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Two Phases of Chromatin Decondensation during Dedifferentiation of Plant Cells

DISTINCTION BETWEEN COMPETENCE FOR CELL FATE SWITCH AND A COMMITMENT FOR S PHASE*

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Jing Zhao, Nadya Morozova, Leor Williams, Laurence Libs, Yigal Avivi, and Gideon Grafi‡

From the Department of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel

Cellular dedifferentiation is the major process underlying totipotency, regeneration, and formation of new stem cell lineages in multicellular organisms. In animals it is often associated with carcinogenesis. Here, we used tobacco protoplasts (plant cells devoid of cell wall) to study changes in chromatin structure in the course of dedifferentiation of mesophyll cells. Using flow cytometry and micrococcal nuclease analyses, we identified two phases of chromatin decondensation prior to entry of cells into S phase. The first phase takes place in the course of protoplast isolation, following treatment with cell wall degrading enzymes, whereas the second occurs only after protoplasts are induced with phytohormones to re-enter the cell cycle. In the absence of hormonal application, protoplasts undergo cycles of chromatin condensation/decondensation and die. The ubiquitin proteolytic system was found indispensable for protoplast progression into S phase, being required for the second but not the first phase of chromatin decondensation. The emerging model suggests that cellular dedifferentiation proceeds by two functionally distinct phases of chromatin decondensation: the first is a transitory phase that confers competence for cell fate switch, which is followed, under appropriate conditions, by a second proteasome-dependent phase representing a commitment for the mitotic cycle. These findings might have implications for a wide range of dedifferentiation-driven cellular processes in higher eukaryotes.

A high proportion of mature plant cells retain characteristic features of totipotent stem cells, *i.e.* they have the capability to dedifferentiate, re-enter the cell cycle, and proliferate, eventually giving rise to the various organs that make up a new plant (1). In multicellular organisms, cellular dedifferentiation is the major process enabling the regeneration of complex tissues and organs as well as the establishment of new stem cell lineages; in animals it is often associated with carcinogenesis. The molecular mechanism(s) underlying re-entry of differentiated animal cells into the cell cycle and reactivation of DNA synthesis have been intensively studied. Yet, little is known about the early events that accompany cellular dedifferentiation, *i.e.* the establishment of competence for cell fate switch and the determination of cell fate.

The tobacco protoplast system provides an outstanding experimental tool for the study of the biochemical and molecular basis for cellular dedifferentiation. The fully differentiated, non-dividing mesophyll cells of tobacco leaves can be separated from their original tissue by cell wall-degrading enzymes. This treatment results in the formation of a large population of protoplasts (plant cells devoid of cell wall) that, following treatment with phytohormones (auxin and cytokinin) can re-enter the cell cycle and proliferate (2–5). This system demonstrates a unique attribute of plant cells, totipotency, *i.e.* the capability for cloning in plants (1).

Dedifferentiation of mature tobacco cells was shown to be accompanied by a sharp increase in ubiquitin gene expression (6), the biochemical tag that marks proteins for degradation by the ubiquitin proteasome system (7). Elevation in ubiquitin gene expression probably represents a critical point in cellular reorganization, namely, selective destruction of proteins involved in maintaining the previous function of a cell and a concomitant activation of proteins that are essential for cell proliferation (8–11). Genetic studies in *Arabidopsis* demonstrated that some of the factors involved in auxin response are components of the cell cycle-regulated ubiquitin-protein-ligase complex (E3), known as SCF (Skp1-Cdc53-F-box protein) (Ref. 12 and references therein). The SCF complex is required for the ubiquitination of specific phosphorylated substrates during G_1 to S transition (9–11).

In the course of cellular dedifferentiation, mature cells undergo remarkable changes in their pattern of gene expression, switching from a program that drives the specific function of a given somatic cell to a new one that directs cell multiplication. How do differentiated cells remodel their gene expression program in a rapid and orderly manner? Conceivably, chromatin reorganization could serve that purpose. Indeed, it has been suggested that one of the first stages in cellular dedifferentiation is the unfolding of chromatin supercoiling (13). We currently study mechanism(s) underlying dedifferentiation of mature plant cells, focusing on changes in chromatin structure. Using FACS[1] and MNase analyses, we found that differentiated tobacco mesophyll cells undergo two distinct phases of chromatin decondensation prior to entry into S phase. We also report the involvement of the ubiquitin proteasome system in chromatin decondensation and entry of cells into S phase.

EXPERIMENTAL PROCEDURES

Protoplast and Nuclei Preparation—Protoplasts were isolated from sterile leaves of *Nicotiana tabacum* ("Samsun NN") essentially as

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‡ To whom correspondence should be addressed: Plant Sciences, The Weizmann Inst. of Science, Rehovot 76100, Israel. Tel.: 972-8-934-3505; Fax: 972-8-934-4181; E-mail: gideon.grafi@weizmann.ac.il.

[1] The abbreviations used are: FACS, fluorescence-activated cell sorter; BAP, 6′-benzylaminopurine; NAA, α−naphthalene acetic acid; PI, propidium iodide; HU, hydroxyurea; MNase, micrococcal nuclease; MES, 4-morpholineethanesulfonic acid; SCF, Skp1-Cdc53-F-box protein.

described (14). Freshly prepared protoplasts were washed and incubated at 22 °C in VKM medium containing 0.5 μg/ml 6′-benzylaminopurine (BAP) and 2 μg/ml α−naphthalene acetic acid (NAA). In certain assays, protoplasts or leaves were incubated in the presence of 100 μM of the ubiquitin proteasome inhibitor MG132 (*N*-carbobenzyloxy (*N*-CBZ)-Leu-Leu-Leu-al, Sigma), or the protease inhibitor leupeptin (acetyl-Leu-Leu-Arg-al, Sigma), or 5 mM hydroxyurea (Sigma). All FACS measurements were performed on isolated nuclei to avoid interference of plastid and mitochondrial DNAs. Nuclei were isolated in a Hamilton buffer essentially as described (15, 16), washed twice with FACS buffer (10 mM MES, 0.2 M sucrose, 0.01% Triton X-100, 2.5 mM EDTA, 2.5 mM dithiothreitol (16)) to remove soluble contaminants, and passed through two layers each of 150- and 100-μm filters to remove cell debris. Nuclei were precipitated (1000 × *g*, 7 min, 4 °C), resuspended in FACS buffer supplemented with 50 μg/ml DNase-free RNase A (Roche Molecular Biochemicals) and 50 μg/ml PI (Sigma), viewed under the microscope, and subjected to FACS analysis using FACSort (Becton Dickinson). The position of PI fluorescence intensity for G_0/G_1 nuclei has been changed from one experiment to another as a result of alteration in the amplifier gains for FL-2, which was necessary to accommodate fluorescence intensity of both G_0/G_1 and G_2 nuclei.

Bromodeoxyuridine (BrdUrd) Labeling—Protoplasts reactivated for S phase (72 h after their preparation) were pulse-labeled with 10 μM BrdUrd (Sigma) for 30 min, after which nuclei were isolated, stained with PI, and analyzed by FACS. Nuclei were then sorted, and DNA was prepared (from about 50,000 nuclei) as described below, resolved on 0.8% agarose gel, transferred onto nitrocellulose membrane, and probed with anti-BrdUrd (Becton Dickinson).

Decondensation Assay—Equal amounts of nuclei (determined by pack volume and relative density) prepared from protoplasts or from tobacco leaves were first permeabilized by incubation in Hamilton buffer containing 0.15% Triton X-100 (15), washed and resuspended in 600 μl of nuclei digestion buffer (50 mM Tris-HCl, pH 8.0, 0.3 M sucrose, 5 mM $MgCl_2$, 1.5 mM NaCl, 0.1 mM $CaCl_2$, and 5 mM β-mercaptoethanol (15)). A sample (80 μl) was removed for untreated control. MNase (1000 units/ml) was then added and at various time points, samples (80 μl) were taken, mixed with 350 μl of stop solution (2 mg/ml proteinase K (Roche Molecular Biochemicals), 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, and 2% SDS, (17)), and incubated overnight at 37 °C. To prepare DNA, each sample was added 140 μl of 5 M potassium acetate, mixed well, incubated on ice for 15 min, and centrifuged (15 min, 12,000 × *g*, 4 °C). The supernatant was collected, extracted once with phenol/chloroform/isoamyl alcohol (25:24:1), and DNA was precipitated by adding 1 ml of 100% ethanol, followed by centrifugation. The DNA pellet was resuspended in 30 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), treated with RNase A (20 μg/ml, 25 min at room temperature), and nuclease digestion products were resolved on 1.6% agarose gels and stained with ethidium bromide.

RNA Preparation and Northern Blot Analysis—Total RNA was prepared from protoplasts by using the Tri-Reagent kit (Sigma) according to the manufacturer's protocol. Equal amounts of RNA were resolved on 1.4% agarose formaldehyde gel, transferred to a BioTrace pure nitrocellulose membrane (Gelman Sciences), and hybridized with ^{32}P-labeled *Arabidopsis* Skp1 antisense RNA. *Arabidopsis* Skp1 was obtained by polymerase chain reaction using PWO DNA polymerase (Roche Molecular Biochemicals) and the following primers: Skp1-sense, 5′-GTCGACGAATTCGAGCTCTCACTCGAGTCATTCAAAAGCCCATTGATTCTCC-3′ flanked with the *Eco*RI and *Bam*HI restriction sites, and Skp1-antisense primer, 5′-GTCGACGAATTCGAGCTCTCACTCGAGTCATTCAAAAGCCCATTGATTCTCC-3′ flanked with *Hinc*II and *Eco*RI sites. As a template we used the BAC clone T4L20 (GenBank™/EBI accession number AL023094), kindly provided by the *Arabidopsis* Biological Resource Center (ABRC). The amplified DNA fragment was digested with *Bam*HI and *Eco*RI, subcloned into the same sites of pBluescript SK, and sequenced to confirm identity. To prepare antisense RNA probes, the plasmid DNA was linearized with *Bam*HI and subjected to *in vitro* transcription using the Riboprobe System (Promega) and T7 RNA polymerase according to the manufacturer's protocol.

RESULTS

First Phase Chromatin Decondensation Occurs in Freshly Prepared Protoplasts—Tobacco leaves were treated with cell wall-degrading enzymes to produce a large population of protoplasts. We compared the FACS histograms of PI-stained nuclei isolated from freshly prepared protoplasts (t-0 protoplasts) *versus* nuclei prepared from leaves. Both t-0 protoplasts and leaf nuclei displayed a single peak corresponding to G_0/G_1 content of DNA (Fig. 1, *A* and *B*). However, nuclei prepared from t-0 protoplasts (Fig. 1*B, G1 nuclei*) reproducibly showed an upward shift in fluorescence intensity compared with leaf nuclei (Fig. 1*A, G0 nuclei*). To verify that these changes in PI fluorescence reflected intrinsic differences in the PI binding capabilities of chromatin, both populations of nuclei were mixed and incubated on ice for 15–30 min prior to FACS analysis. Fig. 1*C* shows that even after nuclei mixing, each population retained its position in the FACS histogram supporting an intrinsic difference in PI binding capabilities of chromatin. Because PI intercalates between the strands of DNA without sequence specificity, its fluorescence intensity is directly proportional to the level of accessible DNA, hence reflecting the condensation state of chromatin. We next applied a biochemical test, namely the MNase assay, to test whether the observed increment in fluorescence intensity of protoplasts nuclei reflected an increase in DNA accessibility to PI conferred by DNA decondensation (17). To this end, permeabilized nuclei from leaves or from t-0 protoplasts were treated with MNase for various time periods, and protected DNA fragments were resolved by agarose gel electrophoresis. As shown in Fig. 1*D*, digestion by MNase resulted in a typical nucleosomal ladder visualized by ethidium bromide staining, where each band represents DNA protected by one or several nucleosomes. Chromatin of t-0 protoplasts showed a higer sensitivity to MNase than chromatin from leaf nuclei (Fig. 1*D*, compare *lane 6* with *lane 11*). This verified that chromatin in nuclei of t-0 protoplasts is at a relatively decondensed configuration compared with chromatin in leaf nuclei. By analyzing the effect of leaf and protoplast nuclear protein extracts on the activity of MNase, we excluded the possibility that the reduced sensitivity of leaf chromatin to MNase resulted from the presence of nuclease inhibitors, otherwise absent from protoplasts nuclei (data not shown). Taken together, we suggest that the removal of the cell wall and the separation of differentiated mesophyll cells from their original tissue induce a stress response leading to conformational changes in chromatin.

Progression of Protoplasts into S Phase Is Accompanied by a Second Phase of Chromatin Decondensation—Following preparation, protoplasts re-enter the cell cycle and reactivate DNA synthesis only upon application of the phytohormones auxin and cytokinin. In the absence of phytohormones protoplasts undergo cycles of chromatin condensation/decondensation and die (Fig. 2). FACS analysis showed that within 24 h chromatin became condensed (Fig. 2*C*), shifting from the G_1 to G_0 position (Fig. 2, *G1* and *G0*), which is typical for leaf nuclei (Fig. 2*A*). The chromatin is then gradually decondensed, and sub-G_1 population, which is typical for cells undergoing apoptotic cell death (18), starts to accumulate (Fig. 2, *D* and *E*), eventually leading to protoplast death (not shown). In the presence of phytohormones, protoplasts re-enter the cell cycle (Fig. 3); division patterns were first evident at 72 h after hormonal application (Fig. 3*D*, *right panel*). FACS analysis showed that most freshly prepared protoplasts (t-0 protoplasts, Fig. 3*A*) as well as those incubated with hormones for 24 h (Fig. 3*B*) had DNA content corresponding to the G_1 phase. By 48 h (Fig. 3*C*), about 10% of protoplasts approached S phase, and by 72 h (Fig. 3*D*) 30–40% were in S and G_2 phases of the cell cycle. An extra G_1 peak (referred to as supra-G_1; Fig. 3, **G1*), revealing an increase in PI fluorescence intensity, was reproducibly evident at 72 h after protoplast preparation (Fig. 3*D*). In contrast, cycling tobacco cells showed monophasic G_1 (see Fig. 4*C*), suggesting that the occurrence of a supra-G_1 peak is unique to differentiated cells re-entering the cell cycle. When protoplasts, however, were incubated for a longer duration (96 h), almost no original G_1 nuclei were apparent; the majority of nuclei shifted

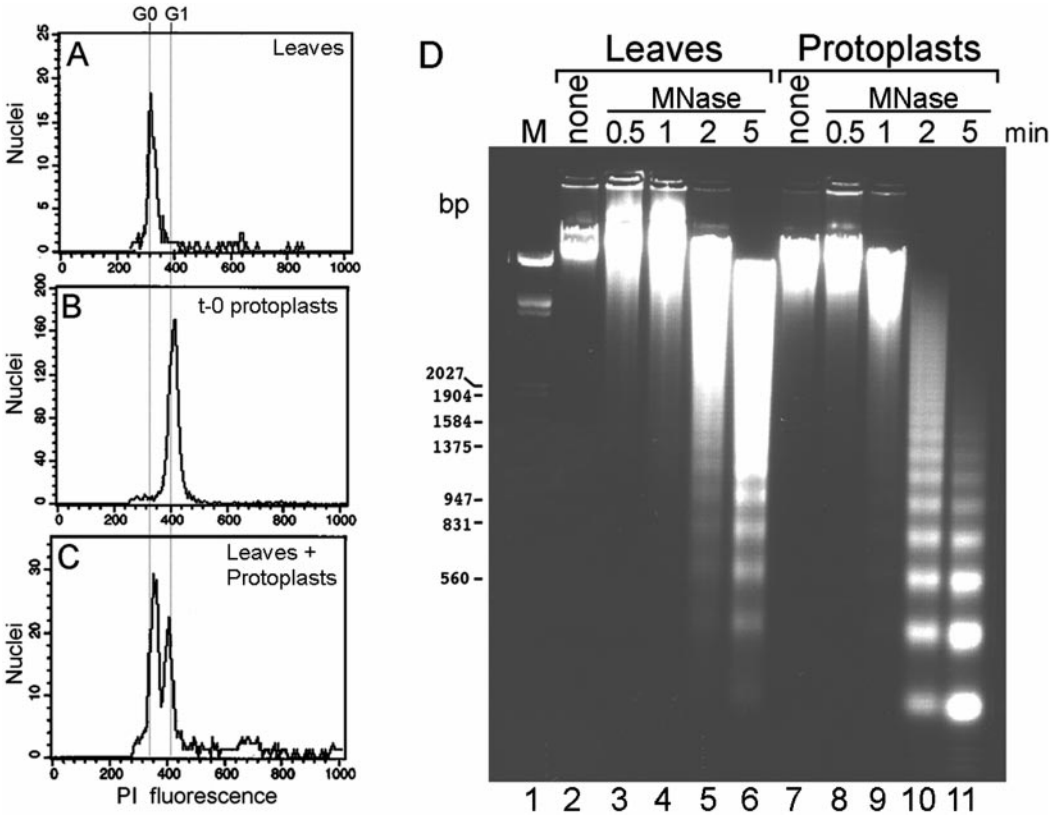

FIG. 1. **First phase chromatin decondensation revealed in freshly prepared protoplasts.** Nuclei were prepared from tobacco leaves (*A*) or from freshly prepared protoplasts (*B*), stained with PI, and subjected to FACS analysis. Note the higher PI fluorescence intensity in protoplast nuclei (*G1*) compared with leaf nuclei (*G0*). *C,* FACS histogram of a mixture of both types of nuclei. *D,* chromatin from freshly prepared protoplasts shows increased sensitivity to MNase. MNase (1000 units/ml) was applied for the indicated time periods (min) to nuclei prepared from tobacco leaves (*lanes 2–6*) or from freshly isolated protoplasts (*lanes 7–11*). DNA was extracted and separated by 1.6% agarose gel, followed by ethidium bromide staining. *None* (*lanes 2* and *7*) is DNA prepared from untreated nuclei. Positions of DNA size markers (*M*, *lane 1*) are indicated in base pairs (*bp*) on the left.

to the supra-G_1 position (Fig. 3*E*). This pattern suggests that the supra-G_1 peak represents a stage at which nuclei are competent to re-enter S phase, and, therefore, may be equivalent to G_1 nuclei of cycling cells. To examine this possibility, we established a cell suspension culture of *N. tabacum* (cycling cells) and compared the FACS histograms of nuclei prepared from this culture with that of *N. tabacum* protoplasts. Fig. 4 shows that G_1 nuclei of cycling cells had a higher fluorescence intensity than nuclei of t-0 protoplasts (Fig. 4, compare *A* with *C*), coinciding with the position of the supra-G_1 nuclei (Fig. 4, compare *B* with *C*). Each population of nuclei retained its position in the histogram after mixing (data not shown). These results indicate that the supra-G_1 peak represents chromatin relaxation rather than increased DNA content. This conclusion is strengthened by the finding that treatment of protoplasts with HU (a blocker of DNA replication) resulted in accumulation of both G_1 and supra-G_1 nuclei (Fig. 5*A, G1 and *G1*), and by the absence of BrdUrd labeling from supra-G_1 nuclei (Fig. 5, *B* and *C*). These results verify that the increased fluorescence intensity of PI-stained nuclei (supra-G_1) prior to entry of cells into S phase resulted from chromatin decondensation, apparently an essential event for replication factors to approach and be assembled onto origins to initiate DNA replication.

The Ubiquitin Proteasome System Is Required for the Second Phase of Chromatin Decondensation and Reactivation of S Phase—The expression of various ubiquitin genes is enhanced in the course of dedifferentiation of mature tobacco cells (6). To investigate the involvement of the ubiquitin pathway in dedifferentiation, we tested the progression of protoplasts into S phase in the presence of MG132, a specific inhibitor of the ubiquitin proteasome system. MG132 had no effect on the occurrence of the first phase chromatin decondensation (data not shown). Freshly prepared protoplasts induced to re-enter the cell cycle were incubated with MG132 at various time points (0, 24, and 48 h), and their progression into S phase was monitored by FACS 72 h after preparation. Addition of MG132 at times 0 and 24 h completely abolished progression of protoplasts into S phase (Fig. 6, *A* and *B*). When MG132 was added after 48 h (the time at which about 10% of the protoplasts approached S phase (see Fig. 3*C*)), the FACS histogram showed two peaks corresponding to G_1 and G_2 content of DNA; no or very small amount of S phase protoplasts could be detected (Fig. 6*C*). Notably, treatment of protoplasts with MG132 diminished the supra-G_1 peak (Fig. 6, compare *C* with *E*). In contrast, the protease inhibitor leupeptin, used as a control in these experiments, enhanced protoplast progression into S phase (Fig. 6*D*). These findings demonstrate the indispensability of the ubiquitin proteolytic system for the second phase chromatin decondensation and for protoplasts entry into S phase; progression from S to G_2 phase is proteasome-independent. Consistent with the requirement for the proteasome system, Skp1 mRNA, a component of the cell cycle-regulated E3 ubiquitin ligase (SCF), is absent in leaves, but its level is markedly increased in protoplasts progressing into S phase (Fig. 6*F*).

DISCUSSION

The results presented here demonstrate that chromatin decondensation is an integral part in cellular dedifferentiation. Tobacco mesophyll cells re-entering the cell cycle displayed two successive phases of increased PI fluorescence intensity of nu-

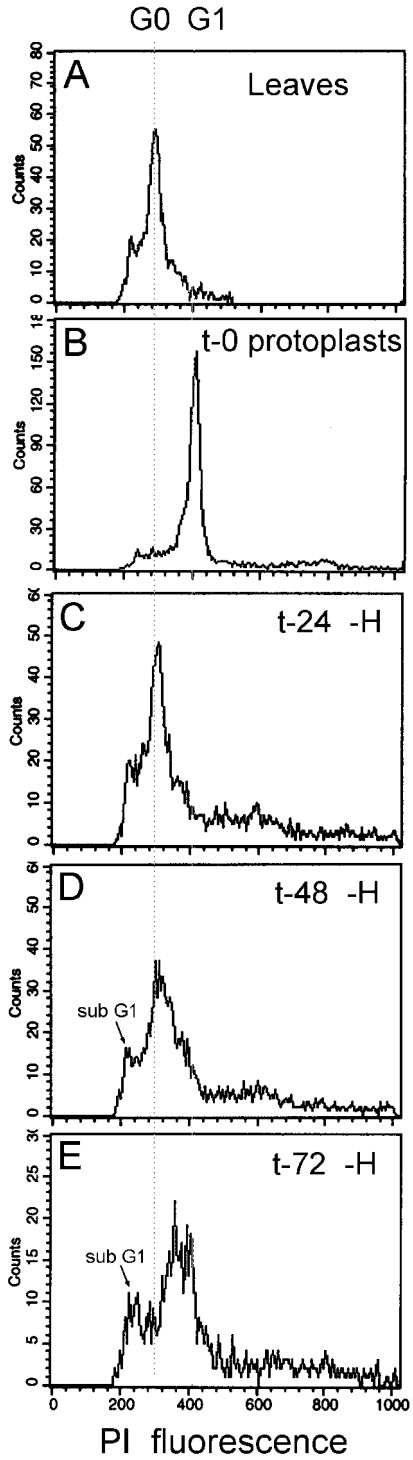

FIG. 2. **Protoplasts undergo cell death in the absence of phytohormones.** Protoplasts grown in the absence of hormones (*−H*) were sampled at time 0, 24, 48, and 72 h (*B-E*, respectively). Nuclei were prepared, stained with PI, and subjected to FACS analysis. The FACS histogram of leaf nuclei (*A*) is shown. The positions of *G0* and *G1* nuclei are marked by *dashed lines* and *sub G1* is indicated by an *arrow*.

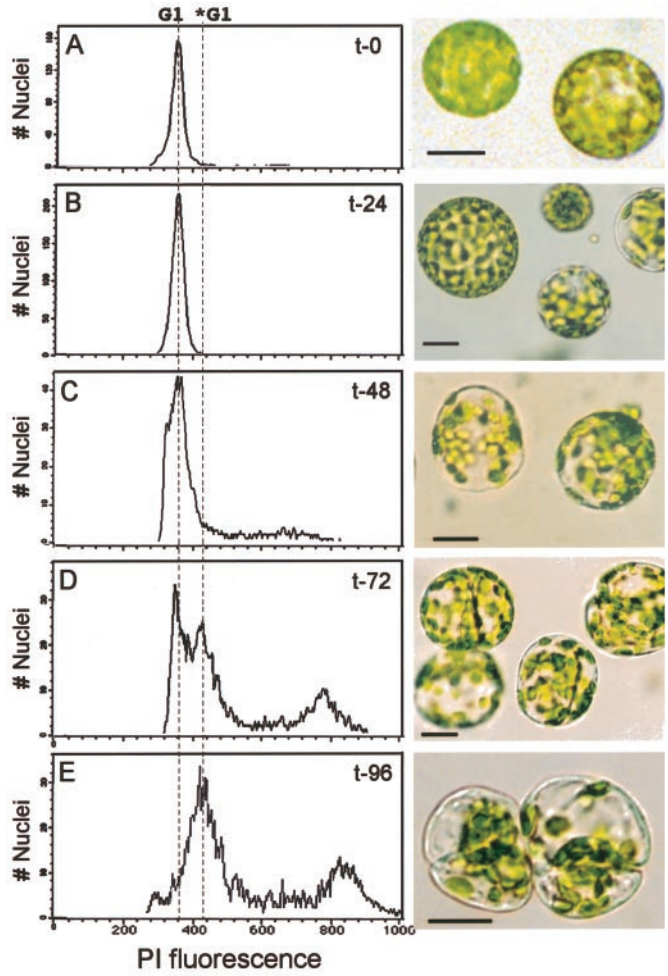

FIG. 3. **Protoplasts approaching S phase display a second phase chromatin decondensation.** Protoplasts were induced to reenter the cell cycle by auxin and cytokinin. At the indicated time points after hormonal induction (*t-0* to *t-96*), nuclei were isolated, and progression of protoplasts into S phase was monitored by FACS analysis (*A-E*). The appearance of protoplasts is shown (*right panels*, *size bars* = 20 μm); cell division patterns are seen at 72 and 96 h. Note the additional G_1 peak designated supra-G_1 (**G1*) at *D*, a characteristic feature of protoplasts re-entering the cell cycle. To compare changes in fluorescence intensity in both G_1 and G_2 nuclei, we changed the amplifier gains for FL-2 to put the G_1 peak at about channel 360.

clei prior to reactivation of S phase. These phases reflect increased accessibility of DNA to PI conferred by chromatin relaxation rather than DNA synthesis. The first phase (transition from G_0 to G_1), which occurs in the course of protoplast isolation, is ubiquitin-independent and does not require exogenous application of auxin and cytokinin. The second phase (transition from G_1 to supra-G_1) is auxin/cytokinin-dependent and occurs just before entry of cells into S phase. In agreement with these findings, an extra G_1 peak, not attributed to DNA synthesis but rather to conformational changes in chromatin, has been recorded by FACS in dedifferentiating red blood cells of frog (13). Also, two phases of chromatin decondensation, prior to reactivation of DNA synthesis, were found in chicken erythrocyte nuclei incubated in *Xenopus* egg extract (17). The mechanism underlying chromatin remodeling of somatic nuclei transplanted into *Xenopus* egg extract has recently been shown to require the activity of the chromatin-remodeling nucleosomal adenosine triphosphatase (ATPase) ISWI (19), a member of the SWI2/SNF2 superfamily. The ISWI is a subunit of several distinct nucleosome remodeling complexes that increase the accessibility of DNA in chromatin (20). Taken together, it appears that chromatin decondensation is a common mechanism underlying cellular dedifferentiation both in plants and animals. We propose that the first phase of chromatin decondensation represents a transitory phase that is necessary for activation of genes whose products are required for the establishment of competence for cell fate switch (Fig. 7). The ensuing cell fate determination may be governed by growth factors and environmental signals. In plant cells, auxin and cytokinin in-

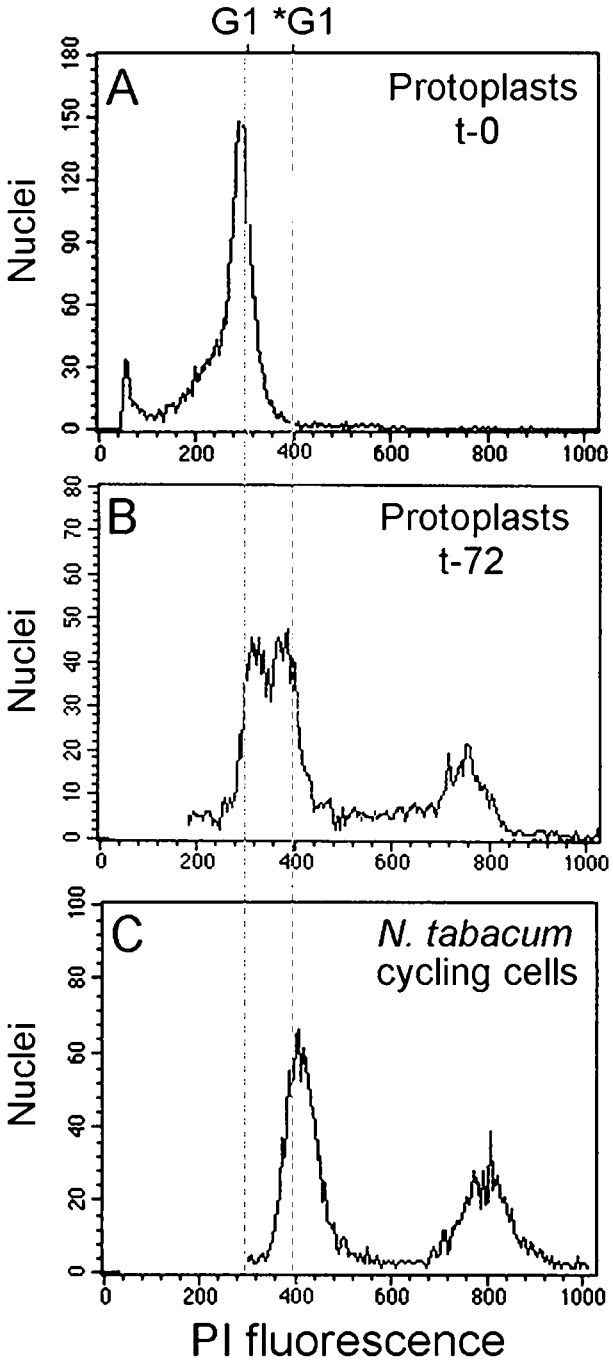

FIG. 4. **Supra-G_1 nuclei converge with the G_1 nuclei of cycling cells.** Nuclei were prepared from t-0 protoplasts (*A*), t-72 protoplasts (*B*), and from *N. tabacum* cycling cells (*C*) and subjected to FACS analysis. To compare changes in fluorescence intensity in both G_1 and G_2 nuclei, we changed the amplifier gains for FL-2 to place the G_1 peak at about channel 300.

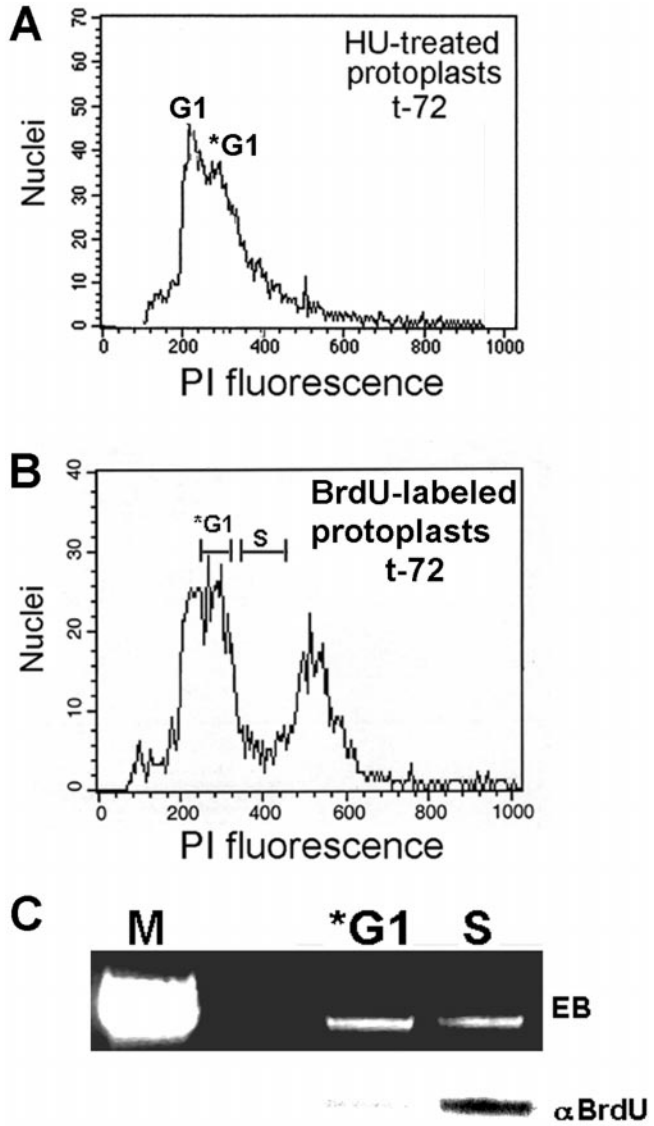

FIG. 5. **The supra-G_1 peak does not reflect initiation of DNA synthesis.** *A*, FACS histogram of HU-treated protoplasts. HU (5 mM) was applied after 36 h and progression into S phase was analyzed 72 h after protoplast preparation. The FACS *histogram* shows accumulation of G1 and supra-G_1 (**G1*) nuclei. *B,* the FACS histogram of BrdUrd-labeled t-72 protoplasts. The sorted fractions of supra-G_1 (**G1*) and S phase nuclei are indicated. *C,* detection of BrdUrd-labeled DNA. DNA was extracted from sorted nuclei and separated on a 0.8% agarose gel containing ethidium bromide (*EB*, *upper panel*). DNA was Southern blotted and subjected to Western analysis using BrdUrd antibody (*αBrdU*, *lower panel*). *M* indicates the *Bam*HI- and *Eco*RI-digested lambda DNA marker (the slow migrating fragment is shown).

duce dedifferentiation and re-entry into the cell cycle, auxin by itself may induce redifferentiation (21), whereas in the absence of phytohormones, cells die.

The occurrence of the supra-G_1 peak appears to be a unique feature of differentiated cells re-entering the cell cycle not found in cycling cells. The accumulation of protoplasts at the supra-G_1, just before entry into S phase, indicates that the transition rate from G_1 to supra-G_1 is faster than from supra-G_1 to S phase. This progression pattern may mark a checkpoint control known as the restriction point in animals or START in yeast, a point of commitment to the mitotic cell cycle (22).

The stepwise manner by which chromatin decondensation occurs points to various levels of heterochromatin compaction, *i.e.* heterochromatin may not be a homogeneous but rather a heterogeneous structure displaying different degrees of chromatin condensation. This assumption is supported by recent findings demonstrating variations in chromatin (de)condensation within heterochromatin segments in meiotic chromosomes of *Arabidopsis* (23). We propose that the first phase of chromatin decondensation occurs in a less condensed heterochromatin, often called facultative heterochromatin, which is likely to be located at the boundary region between eu- and heterochromatin. This border chromatin might have an important biological significance in cellular plasticity, *i.e.* the interplay between differentiation, proliferation, and cell death.

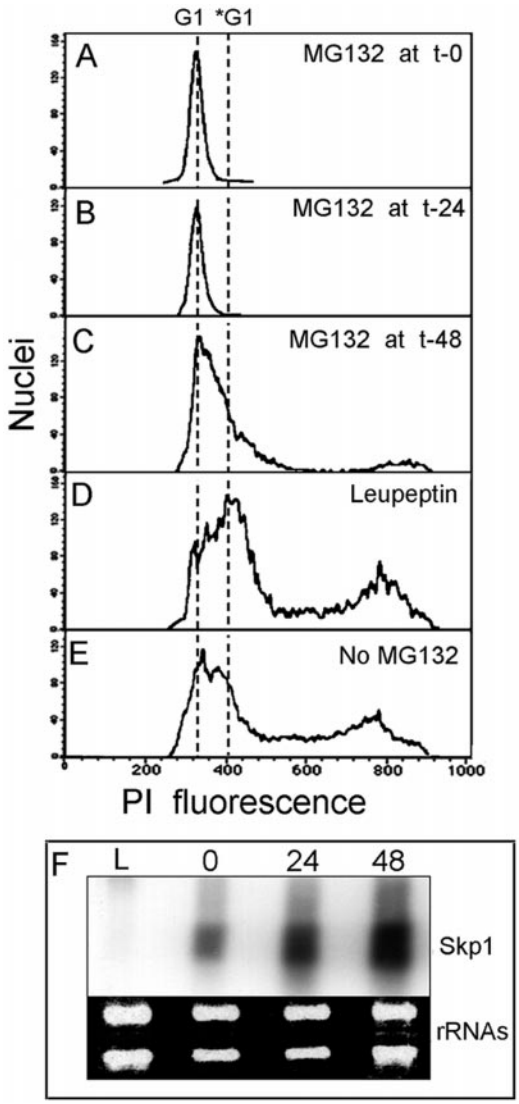

FIG. 6. **The second phase chromatin decondensation and re-entry into the cell cycle require the activity of the ubiquitin proteasome system.** MG132 (100 μM) was added to protoplasts at the indicated time points (0, 24, and 48 h) and progression into S phase was determined by FACS 72 h after preparation (*A-C*). *D*, FACS histogram of protoplasts treated with 100 μM protease inhibitor leupeptin applied at t-0. *E,* untreated protoplasts. The positions of G_1 (*G1*) and supra-G_1 (**G1*) are indicated. *F,* the mRNA level of Skp1 increases during protoplasts progression into S phase. Northern blot analysis of total RNAs (*upper panel*) isolated from tobacco leaves (*L*) or from protoplasts at the indicated times (0, 24, and 48 h). The amount of RNA loaded in each lane was estimated by the relative ethidium bromide staining of ribosomal RNAs (*rRNAs*, *lower panel*).

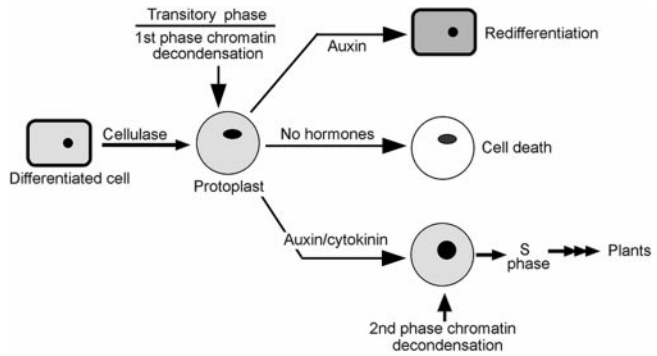

FIG. 7. **A model proposing the distinction between competence for cell fate switch and cell fate determination.** A differentiated plant cell responds to removal of cell wall (cellulase) by decondensing its chromatin (*1st phase*), an event marking a transitory phase, *i.e.* competence for cell fate switch. This phase of chromatin decondensation appears to be essential, though not sufficient, for re-entry of cells into S phase. At this stage, additional signals determine cell fate: auxin and cytokinin induce chromatin decondensation (*2nd phase*), followed by reactivation of S phase and proliferation, eventually giving rise to production of new plants. Auxin by itself may induce redifferentiation (19), whereas in the absence of hormonal application, cells die.

The role played by the ubiquitin system in regulating cell cycle progression has been well documented in animals (10–11) and recently described also in plants (24). By using a specific inhibitor to the proteasome system we demonstrated its role in driving protoplasts into S phase. The fact that treatment of protoplasts with the proteasome inhibitor MG132 eliminated the supra-G_1 and, consequently, entry into S phase supports the notion that DNA decondensation is essential for reactivation of DNA synthesis (25). Furthermore, our results suggest that the activity of the ubiquitin proteolytic system plays a role, either directly or indirectly, in DNA relaxation, thereby enabling the assembly of replication proteins onto origins to initiate DNA replication.

It is long standing that auxin and cytokinin are required to induce cell division in plant tissue culture (26) and are indispensable for the progression of protoplasts into S phase (3–5). Auxin and cytokinin presumably evoke signaling pathway(s) leading to a commitment for the mitotic cell cycle and reactivation of DNA replication (4, 27, 28). Notably, several mutations that confer resistance to auxin were found to encode components of the cell cycle-regulated ubiquitin ligase complex SCF (12) required for G_1-S transition (10, 11). This implies that auxin exerts its effect, at least in part, by inducing an orderly regulated protein destruction *via* the ubiquitin proteasome system. Given that the ubiquitin proteolytic system is required for redifferentiation of *Zinnia* mesophyll cells into tracheary elements (29), we suggest that the ubiquitin proteolytic system is not only involved in cell cycle progression but also in regulating cell fate determination.

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