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Chromatin Reorganization Accompanying Cellular Dedifferentiation Is Associated With Modifications of Histone H3, Redistribution of HP1, and Activation of E2F-Target Genes

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The remarkable regeneration capacity of plant cells is based on their capability to dedifferentiate. We recently reported that cellular dedifferentiation proceeds through two distinct phases, each accompanied by chromatin decondensation: acquisition of competence for fate switch followed by a signal-dependent reentry into S phase (Zhao et al. [2001] J. Biol. Chem. 276:22772-22778). The purpose of this study was to (1) characterize changes in chromatin factors associated with chromatin decondensation, and (2) study the relationship between chromatin decondensation and transcriptional activation of pRb/E2F-regulated genes. We show that plant cells competent for fate switch display a disruption of nucleolar domain appearance associated with condensation of 18S ribosomal DNA, as well as modifications of histone H3 and redistribution of heterochromatin protein 1 (HP1). We further show that the pRb/E2F-target genes *RNR2* and *PCNA* are condensed and silent in differentiated leaf cells but become decondensed, although not yet activated, as cells acquire competence for fate switch; transcriptional activation becomes evident during progression into S phase, concomitantly with pRb phosphorylation. We propose that chromatin reorganization is central for reversion of the differentiation process leading to resetting of the gene expression program and activation of silent genes. *Developmental Dynamics 228:113–120, 2003.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Cellular plasticity signifies the capability of differentiated cells to reverse the differentiation process and acquire new fates. One way by which cellular plasticity is manifested is through dedifferentiation, a process underlying regeneration as well as carcinogenesis. Most studies related to cellular dedifferentiation in animals focused on the G1 to S transition in cultured cells. The question, however, of how cells reverse the differentiation process and acquire competence for fate switch has hardly been addressed, largely because of lack of a suitable experimental system. It appears that plant cells, which naturally exhibit remarkable plasticity, are very suitable for studying early events related to cell fate switch. By using the plant protoplast (plant cells devoid of cell wall) regeneration system (Takebe et al., 1971), we recently demonstrated that dedifferentiation of mature plant cells proceeds through two distinct phases: acquisition of competence for cell fate-switch followed by hormone-dependent reentry into S phase; each of these phases is accompanied by chromatin decondensation (Zhao et al., 2001).

Dynamic changes in chromatin structure are directly influenced by posttranslational modifications of the amino terminal tails of core histone proteins, H2A, H2B, H3, and H4,

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that build up the basic structural unit of chromatin, the nucleosome (van Holde, 1989; Wolffe, 1992). These modifications (e.g., methylation, acetylation, and phosphorylation) generate a "code" for the recruitment of proteins or protein complexes that affect chromatin structure and function (Jenuwein and Allis, 2001; Zhang and Reinberg, 2001). Histone acetylation is often associated with active chromatin, whereas deacetylation and further methylation at specific amino acid residues induce heterochromatinization and transcriptional repression (Jenuwein and Allis, 2001; Lusser, 2002; Eberharter and Becker, 2002). For example, methylation of histone H3 at lysine 9 generates a 'code' for the recruitment of heterochromatin protein 1 (HP1), thereby inducing the assembly of restrictive chromatin and, consequently, gene silencing (Bannister et al., 2001; Lachner et al., 2001). Although HP1 proteins are predominantly distributed in heterochromatic regions (Eissenberg and Elgin, 2000), they can be recruited to promoter regions of euchromatic genes, such as E2F-target genes, through interaction with the retinoblastoma (pRb) protein (Williams and Grafi, 2000; Nielsen et al., 2001). In human and plant cells, pRbs are potent inhibitors of gene transcription mediated by the E2F family of transcription factors. After interaction of hypophosphorylated pRb with E2F, an active transcription repressive complex is formed that constrains expression of E2F-target genes and, consequently, the progression of cells into S phase (Herwig and Straus, 1997; Dyson, 1998; DeGregori, 2002; Shen, 2002). The pRb-E2F repressive complex acts by sequestering transcription activators, and/or by recruiting various chromatin modifiers, such as polycomb proteins, heterochromatin protein 1 (HP1), histone deacetylases (HDACs), as well as DNA and histone methyltransferases (Herwig and Straus, 1997; Dyson, 1998; Brehm and Kouzarides, 1999; Robertson et al., 2000; Williams and Grafi, 2000; Dahiya et al., 2001; Nielsen et al., 2001). Hence, the activity of pRb as a master regulator of cellular differentiation and proliferation may be accomplished through its effect on chromatin configuration of specific genes such as E2F-target genes.

The purpose of the present study was to (1) characterize changes in chromatin factors associated with chromatin decondensation, and (2) study the relationship between chromatin decondensation and transcriptional activation of pRb/E2F-regulated genes during cellular dedifferentiation. By using the plant protoplast system, we showed that chromatin reorganization accompanying cell fate switch involves a disruption in the nucleolar domain appearance and condensation of 18S rDNA, modifications of histone H3, and redistribution of HP1 proteins. pRb/E2F-target genes underwent chromatin decondensation as cells became competent for fate switch. Activation of these genes occurred before entry of cells into S phase, concomitantly with phosphorylation of pRb.

RESULTS

Acquisition of Competence for Cell Fate-Switch Correlates With Chromatin Reorganization, Redistribution of HP1 Protein, and Modifications of Histone H3

The tobacco protoplast (plant cells devoid of cell wall) regeneration system (Takebe et al., 1971) provides a suitable experimental tool for studying the biochemical and molecular basis of cellular dedifferentiation, particularly early events related to fate switch and determination. The fully differentiated, nondividing mesophyll cells of tobacco leaves can be easily separated from their original tissue by cell wall-degrading enzymes. This treatment results in the formation of a large population of fate-switch–competent protoplasts. After treatment with phytohormones (auxin and cytokinin), protoplasts can reenter the cell cycle, and after deposition of a new cell wall, they divide, proliferate, and form a callus from which shoots and roots can be regenerated to form the entire fertile plant (Fig. 1A). Nuclei prepared from differentiated leaf cells were stained with diamidino-phenyl-indole (DAPI) almost evenly and displayed well-organized nucleolar domain (Fig. 1B, left panel). However, the transition into protoplasts resulted in a marked reorganization of chromatin as evidenced by the uneven DAPI staining and the disruption of nucleolar domain appearance (Fig. 1B, right panel). Chromatin reorganization of the nucleolar domain was further assessed by fluorescence in situ hybridization (FISH). Fixed nuclei from leaves and protoplasts were probed with18S rDNA labeled with fluorescein-in-12-dUTP. Results show (Fig. 1C) that in leaf nuclei the 18S rDNA is mainly dispersed within the nucleolar compartment, whereas in fate-switch–competent protoplasts it appears more condensed. These results corroborate our previous findings demonstrating changes in chromatin structure in cells acquiring competence for fate switch (Zhao et al., 2001).

Further support for chromatin reorganization in protoplasts was demonstrated by the redistribution of the HP1 protein. The human HP1γ fused to GFP, when expressed in transgenic tobacco plants or cultured cells, was strictly localized to the nucleus, whereas in fate-switch-competent protoplasts a fraction of the GFP-HP1γ was found in the cytoplasm (Fig. 2A, see also Fass et al., 2002). These results were supported by salt extraction of nuclei showing that, in leaf nuclei, the GFP-HP1γ was tightly bound to chromatin and could not be released even at high salt concentration (Fig. 2B, upper panel). However, the transition into protoplasts resulted in the release of a fraction of GFP-HP1γ from chromatin upon extraction with low concentration of NaCl (Fig. 2B, lower panel). The release of GFP-HP1γ was associated with increase in acetylated histone H3 at K9 and K14 and modifications of K9-methyated histone H3 as demonstrated by the mobility shift of methylated H3 upon transition into protoplasts (Fig. 2C). Although the nature of the modifications in methylated K9 histone H3 is unknown, acetylation of histone H3 has been correlated with chromatin decondensation and transcriptional activation (Spencer and

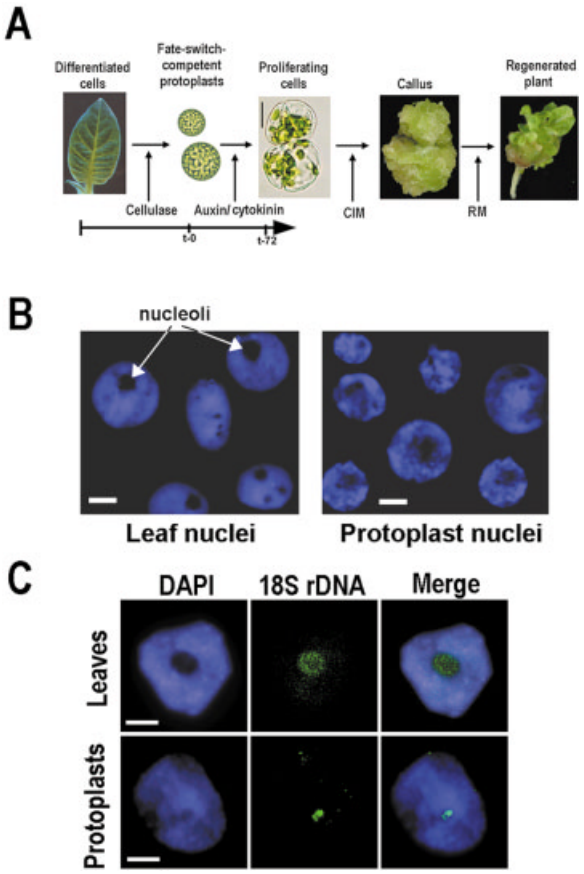

Fig. 1. Acquisition of competence for cell fate-switch is associated with chromatin reorganization. **A**: Representation of the experimental system used to study cellular dedifferentiation. Leaf cells were separated from their tissue by treatment with cell wall-degrading enzymes (cellulase) for 12 hr, yielding a large population of fate-switch–competent protoplasts (plant cells devoid of cell wall). Application of the phytohormones auxin- and cytokinin-induced protoplasts (t-0) to reenter the cell cycle and proliferate. Proliferating cells incubated on callus-inducing medium (CIM) form callus from which roots and shoots can be regenerated to form the entire plant. RM indicates regeneration medium. **B**: Diamidino-phenyl-indole (DAPI) staining of nuclei prepared from differentiated leaf cells and fate-switch–competent protoplasts (P t-0). Note the disruption of nucleolar domains in protoplasts. **C**: The 18S rDNA undergoes condensation in fate-switch–competent protoplasts. FISH analysis of nuclei prepared from leaves (upper panel) and protoplasts (lower panel) using fluorescein-labeled 18S rDNA. DAPI was used as a counterstain. Scale bars = 10 μm in B,C.

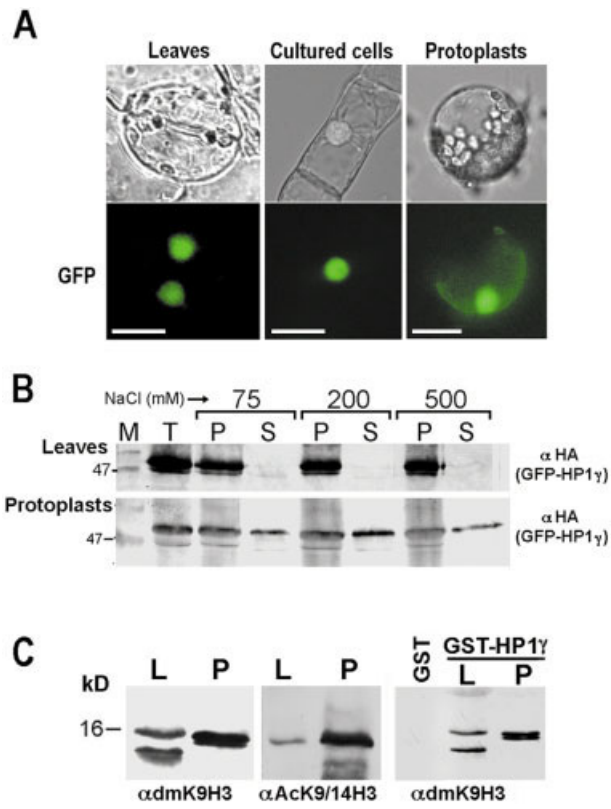

Fig. 2. Redistribution of heterochromatin protein 1 (HP1) and modifications of histone H3 in cells competent for fate switch. **A**: Fluorescence microscopy analysis of GFP-HP1γ distribution in leaf cells (guard cells), cultured cells, and protoplasts. Note the release of GFP-HP1γ into the cytoplasm in fate-switch–competent protoplasts. **B**: Nuclei prepared from leaves (upper panel) or from protoplasts (lower panel) were extracted with increasing concentrations of NaCl, and soluble (S) and insoluble pellet (P) fractions were analyzed for the presence of GFP-HP1γ by immunoblotting using anti-HA. T is total protein extract, and M is molecular weight markers. **C**: Histone H3 is modified when cells become competent for fate switch. Acid soluble fraction prepared from tobacco leaves (L) or protoplasts (P) was separated on 18% sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by immunoblotting either with anti-dimethylated K9 histone H3 (αdmK9H3), or anti-acetylated K9/K14 histone H3 (αAcK9/14H3), or subjected to GST pull-down assay by using GST-HP1γ followed by immunoblotting with αdmK9H3. Scale bars = 40 μm in A.

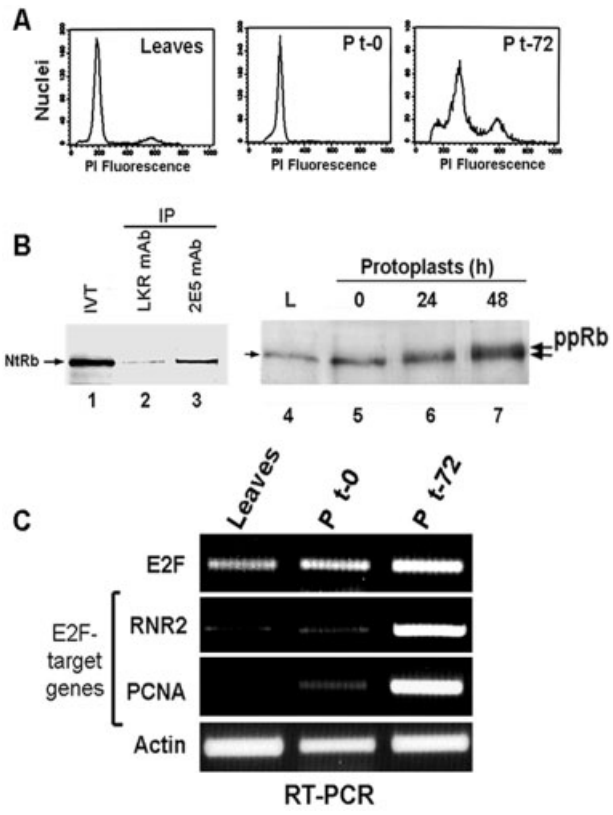

Fig. 3. The E2F-target genes *RNR2* and *PCNA* are down-regulated in differentiated leaf cells and up-regulated during dedifferentiation. **A**: Fluorescence activated cell sorting (FACS) analysis of leaf cells undergoing dedifferentiation. Nuclei prepared from leaves, fate-switch–competent protoplasts (P t-0), or protoplasts approaching S phase (P t-72) were stained with propidium iodide and subjected to FACS analysis. **B**: The retinoblastoma protein undergoes phosphorylation (ppRb) before entry of cells into S phase. The 2E5 monoclonal antibody precipitated the in vitro translated (^{35}S)methionine-labeled *Nicotiana tabacum* Rb (NtRb, lane 3). IVT (lane 1) indicates 50% of the in vitro translated NtRb; LKR mAb (lane 2) is a reference antibody raised to the *Arabidopsis* lysine–ketoglutarate reductase. pRb was immunoprecipitated (2E5 mAb) from 1 mg of total protein extract from tobacco leaves (lane 4), or from protoplasts progressing into S phase (lanes 5-7) and immunoblotted by using 2E5 mAb. Note the occurrence of a diffused pRb bands in cells approaching S phase (lane 7), which is attributed to pRb phosphorylation. **C**: E2F-target genes are down-regulated in differentiated leaf cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of E2F and the E2F-target genes *RNR2* and *PCNA*. Actin was used as a reference. PCNA, proliferating cell nuclear antigen.

Davie, 2001). Because HP1 proteins can interact with K9-methylated histone H3 (Bannister et al., 2001; Lachner et al., 2001), we hypothesized that the modifications in K9-methylated histone H3 render GFP-HP1γ incapable of binding modified histone H3. However, Figure 2C shows that histone H3 modifications cannot account directly for the release of GFP-HP1γ from chromatin inasmuch as HP1γ was still capable of binding modified K9-methylated histone H3 in a GST pull-down assay.

pRb/E2F-Target Genes Are Down-regulated in Differentiated Leaf Cells and Up-regulated Before Entry of Cells Into S Phase

The capability of pRb to bind HP1γ led to the hypothesis that pRb could bring euchromatic genes (e.g., E2F-target genes) into close proximity with heterochromatin, thereby inducing gene compaction and silencing (Williams and Grafi, 2000; Nielsen et al., 2001). We, therefore, studied the relationship between chromatin reorganization accompanying cellular dedifferentiation and the activation of pRb/E2F-regulated genes by using the protoplast system. Analyzing the progression of protoplasts into S phase by fluorescence activated cell sorting (FACS) (Fig. 3A), we found that fate-switch–competent protoplasts reentered the cell cycle 72 hr after hormone application (P t-72, Fig. 3A, right panel). To study the expression pattern of pRb in differentiated cells and during cellular dedifferentiation, we used 2E5 monoclonal antibody raised to maize Rb (Grafi et al., 1996), shown to immunoprecipitate the tobacco Rb (NtRb; Fig. 3B, lanes 1-3). NtRb was hypophosphorylated in differentiated leaf cells (Fig. 3B, lane 4; pRb appearing as a sharp band) but became phosphorylated during progression of cells into S phase, as evident by the diffused protein band (Fig. 3B, lanes 6-7), a typical appearance of phosphorylated pRb in plants (Grafi et al., 1996). These results are consistent with previous findings showing that dedifferentiation of animal cells is associated with phosphorylation of pRb (Tiainen et al., 1996; Tanaka et al., 1997). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed (Fig. 3C) that the tobacco E2F is expressed both in tobacco leaf cells and in protoplasts progressing into S phase, consistent with previous reports (Ramirez-Parra et al., 1999; Albani et al., 2000). The expression of E2F coupled with the hypophosphorylated form of pRb in differentiated leaf cells suggest that these proteins may cooperate in silencing E2F-target genes. Indeed, RT-PCR analysis showed that the small subunit of ribonucleotide reductase (RNR2, Chaboute et al., 2000) and the proliferating cell nuclear antigen (PCNA, Egelkrout et al., 2002), two plant E2F-regulated genes, are down-regulated in differentiated leaf cells. These genes did not show significant expression in fate-switch–competent protoplasts (P t-0), but were up-regulated in protoplasts approaching S phase (P t-72) (Fig. 3C), concomitantly with pRb phosphorylation.

pRb/E2F-Target Genes Become Less Condensed in Cells Acquiring Competence for Fate Switch or Progressing Into S Phase

Increasing evidence implicate chromatin structure in the repression of E2F-regulated genes in quiescent mammalian cultured cells. Such silencing may be brought about in a pRb-dependent or -independent manner through the recruitment of multiple chromatin-associated factors such as histone and DNA methyltransferases (Ogawa et al., 2002; Rayman et al., 2002). By using the MNase assay, we studied the chromatin configuration of E2F-regulated genes in differentiated cells and in cells undergoing dedifferentiation. Nuclei from tobacco leaf cells, fate-switch–competent protoplasts (P t-0), and protoplasts entering S phase (P t-72) were first incubated with MNase for various durations, and the DNA was resolved on agarose gel. MNase treatment resulted in a classic nucleosomal ladder (Fig. 4A). As previously shown, chromatin of protoplast nuclei (t-0 and t-72) was more sensitive than chromatin of leaf cell nuclei to MNase (Zhao et al., 2001). PCR analysis showed that both *RNR2* and *PCNA* were more resistant to MNase in leaf cells than in protoplasts (Fig. 4B). Southern blot analysis of the MNase-digested nuclei with ^{32}P-labeled PCNA confirmed that the *PCNA* gene was more protected in leaf nuclei than in protoplast nuclei (Fig. 4C). These results suggest that repression of *RNR2* and *PCNA* in differentiated leaf cells (Fig. 3C) is achieved, at least partly, by heterochromatinization. The decondensation of the *RNR2* and *PCNA* loci during cellular dedifferentiation appears to be specific inasmuch as a repetitive DNA sequence 3-19 (RS 3-19, Yang and Chen, 2001) remained relatively resistant to MNase both in differentiated leaf cells and in protoplasts (Fig. 4B).

DISCUSSION

Chromatin reorganization accompanying acquisition of competence for cell fate-switch has been demonstrated in the present work in various ways. DAPI staining of nuclei clearly demonstrated the reorganization of chromatin and the disruption of the nucleolar domain, which is accompanied by condensation of the 18S rDNA; the latter is involved in the biogenesis of ribosomes (Leary and Huang, 2001). Expression of pre-rRNAs is regulated, at least partly, by the silencing of individual rDNA genes by means of alterations in chromatin structure (Pikaard, 1999; Leary and Huang, 2001). Hence, the observed chromatin condensation of 18S rDNA in cells competent for fate switch might lead to gene silencing and consequently to a reduction in ribosome biogenesis. This sequence of events is consistent with a previous report showing that the number of ribosomes is dramatically reduced at or soon after protoplast isolation (Gigot et al., 1975). The reduction in ribosome production together with an increase in ribonuclease level (Lazar et al., 1973) in fate-switch–competent protoplasts may point to a state whereby existing transcription and translation of genetic products is being erased in preparation for a new cell function (Galun, 1981). Notably, the picture obtained by FACS and MNase analyses clearly pointed to chromatin decondensation as the major process accompanying acquisition of competence for cell fate-switch (Zhao et al., 2001). However, the observed condensation of the 18S rDNA suggests that changes in chromatin in cells competent for fate-switch may be better defined as chromatin reorganization.

Chromatin reorganization accompanying cell fate-switch may be achieved by modifications of histone H3 (e.g., acetylation) and redistribution of HP1 protein. We observed increased levels of acetylated histone H3 in cells competent for fate switch, a modification often associated with chromatin relaxation and gene transcription (reviewed by Dillon and Festenstein, 2002). We also found changes in the electrophoretic mobility of K9-methylated histone H3, which are attributed to posttranslational modifications. Because methylation of histone H3 at lysine 9 generates a

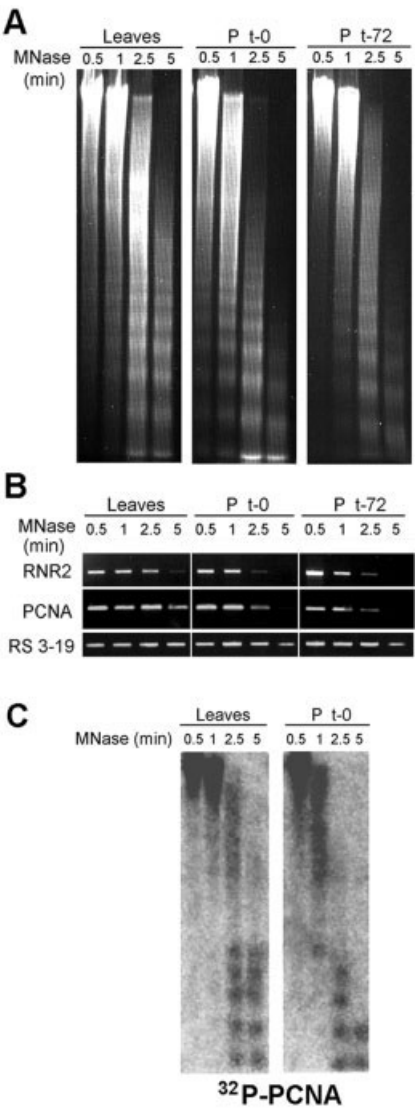

Fig. 4. E2F-target genes are in a condensed chromatin configuration in differentiated cells and undergo decondensation during cellular dedifferentiation. **A**: Nuclei prepared from leaves, fate-switch–competent protoplasts (P t-0), or protoplasts approaching S phase (P t-72) were treated with MNase for the indicated time periods. DNA was resolved by 1.4% agarose gel and stained with ethidium bromide. **B**: Assessment of chromatin configuration of *RNR2* and *PCNA* was performed by polymerase chain reaction (PCR) using as a template DNA isolated from MNase-treated nuclei. RS 3-19 indicates the PCR product of a repetitive DNA used as a reference. Note that, in differentiated leaf cells, *PCNA* is more protected than *RNR2* from MNase digestion. **C**: *PCNA* is more condensed in differentiated cells than in fate-switch–competent protoplasts. DNA from MNase-treated nuclei was resolved as in A, Southern blotted, and probed with ^{32}P-labeled PCNA. PCNA, proliferating cell nuclear antigen.

binding site for HP1 (Bannister et al., 2001; Lachner et al., 2001), we addressed the possibility that these modifications may account for the release of a fraction of GFP-HP1γ from chromatin. However, our results showed that modifications of K9-methylated histone H3 cannot directly account for this detachment inasmuch as modified K9-H3 was capable of binding GST-HP1γ in a GST pull-down assay. In view of the stable nature of lysine methylation, it is possible that modifications of K9-methylated H3 during acquisition of competence for cell fate-switch generate a "code" (Jenuwein and Allis, 2001) that is read by as yet an unknown factor(s) or protein complexes that compete out GFP-HP1γ from chromatin, leading to chromatin reorganization.

Accumulating data suggest that the transition from differentiation to proliferation is controlled by pRb; inactivation of pRb either by overexpression of viral oncogenes, reconstitution of cyclin D/CDK, or by phosphorylation induces differentiated cells to reenter S phase (Iujvidin et al., 1990; Crescenzi et al., 1995; Tanaka et al., 1997; Endo and Nadal-Ginard, 1998; Latella et al., 2001). The Rb protein appears to function as a molecular switch controlling the interplay between differentiation and proliferation, at least partly, through interaction with members of the E2F family of transcription factors; the latter regulate the expression of various genes involved in DNA replication and cell cycle progression (Dyson, 1998; DeGregori, 2002). Our results suggest that E2F-regulated genes are controlled in differentiated plant cells at the chromatin level by heterochromatinization. Their activation during cellular dedifferentiation occurs gradually: they become decondensed, although not yet activated, as cells become competent for fate-switch, and transcriptionally activated just before entry of cells into S phase, concomitantly with phosphorylation of pRb. Indeed, promoter occupancy of E2F-regulated genes in quiescent cultured cells points to pRb-mediated regulation of E2F-target genes at the chromatin level. Accordingly, a corepressor containing histone deacetylase complex HDAC1/mSin3B is specifically recruited, in a "pocket" protein-dependent manner, to promoters of E2F-target genes in quiescent cells but is released as cells reach late G1 (Rayman et al., 2002).

Taken together, chromatin reorganization appears to be a fundamental theme in cellular plasticity and dedifferentiation providing the means for reversion of the differentiation process and resetting the gene expression program. This mechanism leads to activation of silent genes, such as E2F-target genes, whose products may induce fate-switch and reentry into S phase. Is this mode of dedifferentiation unique to plants, or does it have bearing to dedifferentiation of mature animal cells as well?

The *Xenopus* egg cell-free extract was used extensively to study reactivation of S phase in somatic nuclei, as it provides a suitable biochemical environment that support all the nuclear events necessary for cell division cycle (Lohka and Masui, 1983). Similarly to the plant protoplast system (Zhao et al., 2001), chicken erythrocyte nuclei incubated in *Xenopus* egg cell-free extract displayed two distinct phases of chromatin decondensation before reactivation of DNA synthesis (Blank et al., 1992). The mechanism underlying chromatin remodeling of somatic nuclei transplanted into *Xenopus* egg extract has been shown to require the activity of the chromatin-remodeling nucleosomal adenosine triphosphatase (ATPase) ISWI (Kikyo et al., 2000), 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 (Varga-Weisz and Becker, 1998). In view of the wide occurrence of chromatin reorganization in a variety of dedifferentiating eukaryotic cells (Chiabrera et al., 1979; Fontes et al., 1980; Blank et al., 1992), and the high similarity in the regulation of chromatin structure between plants and animals, chromatin dynamics might be important for the regenerative capacity not only of plant cells but of animal cells as well.

EXPERIMENTAL PROCEDURES

Protoplast Isolation, Nuclei Preparation, FACS, and Micrococcal Nuclease Analysis

Protoplasts were isolated from leaves of *Nicotiana tabacum* ("Samsun NN") or from transgenic tobacco expressing GFP-HP1γ (Fass et al., 2002) essentially as described (Zelcer and Galun, 1976). The distribution of GFP-HP1γ in leaf cells, cultured cells, and protoplasts was inspected by a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonics) using Olympus filter U-MWIBA2 to detect GFP.

Freshly prepared tobacco protoplasts were washed and induced by phytohormones to reenter the cell cycle as described (Zhao et al., 2001). Nuclei from mature leaf cells, freshly prepared protoplasts (t-0), and protoplasts progressing into S phase (t-72) were prepared as described (Zhao et al., 2001). Nuclei were resuspended in a FACS buffer (10 mM MES, 0.2 M sucrose, 0.01% Triton X-100, 2.5 mM EDTA, 2.5 mM dithiothreitol) and subjected to FACS analysis by using FACSort (Becton, Dickinson). In addition, nuclei were stained with 10 μg/ml DAPI (Sigma) and inspected by a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonics) using Olympus filter U-MNU. Equal amounts of nuclei (determined by pack volume and relative density) were subjected to MNase digestion, and DNA was extracted and precipitated as previously described (Zhao et al., 2001). The DNA samples were resuspended in 30 μl of H_2O, treated with RNase A (20 μg/ml, 25 min at room temperature), and resolved by 1.6% agarose gels followed by staining with ethidium bromide. The chromatin configuration of various genes was assessed either by Southern blot of the MNase-digested DNA using ^{32}P-labeled RNR2 or PCNA or by PCR using the following primers: NtRNR2-Sense (S) (5′-ATGCCTCTAATTCCAGAAGAGCC-3′); NtRNR2-Antisense (AS) (5′-GAAGTCTTCATCCAACTTGAACTC-3′) (RNR2 accession no. X92443); NtPCNA-S (5′-GTCCTTTCCCTCCTCCCCATTTCAG-3′); NtPCNA-AS (5′-CAAACATGAA-

AGTGACGGTGTCAC-3′) (PCNA accession no. AB025029); NtRS-3-19-S (5′-CATCTCTGTATAACGATCCGATCG-3′); and NtRS-3-19-AS (5′-CAACAATTTGAATCCCATGAAATCG-3′). PCR fragments were separated in 1% agarose gel containing ethidium bromide. Each PCR experiment was performed two to three times to ensure accuracy and reproducibility of the assays.

Salt Extraction of Nuclei, Histone Extraction, Immunoblotting, Immunoprecipitation, and GST Pull-down Assay

Nuclei were extracted with increasing concentrations of NaCl as described (Remboutsika et al., 1999). Core histone proteins were extracted with 2% trichloroacetic acid as described (Zhao and Grafi, 2000). Proteins were resolved by SDS/PAGE and immunoblotted with the following antibodies: anti-HA (Covance, BAbCO) to detect GFP-HP1γ, anti-dimethylated (K9) histone H3, and anti-acetylated (K9 and K14) histone H3 (Upstate Biotechnology). Immunoprecipitation of pRb was performed with 2E5 monoclonal antibody (100 μl of supernatant hybridoma cells) and 1 mg of total protein extract from various tissues essentially as described (Grafi et al., 1996). GST pull-down assay was performed using the GST-HP1γ previously described (Fass et al., 2002).

RT-PCR Analysis

Total RNA was extracted from tobacco leaves, freshly prepared protoplasts (t-0), and protoplasts progressing into S phase (t-72) by using EZ-RNA kit (Biological Industries, Bet Haemek, Israel). RNA was treated with DNase for 30 min followed by phenol/chloroform extraction and ethanol precipitation. First-strand cDNA synthesis was performed with 2 μg of total RNA primed with oligo $(dT)_{18}$ by using the Superscript RT II kit (Gibco BRL) according to the manufacturer's protocol. First-strand DNA was used as a template for semiquantitative PCR amplification, and the resultant fragments were resolved in 1% agarose gel containing ethidium bromide. The following primers were used: NtRNR2-S; NtRNR2-AS; NtPCNA-S; NtPCNA-AS; NtE2F-S (5′-GAAGTTGATGATAGTGTTACAAG3′); NtE2F-AS (5′-CTCCTCCATTCTCCTCAGTAG-3′) (E2F accession no. AB025347); Actin-S (5′-GGTTTTGCTGGGGATGATGC-3′); and Actin-AS (5′-CATGGCTGGCACATTGAATGTCTC-3′). PCR fragments were resolved in 1% agarose gel containing ethidium bromide.

Slide Preparation, Probe Labeling, and Fluorescence In Situ Hybridization (FISH) Assay

Fixed nuclei (5-10 μl) either from tobacco leaves or protoplasts (kept at -20°C in ethanol:acetic acid /3:1) were spread on a slide, air-dried, and incubated in 100% ethanol for 1 hr at room temperature. Slides were then air-dried and incubated for 6 min in a fixative solution containing freshly prepared 2% paraformaldehyde in 1 $\times$ SSC. Slides were washed three times, 5 min each, with 2 $\times$ SSC and subjected to denaturation solution containing 70% formamide in 1 $\times$ SSC at 60°C for 3 min followed by sequential washes, 3 min each, in 70%, 95%, and 100% cold ethanol. Slides were air-dried and either used immediately for hybridization or kept at room temperature for 1-2 days before hybridization. The probe, 18S ribosomal DNA from *Arabidopsis thaliana*, was amplified by PCR using genomic DNA as a template and the following primers: 18S-sense (5′ GTCACCTGGTTGATCCTGCCAGTAGTC-3′) and 18S-antisense (5′-GAGAAGATCTGAGACTAGGACGGTATCTGATCG-3′). This probe was labeled directly with fluorescein-12-dUTP (Roche) using Nick Translation kit (Roche), mixed with a hybridization solution (final volume of 100 μl) containing 10% (w/v) sodium dextran sulphate, 50% deionized formamide, and 2 $\times$ SSPE, denatured at 80°C for 5 min, and cooled on ice. The probe was added to slides, covered with coverslips, and incubated at 37°C, in the dark, for 16-20 hr followed by washing essentially as described (Fransz et al., 1996). Next, slides were stained with 10 μg/ml DAPI and mounted in Vectashield. Hybridization signals were visualized by a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonics) using Olympus filters U-MNU and U-MWIBA2 to detect DAPI and fluorescein, respectively. Images were pseudocolored and merged by using TILL Vision version 3.3 software. All images were processed by using Adobe Photoshop software.

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