). These comparisons point out the high conservation of primary amino acid sequence in the histone H2A.F/Z subfamily. A high degree of similarity is observed in a large central part (amino acids 60–160) of the proteins, whereas the amino- and carboxyl-terminal tails appear to vary in both composition and length.

A nine-amino acid motif, the H2A box, which is strictly conserved in major H2A histones and histone H2A variants (e.g. van Daal et al., 1988), was also identified in the two sequences from Arabidopsis (Fig. 4), although an L-to-I substitution was found in H2AvAt.

Despite a high sequence homology between the two polypeptides from Arabidopsis, nucleotide sequence identity in the coding sequence is rather reduced (73%) and the 3′ untranslated regions of the two cDNAs appear completely unrelated to each other (data not shown). These data indicate that the two Arabidopsis genes diverged early in evolution.

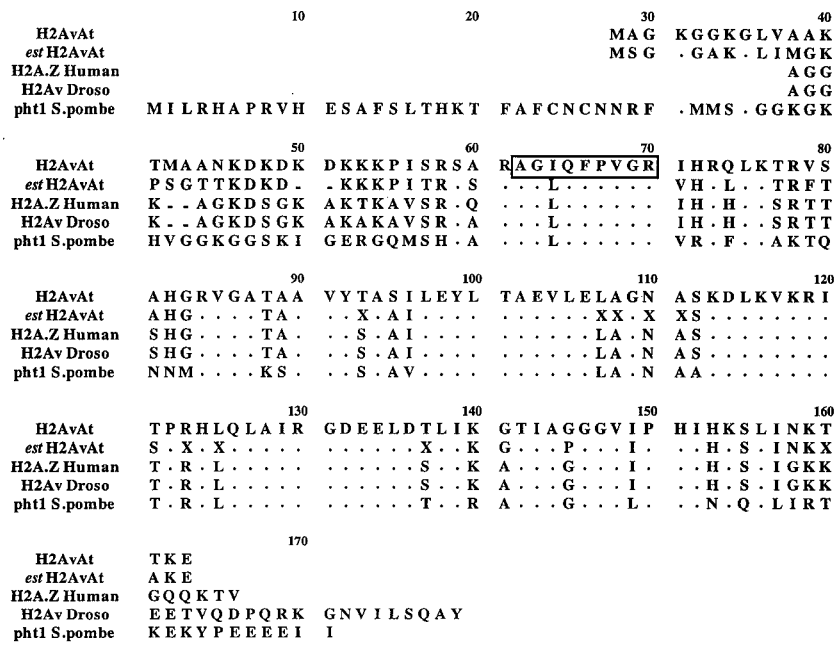

Figure 4. Alignment of the H2AvAt polypeptide with known members of the histone H2A.F/Z variant subfamily. The deduced H2AvAt protein sequence is compared with protein sequences derived from an Arabidopsis expressed sequence tag (*est*H2AvAt; accession no. Z26465), human H2A.Z (accession no. P17317; the protein sequences from human, cow, and rat are identical), *Drosophila melanogaster* (Droso) H2AvD (accession no. P08985), and *Schizosaccharomyces pombe* (S. pombe) pht1 protein (accession no. P48003). Numbering is based on the sequence of the pht1 protein. Alignments were maximized by introducing gaps denoted by dashes. Dots indicate strict identity with the amino acid. X residues in the *est*H2AvAt sequence represent unidentified amino acids resulting from nucleotide ambiguities in the corresponding sequence. The H2A motif is boxed in H2AvAt.

Cell-Proliferation-Dependent Expression of the *H2AvAt* Gene

The expression of the *H2AvAt* gene in relation to cell proliferation was further investigated. We first compared its relative transcript levels in exponentially growing cells and in cells that had stopped dividing, either at the late phase of the growth curve in standard culture conditions or as a consequence of nutrient starvation. In Suc- or Pi-limiting culture medium, growth arrest preceded by a severe decrease in DNA synthesis activity was observed after 6 d of culture (results not shown). Cells resumed proliferation after the addition of the limiting nutrient, confirming that the exhaustion of this nutrient was the primary factor limiting cell growth in these experiments.

As shown in Figure 5, *H2AvAt* gene expression was highly reduced in growth-arrested cells in the different culture conditions tested, and H2AvAt mRNA accumulation was nearly restricted to actively growing cells. The *H2BAt* gene was also strongly expressed in exponentially growing cells. However, the transcript levels appeared to be only moderately reduced in growth-arrested cells. The Arabidopsis histone H4 gene showed intermediate expression profiles, with reduced expression in growth-arrested cells, and strong mRNA accumulation in exponentially growing cells.

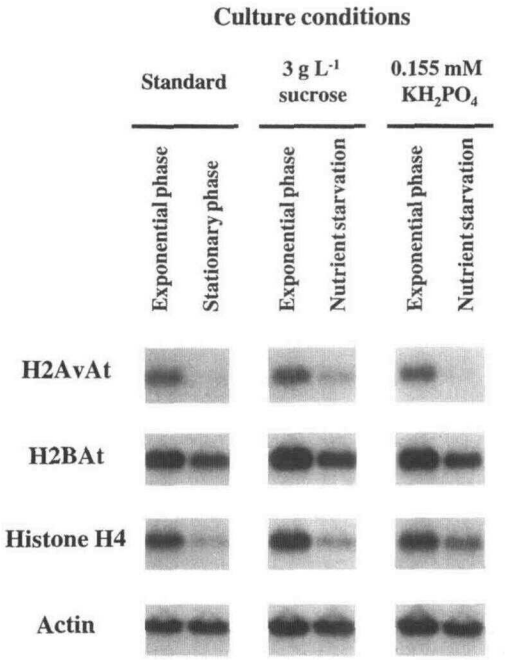

Figure 5. Northern-blot analysis showing that the expression of the *H2AvAt* gene is strongly correlated with cell proliferation. Cells were cultured in nutrient-limiting conditions (3 g L^{-1} Suc or 0.155 mM KH_2PO_4) or in standard Gamborg B5 medium. Twenty micrograms of total RNA extracted from the cells was subjected to northern-blot analysis and hybridized sequentially to the cH2AvAt, cH2BAt, histone H4, and actin (constitutive control) cDNA probes. Exponential phase, Actively growing cells collected 2 d after subculturing in each culture condition; Stationary phase, 9-d-old growth-arrested cells; Nutrient starvation, growth-arrested cells as a result of Suc or Pi starvation.

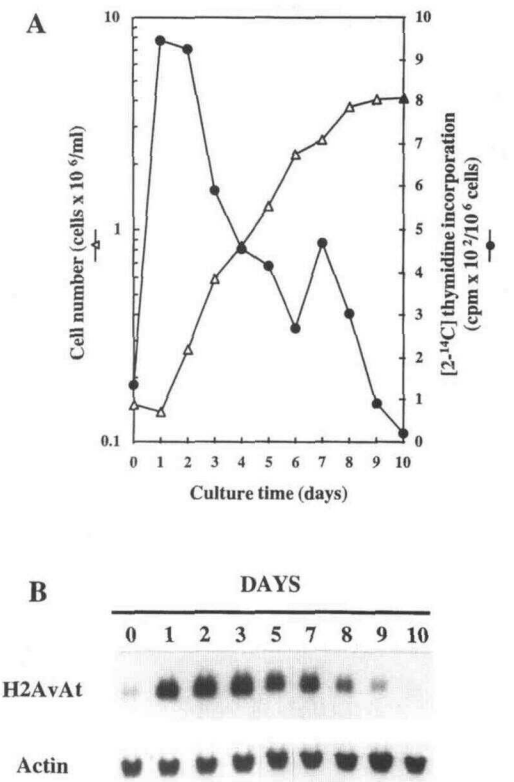

Figure 6. Time course of *H2AvAt* gene expression during the growth cycle of Arabidopsis At112 cell suspension. A, Growth curve of Arabidopsis At112 cell culture. Cells in the growth-arrest phase were transferred to fresh medium and cultured for 10 d. [2-^{14}C]Thymidine incorporation into the DNA fraction and cell number were monitored by sampling at 1-d intervals. B, Northern-blot analysis of the H2AvAt transcript levels. RNA was extracted from the At112 cells at the indicated times. Twenty micrograms of total RNA was subjected to electrophoresis, transferred to nylon membranes, and hybridized to the cH2AvAt cDNA probe. As a control for constitutive expression, the northern blot was hybridized to the Arabidopsis actin cDNA.

The expression of the *H2AvAt* gene during the complete growth cycle of asynchronous cell cultures was also studied. Nine-day-old S-phase cells were transferred into new culture medium and DNA synthesis activity and cell number were determined each day (Fig. 6A). After a 1-d lag the cell number increased exponentially until d 6. Thereafter, the ratio of actively dividing cells gradually decreased and on d 9 all of the cells had stopped dividing. A rapid increase in the rate of thymidine incorporation was observed after the transfer of cells into fresh medium, preceding cell division. After d 2, overall DNA synthesis activity in the cell culture decreased progressively to reach basal levels 9 d after subculture.

As shown in Figure 6B, the H2AvAt transcript level rapidly increased to maximal levels after transfer of the cells into new medium, and remained high and weakly modulated in actively growing cells. It then decreased in parallel with cell proliferation, to become barely detectable in growth-arrested cells (0-, 9-, and 10-d-old cells). These results confirmed that H2AvAt expression is restricted to actively dividing cells.

DISCUSSION

The pioneering work of Kodama et al. (1991a) indicated that searching for changes in mRNA content correlated with cell-cycle induction and progression in synchronized cell-suspension cultures could represent an additional selection method to identify new genes involved in the cell-division process.

In this study we have isolated genes differentially expressed upon induction and progression through the cell cycle in *A. thaliana* cell-suspension cultures. First, culture conditions for cell-cycle arrest and partial synchronization were established. The retained conditions make use of Pi starvation, which efficiently blocks the cells in the G1 phase and allows rapid recovery of the cells, as observed in other systems (Amino et al., 1983; Magyar et al., 1993). Transient treatment with aphidicolin is subsequently used to induce a synchronous release of the Arabidopsis cells into the S and G2 phases of the cell cycle.

A survey by the differential display technique of mRNA populations of cells collected under growth-arrest conditions and at different phases of the cell cycle revealed that the qualitative changes in gene expression are very limited and only a few modifications of display patterns could be observed with the 20 primer combinations tested. These results appear to be in agreement with those previously obtained in *Catharanthus roseus* (Kodama et al., 1989).

Six cDNA sequences corresponding to genes that are highly induced in proliferating cells were characterized. The display profiles obtained with the GGN9a PCR product strongly suggest that this cDNA corresponds to a gene that is preferentially expressed at the S/G2 boundary and in the G2 phase. The inability to detect the corresponding mRNA in our northern-blot experiments indicates that this gene is expressed at low levels. A number of genes, some of which code for essential components of the cell cycle machinery, such as cyclin A or the cdc25 phosphatase, had previously been found to be induced in the late S and the G2 phase in yeast and mammalian cells (McKinney and Heintz, 1991). No sequence related to GGN9a could be identified in the databases, and therefore the identity and function of the corresponding protein is currently unknown.

The other identified PCR products can be classified into two groups with respect to their transcript-accumulation profiles. The first group, which includes GGA8 and GGL17, corresponds to genes that are preferentially expressed in the S phase and that are strongly down-regulated when DNA synthesis is inhibited by aphidicolin treatment. The second group, including GCK7, GGN9b, and GCK12, corresponds to genes that are expressed at the G1/S boundary and the S phase of the cell cycle, and appear insensitive to the inhibition of DNA replication by aphidicolin. Computer searches did not reveal any sequence homologous to GGL17, GGN9b, or GCK12. Because no long open reading frames were found in these sequences, the cloned display products may correspond mainly to the 3′ untranslated region of the corresponding mRNAs, which are usually poor in sequence information. This is a limitation of the display approach (e.g. Sompayrac et al., 1995) and restricts the initial characterization of the identified genes.

Identification of a New Arabidopsis Histone H2B Gene

The existence of primary sequence variants of H2B histones has been reported in a wide variety of organisms (Wu et al., 1986). Here we showed that at least three H2B histone variants can be identified in Arabidopsis. As illustrated in Figure 3, the *H2BAt* gene showed replication-dependent expression in the partially synchronized Arabidopsis cells. Preferential histone gene expression during the S phase, both in plant tissues and in cell-suspension cultures, has been demonstrated in a large number of systems (e.g. Kapros et al., 1992; Tanimoto et al., 1993; Reichheld et al., 1995).

Significant amounts of H2B transcripts were also detected in growth-arrested cells (Fig. 5), indicating that, as is usually observed with major histone genes (e.g. Lepetit et al., 1992; Chaubet et al., 1996), the gene is expressed at basal levels in nonproliferating cells.

As observed with histone genes from animals, H2BAt mRNA levels were highly reduced in the presence of aphidicolin (Fig. 3). Sensitivity of histone H3 gene expression to aphidicolin treatment was also observed in cell cultures of alfalfa (Meskiene et al., 1995) and rice (Ohtsubo et al., 1993). In tobacco BY-2 cell suspensions, however, aphidicolin treatment did not significantly reduce the steady-state mRNA levels of histone H4 (Reichheld et al., 1995; Shaul et al., 1996). The H4 gene, used as a control in our experiments, appeared strongly inhibited under the conditions of this study. The reason for this apparent discrepancy between results remains unclear, but could be related to differences in the blocking procedures. The elucidation of the mode of activation of histone genes in relation to the onset and/or progression of DNA replication should help to clarify this point.

Characterization and Expression Studies of a New H2A.F/Z Histone Variant

Three histone H2A subfamilies differing both in length and primary sequence have been identified in animals. In addition to the major histone type, there are two other subfamilies, H2A.F/Z and H2A.X, the proteins of which are encoded by single-copy genes and represent 5 and 10%, respectively, of the H2A content in chromatin from vertebrates (Hatch and Bonner, 1990). In contrast to H2A.X variants, H2A.F/Z variants appeared very early during evolution (Thatcher and Gorovsky, 1994). These proteins have evolved more slowly than the major H2A histones, indicating that they must perform essential functions distinct from those of major H2A histones.

Genetic studies have previously shown that deletion of the gene coding for the H2A.F/Z variant is lethal in *D. melanogaster* (van Daal and Elgin, 1992) and results in a number of deleterious pleiotropic effects in *S. pombe* (Carr et al., 1994). It has been proposed that H2A.F/Z variants could modify chromatin structure and in some way control the transcriptional competence of chromatin in response to extracellular signals and during development (Stargell et al., 1993; Liu et al., 1996).

With the notable exception of *Saccharomyces cerevisiae* (Liu et al., 1996), H2A.F/Z variants have been identified in a number of highly evolutionarily distant organisms. The reported characterization of the Arabidopsis cH2AvAt cDNA demonstrates that H2A.F/Z histone variants have been conserved in plants and are highly homologous to their animal and lower eukaryote counterparts, indicating that these proteins evolved under similar strong selection pressure.

A selective accumulation of H2AvAt mRNA in proliferating cells (Figs. 5 and 6) and a lack of sensitivity to aphidicolin treatment of the corresponding gene (Fig. 3) was observed in our system. These results are in close agreement with those of Hatch and Bonner (1990), who showed in cultured mammalian cells that H2A.Z expression is insensitive to the inhibition of DNA replication by aphidicolin, but is strictly correlated with proliferative activity. Together these results suggest that H2AvAt proteins perform evolutionarily conserved functions associated with the switch from a quiescent to an actively replicating state.

A preferential accumulation of the H2AvAt mRNAs was observed in the G1/S boundary and in the S phase in Arabidopsis cells (Fig. 3). Although H2A.F/Z gene expression was previously reported to be constitutive during the cell cycle in vertebrates (Dalton et al., 1989), expression of the *pht1* gene of *S. pombe* was found to be cell-cycle-regulated, peaking at the G1/S boundary (Durkacz et al., 1986; Carr et al., 1994). Studies in *D. melanogaster* also suggested that H2AvD expression may be maximal when the genome is replicated (van Daal and Elgin, 1992). Our results agree with this and reinforce previous suggestions regarding the possible existence of different modes of regulation of H2A.F/Z genes among eukaryotes (Carr et al., 1994).

Southern-blot analysis of genomic DNA isolated from two different ecotypes of Arabidopsis indicated that, as previously reported for other H2A.F/Z genes, the *H2AvAt* gene is present as a single copy in the genome (data not shown). An unexpected result was the identification in the database of an expressed sequence tag corresponding to a cDNA encoding a second, closely related H2A.F/Z polypeptide. Because these clones were obtained from cells from the same ecotype (Columbia), these two genes must correspond to nonallelic variants. The observed high divergence in nucleotidic sequence between the two genes is in striking contrast with previously reported data showing that the H2A.F/Z genes from three mammalian species are highly homologous, sharing 94 to 97% and 98% identity in the coding and 3′ untranslated regions, respectively (Hatch and Bonner, 1988). This indicates that the two Arabidopsis genes duplicated and diverged very early in evolution and might have acquired independent functions.

The characterization of two H2A.F/Z variants in Arabidopsis raises the question, previously asked regarding yeast (Carr et al., 1994), of the coexistence of several primary sequence variants of H2A.F/Z histones in other organisms as well, each variant having acquired specialized essential functions in the cell. The availability of the two Arabidopsis sequences should allow this hypothesis to be further investigated.

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