DEVELOPMENTAL DYNAMICS 230:12–22, 2004

RESEARCH ARTICLE

Reorganization of Specific Chromosomal Domains and Activation of Silent Genes in Plant Cells Acquiring Pluripotentiality

Yigal Avivi,[1] Vered Morad,[1] Hagit Ben-Meir,[1] Jing Zhao,[1] Khalil Kashkush,[1] Tzvi Tzfira,[2] Vitaly Citovsky,[2] and Gideon Grafi[1]*

The transition from leaf cells to protoplasts (plant cells devoid of cell walls) confers pluripotentiality coupled with chromatin reorganization. Here, we sought to identify remodeled chromosomal domains in *Arabidopsis* protoplasts by tracking DNA sequences undergoing changes in DNA methylation and by identifying up-regulated genes. We observed a reduction in DNA methylation at a pericentromeric region of chromosome 1, and up-regulation of several members of the NAC (NAM/ATAF1/CUC2) domain family, two of which are located near the telomeric region of chromosome 1. Fluorescence in situ hybridization (FISH) analysis demonstrated that both pericentromeric and telomeric subdomains underwent chromatin decondensation. This decondensation is subdomain-specific inasmuch as centromeric repeats remained largely unchanged, whereas the 18S rDNA underwent condensation. Within the pericentromeric subdomain, *VIP1*, a gene encoding a b-Zip nuclear protein required for *Agrobacterium* infectivity, was transcriptionally activated. Overexpression of this gene in tobacco resulted in growth retardation and inhibition of differentiation and shoot formation. Altogether, our data indicate that acquisition of pluripotentiality involves changes in DNA methylation pattern and reorganization of specific chromosomal subdomains. This change leads to activation of silent genes whose products are involved in acquisition or maintenance of pluripotentiality and/or the ensuing fate of the cell. *Developmental Dynamics 230:12–22, 2004.* © 2004 Wiley-Liss, Inc.

Key words: dedifferentiation; pluripotentiality; chromatin remodeling; regeneration; VIP1; NO APICAL MERISTEM (NAM); *Arabidopsis thaliana*

Received 10 September 2003; Revised 14 October 2003; Accepted 5 November 2003

INTRODUCTION

Plant cells are highly dynamic biological entities that constantly adapt to the changing environment. They often retain plasticity and have the capability to reverse the differentiation process and change their fate. The remarkable plasticity of plant cells is well exemplified by the capability of differentiated leaf cells to retain pluripotency and give rise to whole plants (Takebe et al., 1971). It has been suggested that animal cells, once committed to a specific lineage, can no longer change their fate. Yet, a wide variety of differentiated animal cells appear to retain plasticity and can be induced to assume new cell fates (Graf, 2002; Odelberg, 2002; Echeverri and Tanaka, 2002; Liu and Rao, 2003). This finding is well exemplified by nuclear cloning whereby somatic nuclei transplanted into enucleated oocytes undergo epigenetic reprogramming leading to re-establishment of pluripotency (Wilmut et al., 1997).

Cellular plasticity is often manifested through dedifferentiation, a process underlying regeneration (Sanchez-Alvarado, 2000; Tosh and Slack, 2002; Odelberg, 2002; Echeverri and Tanaka, 2002; Brockes and Kumar, 2002), nuclear cloning (Wilmut et al., 1997), as well as carcino-

[1]Department of Plant Sciences, The Weizmann Institute of Science, Rehovot, Israel
[2]Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York
Grant sponsor: Israel Science Foundation (ISF).
*Correspondence to: Gideon Grafi, Plant Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel.
E-mail: gideon.grafi@weizmann.ac.il

DOI 10.1002/dvdy.20006
Published online 26 March 2004 in Wiley InterScience (www.interscience.wiley.com).

genesis (Sell, 1993). Most biochemical studies related to cellular dedifferentiation focused on the G1 to S transition in cultured animal cells, largely ignoring the question of how mature cells acquire competence to switch fate, thus becoming pluripotent. This question has hardly been addressed, largely because of lack of a suitable experimental system. Plant protoplasts (plant cells devoid of cell walls) provide a suitable experimental tool for studying the biochemical and molecular basis underlying cellular dedifferentiation, particularly early events related to acquisition of pluripotentiality. The fully differentiated, nondividing leaf mesophyll cells can be separated from their original tissue by cell wall-degrading enzymes generating a large population of protoplasts that are not yet committed to any fate but have acquired pluripotentiality (Zhao et al., 2001). Only upon application of phytohormones (auxin and cytokinin) can these protoplasts reenter the cell cycle, proliferate, and form calli (masses of dividing cells) from which shoots and roots can be regenerated to form the entire fertile plant (Takebe et al., 1971). This system demonstrates a common attribute of plant cells, pluripotency—the capability of cloning in plants. By using the protoplast system, we recently resolved the process of cellular dedifferentiation into two distinct phases, each accompanied by a broad chromatin decondensation: acquisition of competence for pluripotentiality, followed by a signal-dependent reentry into S phase (Zhao et al., 2001; Williams et al., 2003).

Dynamic changes in chromatin structure are directly influenced by multiple reactions that modify either DNA, mostly by methylation (Cedar, 1988; Martienssen and Colot, 2001), or DNA-interacting proteins such as core histones, e.g., by acetylation or methylation (van Holde, 1989; Wolffe, 1992; Jones and Wolffe, 1999; Jenuwein and Allis, 2001). Such modifications and the ensuing changes in chromatin organization are critical for the control of the multiple functions carried out by the eukaryotic cell and, hence, for growth and development of the organism. Methylation of cytosine residues, a common modification of DNA in higher eukaryotes, has been implicated in regulating various cellular processes such as gene expression, genomic imprinting, as well as chromatin structure (Cedar, 1988; Jones and Wolffe, 1999; Brannan and Bartolomei, 1999; Alleman and Doctor, 2000). In general, although not as a rule, actively transcribed genes are hypomethylated, whereas transcriptionally repressed genes are highly methylated. Most transcriptional inhibition associated with DNA methylation involves changes in chromatin structure (Keshet et al., 1986; Buschhausen et al., 1987; Kass et al., 1997; Jones and Wolffe, 1999).

Here, we attempted to pinpoint specific chromosomal domains that undergo reorganization during the transition from leaf cells to protoplasts and the acquisition of pluripotentiality. We identified such chromosomal domains in *Arabidopsis thaliana* either by tracking DNA sequences undergoing changes in methylation pattern using the methylation-sensitive amplified polymorphism (MSAP) assay (Reyna-Lopez et al., 1997; Xiong et al., 1999) or by identifying up-regulated genes using DNA chip technology. We noted a reduction in DNA methylation at the *Athila* retroelement located at the pericentromeric region of chromosome 1. Chip analysis revealed multiple genes that became up-regulated during acquisition of pluripotentiality, among them are several NAC-domain containing genes located along the arms of chromosome 1 and near its telomere. By using fluorescence in situ hybridization (FISH), we demonstrated reorganization at specific chromosomal domains, including pericentromeric, telomeric, and nucleolar domains. A gene adjacent to the *Athila* retroelement, *VIP1*, encoding a b-Zip nuclear protein required for *Agrobacterium* infectivity (Tzfira et al., 2001, 2002), was transcriptionally activated; overexpression of this gene inhibited differentiation and shoot formation. Our results suggest that acquisition of pluripotentiality is driven by reorganization of specific chromosomal domains that leads to resetting of the gene expression program and activation of silent genes.

RESULTS

Acquisition of Pluripotentiality Is Associated With Changes in DNA Methylation Patterns and Gene Activation

To identify chromatin subdomains undergoing reorganization, we first followed DNA sequences undergoing changes in methylation pattern. DNA was extracted from leaves and pluripotent protoplasts of *Arabidopsis thaliana* and subjected to the MSAP assay using the methylation-sensitive isoschizomers *Hpa*II and *Msp*I, which recognize the CCGG restriction site. While *Hpa*II is sensitive to methylation of either cytosine (except when the external one is hemimethylated), *Msp*I is sensitive only when the external cytosine is methylated. Hence, methylation of the internal cytosine (C^mCGG) leads to differential cleavage by the two isoschizomers and to the appearance of polymorphic polymerase chain reaction (PCR) fragments. Reproducibility of the method was confirmed by subjecting samples from a given restriction-ligation-amplification reaction to the same selective amplification conditions (data not shown). Figure 1 depicts representative results of one pair of primers showing demethylation of a CCGG site during the transition from leaf cells to protoplasts. Altogether, 11 pairs of selective primers produced 667 bands of which 580 (87%) were not methylated, whereas 87 (13%) were associated with cytosine methylation at CCGG sites (Table 1). Nine different methylation patterns were observed and divided into two classes: (1) 76 bands (87% of all methylated sites) showed the same methylation pattern in leaves and protoplasts, and (2) 11 bands (13% of all methylated sites) showed methylation alterations between leaves and protoplasts (Table 1). Six of the polymorphic fragments underwent demethylation and five fragments underwent methylation. These results suggest that acquisition of pluripotentiality is correlated with changes in methylation pattern fea-

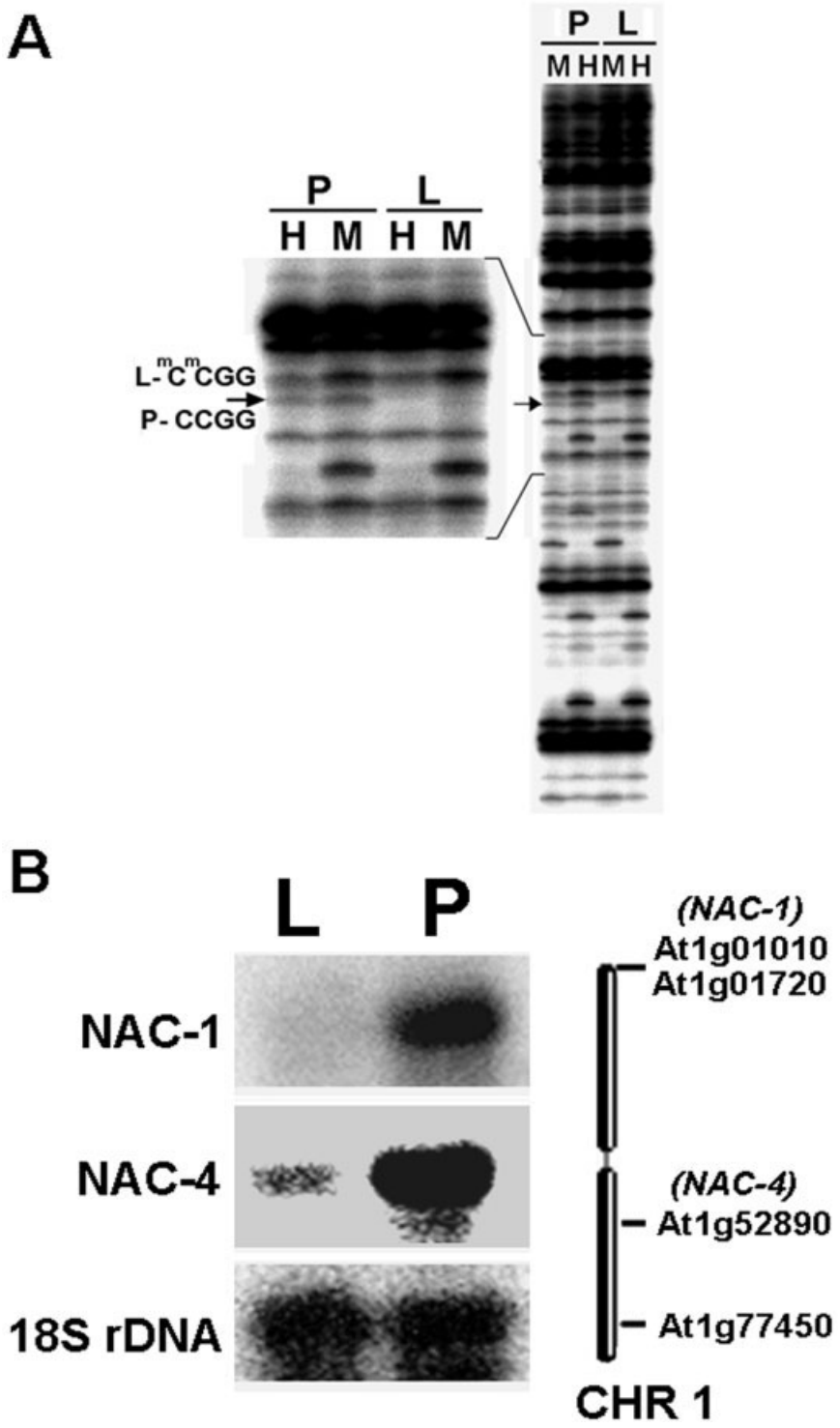

Fig. 1. Protoplasts display changes in DNA methylation patterns. **A:** Results of methylation-sensitive amplified polymorphism (MSAP) analysis using one primer combination. H and M refer to digestion with *Eco*RI+*Hpa*II and *Eco*RI+*Msp*I, respectively. A region showing alteration in methylation pattern between leaf cells (L) and protoplasts (P) is enlarged. Polymorphic fragments are indicated by arrows, and the methylation status at the CCGG site is shown. **B:** Northern blot analysis of *NAC*-like genes (At1g01010 and At1g52890, designated *NAC-1* and *NAC-4*, respectively) in leaf cells (L) vs. pluripotent protoplasts (P). The 18S ribosomal RNA was used as a reference probe. The chromosomal location of *NAC*-domain containing genes that were up-regulated in protoplasts is indicated at the right.

turing both de novo DNA methylation and demethylation. Four polymorphic fragments could be recovered from the gel, cloned, and sequenced. Blast analysis revealed that these sequences are located at different chromosomal locations and possess coding regions, chloroplast DNA, and transposable elements (Table 2). One of the isolated fragments that underwent DNA demethylation was the *Athila* retroelement located at the pericentromeric region of chromosome 1, flanking the centromeric repeats CEN180 (Pelissier et al., 1996). Bisulfite genomic sequencing of the *Athila* coding region (BAC F2J6, 86029-86144) indeed showed reduction in cytosine methylation at this region in protoplasts compared with leaf cells (data not shown).

We next used DNA chip technology to identify genes that are activated during acquisition of pluripotentiality. In preliminary experiments (three repeats), we compared mRNA of leaf cells with that of pluripotent protoplasts. We used slides

Fig. 4. Overexpression of VIP1 in tobacco suppresses differentiation and shoot formation. **A:** Gene organization at the pericentromeric region of chromosome 1 adjacent to the *Athila* retroelements (BAC F2J6). Arrow points to the chromosomal location of the DNA fragment near *Athila* that was found to undergo demethylation (dm). *VIP1*, a gene encoding a b-ZIP protein required for *Agrobacterium* infectivity, is indicated (green box). *Athila* retroelements are indicated (red boxes). RT, reverse transcriptase. **B:** *VIP1*, a b-Zip gene at the pericentromeric region of chromosome 1, is transcriptionally activated in pluripotent protoplasts and dividing cells in culture. The expression of *VIP1* and *Athila* retroelement in leaves (L), protoplasts (P), and dividing cells (DC) was analyzed by semi-quantitative RT-polymerase chain reaction. Actin was used as a reference mRNA. **C:** VIP1 is localized to the nucleus in transgenic tobacco plants expressing VIP1 fused to GFP. Guard cells are shown. **D:** Northern blot analysis showing overexpression of *VIP1* in transgenic tobacco lines 202I and 202D. WT indicates wild-type tobacco (cv. Turk) plants. **E:** Inhibition of shoot regeneration in detached leaves or calli expressing *VIP1* (two independent lines, 202D and 202I). Detached leaves or calli from wild-type (WT/Turk) or *VIP1*-over-expressig plants were incubated on shoot inducing medium and results were scored after 3 weeks.

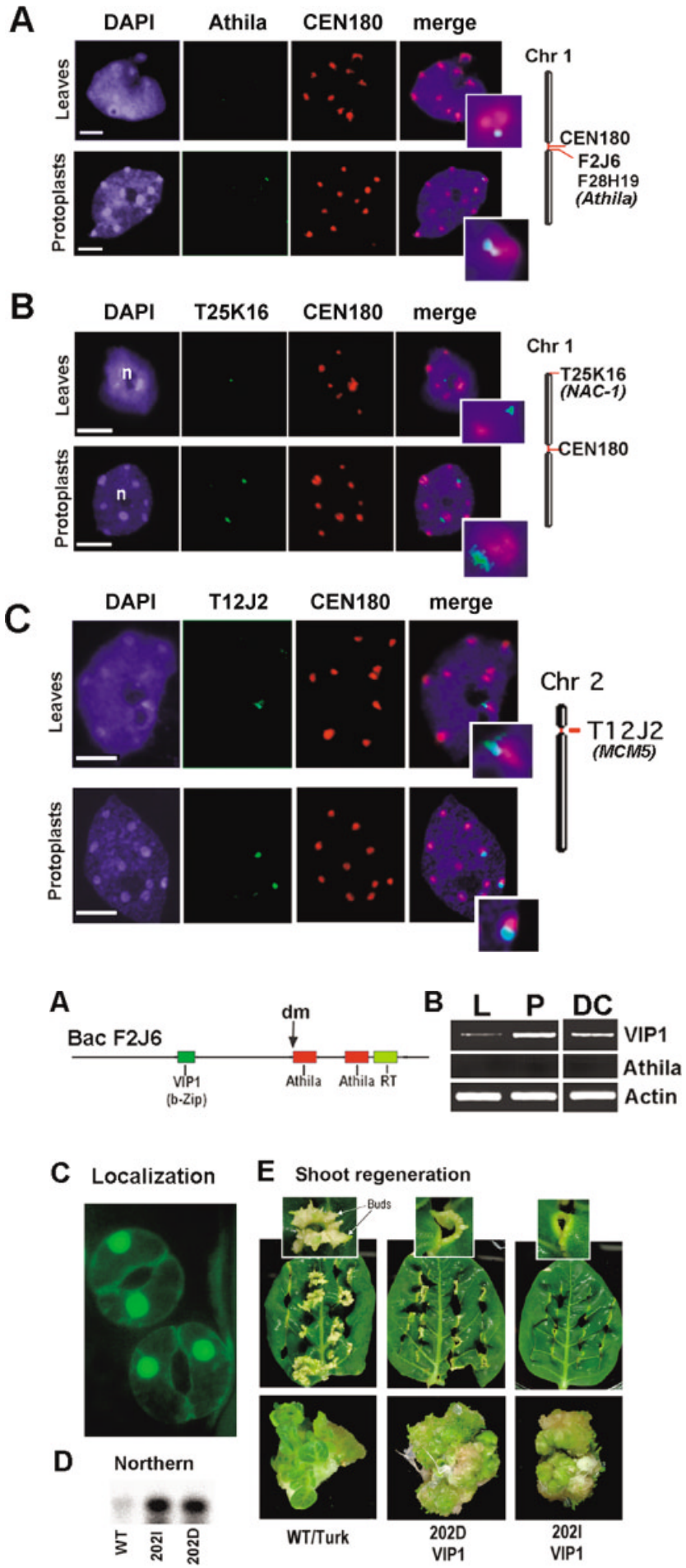

Fig. 4.

Fig. 2. Decondensation of pericentromeric and telomeric subdomains of chromosome 1. **A:** Nuclei prepared from *Arabidopsis* leaves or protoplasts were subjected to fluorescence in situ hybridization (FISH) by using rhodamine-CEN180 (red) as a reference centromeric marker and fluorescein-BAC-F2J6/BAC-F28H19 (green) for the detection of *Athila* locus. Note the extended, decondensed appearance of the *Athila* subdomain in protoplast nuclei compared with leaf nuclei, whereas CEN180 remains relatively unchanged. **B:** FISH analysis with fluorescein-BAC-T25K16 (green) to detect a telomeric subdomain of chromosome 1. Note, in protoplast nuclei, the extended configuration of the telomeric region and its proximity to CEN180. Small "n" indicates nucleolus. **C:** FISH analysis with fluorescein-BAC-T12J2 (green) to detect a pericentromeric subdomin in chromosome 2. Note that both leaf and protoplast nuclei displayed extended, relaxed chromatin configuration of this region. The chromosomal locations of the BACs and relevant genes therein are indicated at the right. DAPI was used as a counterstain. Scale bars = 5 μm in A–C.

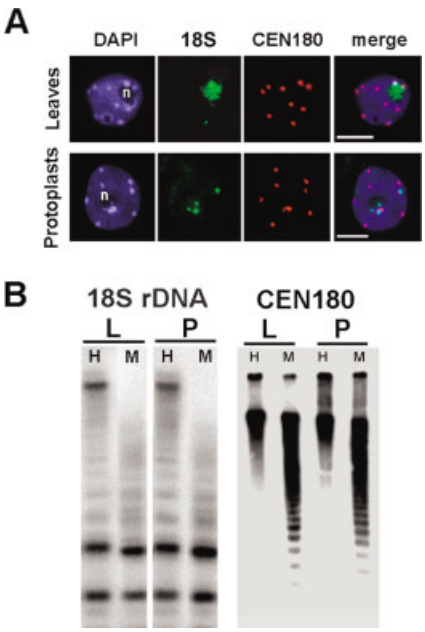

Fig. 3. Acquisition of pluripotentiality is associated with condensation of the 18S rDNA. **A:** Double-labeling fluorescence in situ hybridization (FISH) analysis of nuclei from leaves and protoplasts by using fluorescein-18S rDNA (green) and rhodamine-CEN180 (red). Note that the dispersed configuration of the 18S rDNA in leaf nuclei is lost in protoplast nuclei. Small "n" indicates nucleolus. **B:** The 18S rDNA gene and the centromeric 180 bp repeats (CEN180) show no changes in methylation patterns. Southern blot analysis of genomic DNA from leaves (L) and pluripotent protoplasts (P) after digestion with *Hpa*II (H) or *Msp*I (M). Note that CEN180 and 18S rDNA are predominantly methylated at CpG sites. Scale bars = 5 μm in A.

TABLE 1. Summary of Cytosine Methylation Patterns at CCGG Sites in *Arabidopsis* Leaf Cells and Pluripotent Protoplasts

	Leaves[a]		Protoplasts[a]		No. of	
Methylation	M	H	M	H	sites	Total (%)
No methylation	+	+	+	+	580	580 (87)
Same pattern	−	+	−	+	32	76 (11.4)
	+	−	+	−	44	
Different pattern	−	+	+	+	1	11 (1.6)
	+	−	−	−	1	
	−	+	−	−	3	
	−	−	+	−	1	
	−	−	−	+	2	
	+	+	−	−	1	
	−	−	+	+	2	

[a]Each row represents a different MSAP banding pattern in leaf cells versus protoplasts. Presence (+) or absence (−) of a band indicates DNA demethylation or methylation at CCGG sites, respectively. M, *Msp*I; H, *Hpa*II.

TABLE 2. Summary of Sequence Homology of Fragments Displaying Differential Methylation Patterns During the Transition From *Arabidopsis* Leaf Cells to Protoplasts

				Methylation pattern[a]			
				Leaves		Protoplasts	
	Primer pairs	Size (bp)	Sequence homology	M	H	M	H
1	E + AAG/HM + TCAA	242	Athila retroelement Chr 1 / BAC F2J6	−	−	+	+ (dm)
2	E + AGC/HM + TCAA	290	NADH dehydrogenase ND4 *At* Chloroplast	−	+	+	+ (dm)
3	E + ACG/HM + TCAA	66	Proline-rich protein Chr 3/ BAC T16O11	−	−	−	+ (dm)
4	E + AAC/HM + TCAA	145	Chr 2 / BAC T17H1 Homology to mtDNA	+	−	−	− (m)

[a]Each row represents a different MSAP banding pattern observed in leaf cells versus protoplasts. Presence (+) or absence (−) of a band indicates DNA demethylation (dm) or methylation (m) at CCGG sites, respectively. M, *Msp*I; H, *Hpa*II. Primer pairs are indicated where E and HM represent the *Eco*RI and the *Hpa*II/*Msp*I adapter sequences, respectively.

containing 12,000 *Arabidopsis* expressed sequence tags (ESTs; Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT), corresponding to approximately 6,000 unique genes. The transition from leaf cells to pluripotent protoplasts resulted in a highly modified transcriptome profile (detailed results will be published elsewhere). Transcripts showing a 10-fold increase in protoplasts in all three repeats were selected and found to represent 66 different genes. Of particular significance was the identification of four NAC-domain containing genes (At1g01010; At1g01720; At1g52890; At1g77450) that were up-regulated in pluripotent protoplasts. These genes are localized to chromosome 1 (Fig. 1B) and have been implicated in meristem functionality and establishment/maintenance of stem cell lineages in plants (Souer et al., 1996; Duval et al., 2002). Northern blot analysis showed that the *NAC*-like genes At1g01010 (designated *NAC-1*) and At1g052890 (designated *NAC-4*) are up-regulated in pluripotent protoplasts compared with differentiated leaf cells (Fig. 1B). The identification of pericentromeric and telomeric subdomains in these assays suggest that these otherwise heterochromatic chromosomal regions could be among the chromosomal domains that undergo reorganization in pluripotent cells.

Reorganization of Chromosomal Domains During Acquisition of Pluripotentiality

We next used FISH to examine the chromatin configuration at the pericentromeric and telomeric regions of chromosome 1 during the transition from differentiated leaf cells to pluripotent protoplasts. Fixed nuclei were double labeled with fluorescein-*Athila* (BAC-F2J2 and BAC-F28H19) and rhodamine-CEN180 (as a reference centromeric marker) and tested for the chromatin organization of these BAC sequences in nuclei prepared from leaf cells and pluripotent protoplasts. We noted that the majority of labeled leaf cell nuclei exhibited the *Athila* region as a dot adjacent to CEN180, whereas in protoplast nuclei this region was extended (Fig. 2A). FISH analysis with fluorescein-BAC T25K16 (containing *NAC-1* gene At1g01010), located at the telomeric subdomain of the top arm of chromosome 1, revealed that this telomeric region became highly extended in protoplasts compared with leaf cells (Fig. 2B). This extended chromatin configuration of BAC T25K16 was displayed by most labeled protoplast nuclei, thus relating *NAC-1* transcriptional activation to chromatin decondensation. We also found a high propor-

tion of protoplast nuclei where this telomeric region came into close proximity with the chromocenter (Fig. 2B), suggesting spatial reorganization of this telomeric region during acquisition of pluripotentiality. Thus, the MSAP and DNA chip assays were instrumental in identifying marker genes pointing to specific chromosomal domains undergoing reorganization.

Chromatin remodeling observed at pericentromeric and telomeric subdomains of chromosome 1 does not necessarily indicate a similar propensity in pericentromeric or telomeric subdomains of other chromosomes. For example, FISH analysis using BAC T12J2 (containing the cell cycle regulator *MCM5* gene) that maps to a pericentromeric subdomain of chromosome 2 showed no differences in chromatin configuration between leaf and protoplast nuclei; both displayed extended, relaxed configuration of this region (Fig. 2C).

Chromatin decondensation observed at chromosome 1 pericentromeric and telomeric subdomains appears to be domain-specific inasmuch as other chromosomal regions either remained largely unchanged or underwent chromatin condensation. Thus, FISH analysis with CEN180 revealed no discernible alterations in chromatin structure between leaf and protoplast nuclei (see Figs. 2, 3A). In addition, we analyzed the chromatin configuration of the 18S rDNA using fluorescein-18S rDNA as a probe and rhodamine-CEN180 as a reference centromeric marker. The nucleolar organizing regions (NORs) in *Arabidopsis* are located at the short arms of the acrocentric chromosomes 2 and 4, adjacent to the centromeric regions. FISH analysis revealed that in leaf nuclei the 18S rDNA was dispersed within the nucleolus, or, as expected from the chromosomal location of NORs, concentrated as three to four spots adjacent to CEN180 (Fig. 3A). The dispersed configuration of the 18S rDNA could not be detected in protoplast nuclei; protoplasts displayed only condensed spots adjacent to centromeric regions (Fig. 3A). As revealed by Southern blot analysis (Fig. 3B), condensation of the 18S rDNA gene cluster was independent of DNA methylation.

b-Zip *VIP1* Gene Adjacent to *Athila* Undergoes Transcriptional Activation in Pluripotent Protoplasts

The pericentromeric region possesses genes, although at a low density, that can be transcriptionally activated. Adjacent to the *Athila* retroelements (Fig. 4A) is a b-Zip gene, *VIP1*, encoding an *Agrobacterium* VirE2-interacting protein necessary for VirE2 nuclear import and *Agrobacterium* infectivity (Tzfira et al., 2001, 2002). We hypothesized that the reduction in DNA methylation and decondensation at the pericentromeric *Athila* region may facilitate expression of the *Athila* retroelement as well as of *VIP1*. To this end, we analyzed the expression pattern of *VIP1* in leaves, protoplasts, and dividing cells by semiquantitative reverse transcriptase-PCR (RT-PCR; Fig. 4B). *VIP1* was down-regulated in differentiated leaf cells but transcriptionally activated in pluripotent protoplasts as well as in dividing cells. The expression pattern of *VIP1* was confirmed by Northern blot analysis (data not shown); no activation of the *Athila* retroelement could be detected (Fig. 4B), suggesting that chromatin decondensation while essential is not sufficient for transcriptional activation.

VIP1 Is a Nuclear Protein That Suppresses Differentiation and Shoot Formation

The finding that VIP1 is required for *Agrobacterium* infectivity prompted us to investigate its involvement in cellular dedifferentiation and plant regeneration. By using tobacco transgenic plants expressing VIP1 fused to GFP, we first established that the VIP1 protein is exclusively localized to the nucleus (Fig. 4C), suggesting that it may function as a transcriptional regulator.

We next studied shoot formation capacity in detached leaves or in calli derived from wild-type tobacco and two independent lines of *VIP1*-expressing transgenic tobacco plants (Tzfira et al., 2001, 2002). Both transgenic lines displayed similar levels of *VIP1* expression (Fig. 4D), their growth was retarded, and they appeared to lose apical dominance as demonstrated by the enhanced axillary bud growth (data not shown). While callus formation was not significantly affected, shoot formation was strongly inhibited in leaves and calli-expressing VIP1 (Fig. 4E). We also tested the possibility that VIP1 inhibitory effect is mediated by the phytohormone gibberellin, first, because VIP1 shares high amino acid sequence similarity with the tobacco REPRESSION OF SHOOT GROWTH (RSG), a b-Zip protein involved in gibberellin biosynthesis (Fukazawa et al., 2000), and second, because gibberellin is known to inhibit shoot formation (Ezura and Harberd, 1995). Application of paclobutrazol, an inhibitor of gibberellin biosynthesis, however, did not relieve the inhibitory effect exerted by VIP1(data not shown).

DISCUSSION

Acquisition of pluripotentiality in *Arabidopsis thaliana* has been shown here to be associated with chromatin reorganization at specific domains, i.e., condensation of the nucleolar 18S rDNA gene and decondensation at the pericentromeric and telomeric subdomains of chromosome 1. We postulated that decondensation of specific chromosomal domains might lead to the exposure and transcriptional activation of silent genes whose products are involved in determining the pluripotent state of the cell and/or the cell new function. By using MSAP and DNA microarray analysis, we identified marker genes directing us to specific chromosomal regions, namely, the pericentromere and the telomere. Within the pericentromeric subdomain of chromosome 1, we identified the *VIP1* gene whose product appears to be involved in differentiation and shoot formation. The telomeric region also contains genes that might be involved in determining the pluripotent state of the cell, such as NAC-domain containing genes whose products have been implicated in meristem func-

tionality (Souer et al., 1996; Duval et al., 2002). Notably, the microarray analysis identified four NAC-domain containing genes whose expression was significantly up-regulated in pluripotent protoplasts, suggesting that these gene products may act in concert in determining the pluripotent state of the cells.

The loss of the dispersed configuration of the 18S rDNA in pluripotent *Arabidopsis* protoplasts points to compaction and silencing of this gene, which could lead to a decrease in biogenesis of ribosomes (Pikaard, 1999; Leary and Huang, 2001). The compaction of the 18S rDNA was independent of DNA methylation inasmuch as no changes in methylation pattern of this gene could be observed during the transition from differentiated leaf cells to pluripotent protoplasts. Notably, despite the compaction of the 18S rDNA gene cluster, we could not detect changes in the steady state level of the 18S rRNA (as shown by Northern blot analysis, Fig. 1B), most likely owing to the high stability of the 18S rRNA. This seems to contradict a previous report showing a dramatic reduction in the number of ribosomes during protoplast isolation (Gigot et al., 1975), which could be attributed to inactivation of multiple factors involved in ribosome assembly (Fromont-Racine et al., 2003). The reduction in ribosome production combined with increased levels of RNase and ubiquitin gene expression (Lazar et al., 1973; Jamet et al., 1990) may mark a point at which the "old" gene expression program is being erased in preparation for a new cell function (Galun, 1981). The results obtained in *Arabidopsis* are similar to those previously reported in tobacco protoplasts where the 18S rDNA undergoes chromatin compaction and nucleolar reorganization (Williams et al., 2003). Similarly, somatic nuclei transplanted into *Xenopus* egg extract showed rapid disassembly of nucleoli (Kikyo et al., 2000; Gonda et al., 2003), suggesting that structural reorganization of nucleoli may be a common feature of dedifferentiating cells both in plants and animals. It remains to be confirmed whether chromatin configuration of the 18S rDNA could serve as a marker for cells acquiring pluripotentiality.

The data presented suggest that chromatin reorganization is achieved, at least in part, by de novo methylation and demethylation of nuclear DNA. Numerous data demonstrated the relationship between DNA methylation and chromatin structure in a variety of eukaryotes. In general, although not as a rule, unmethylated DNA is a characteristic feature of transcriptionally active chromatin while methylated DNA is associated with heterochromatin and transcriptional repression (Keshet et al., 1986; Kass et al., 1997; Jones and Wolffe, 1999). However, condensation of the 18S rDNA gene cluster occurred without any detectable changes in DNA methylation pattern, consistent with a recent report (Georgel et al., 2003). Sequence analysis of fragments displaying differential methylation patterns revealed DNA sequences related to coding and intergenic noncoding regions, chloroplast DNA, as well as retrotransposons. One of the isolated fragments that underwent demethylation showed sequence homology to the *Athila* retroelement, located at the pericentromeric, heterochromatic region of chromosome 1. This region was found more extended, hence, decondensed during acquisition of pluripotentiality. A reduction in pericentromeric heterochromatin due to relaxation of dispersed repeats was reported in hypomethylated *Arabidopsis* mutants *ddm1* and *met1* (Soppe et al., 2002). Thus, demethylation at the *Athila* retroelement locus is likely to be part of the mechanism involved in chromatin reorganization-induced transition from a differentiation state to pluripotentiality. The role played by DNA demethylation in acquisition of pluripotentiality is demonstrated by the conversion of cultured fibroblast mouse cells into three new mesenchymal phenotypes (striated muscle cells, adipocytes, and chondrocytes) upon treatment with 5-azacytidine (Taylor and Jones, 1979); the latter reduces methylation after being incorporated into DNA. While reorganization of chromatin may facilitate activation of silent retrotransposons (Hirochika et al., 2000; Miura et al., 2001), we could not detect activation of the *Athila* retroelement, despite reduction in methylation and chromatin decondensation. This finding suggests that chromatin configuration is essential but not sufficient for transcriptional activation; *Athila* might be subjected to additional controls restricting its expression. On the other hand, chromatin reorganization associated with acquisition of pluripotentiality was shown here to bring about activation of silent genes such as NAC-domain genes whose products may be involved in establishment/maintenance of stem cell lineages in plants, and the b-Zip gene *VIP1*, whose product inhibits differentiation and shoot formation.

We showed that overexpression of *VIP1* in tobacco cells significantly inhibited shoot formation; callus and root formation were not significantly affected. The localization of VIP1 to the nucleus suggests that it may function as a transcriptional regulator. It has been reported that overexpression of *VIP1* increased susceptibility of plants to *Agrobacterium* infection, at least partly by facilitating nuclear uptake of T-complexes, and consequently, significantly induced shoot regeneration (Tzfira et al., 2002). These *Agrobacterium* infection experiments were carried out in the absence of exogenous application of growth regulators. This finding suggests that nuclear import and integration of T-DNA induced by VIP1 either bypassed the need for exogenous application of growth factors and/or reduced the negative effect of VIP1 on shoot formation. VIP1 shares high amino acid sequence similarity with the tobacco REPRESSION OF SHOOT GROWTH (RSG), a b-Zip protein involved in the biosynthesis of the plant growth regulator gibberellin (Fukazawa et al., 2000), as well as with PKSF1, a b-Zip protein implicated in adventitious shoot regeneration in *Paulownia kawakamii* (Low et al., 2001). Based on the possible function of RSG in gibberellin biosynthesis, we addressed the possibility that the function of VIP1 is mediated by gibberellin, an inhibitor of shoot formation (Ezura and Harberd, 1995). Our results, however, showed that VIP1 inhibits shoot formation independently of gibberellin (data not shown). In *Paulownia*, *PKSF1* is up-regulated at a stage preceding visible shoot

buds, a stage characterized mostly by cell division; at later stages, the PKSF1 RNA is dramatically reduced below basal level (Low et al., 2001). Consistent with this observation, the *VIP1* gene is up-regulated in dividing cultured cells (data not shown). Thus, it appears that VIP1/PKSF1 may be required for the initial stages of shoot formation to retain pluripotentiality, probably by rendering proliferating cells incapable of differentiating into various types of cells that make up shoots.

We conclude that acquisition of pluripotentiality proceeds through reorganization of specific chromosomal domains eventually leading to activation of silent genes whose products may be involved in maintaining the state of pluripotentiality and in driving the new function of the cell. Considering that chromatin decondensation has been reported in a variety of dedifferentiating eukaryotic cells (Chiabrera et al., 1979; Fontes et al., 1980; Blank et al., 1992), and the great similarity in the regulation of chromatin structure between plants and animals, our findings may have bearing on various dedifferentiation-driven cellular processes in animals such as regeneration, establishment of new stem cell lineages, as well as nuclear cloning.

EXPERIMENTAL PROCEDURES

Protoplast Preparation and DNA Extraction

Nicotiana tabacum ("Samsun NN") protoplasts were prepared by treating fully expanded fresh leaves with an enzyme mixture containing 0.082% (w/v) cellulase Onozuka R-10 (Yakult Honsha), 0.041% (w/v) driselase (Sigma), and 0.16% (w/v) macerozyme (Yakult Honsha) essentially as described (Zelcer and Galun, 1976). *Arabidopsis thaliana* (ecotype Columbia) protoplasts were isolated from 3- to 4-week-old leaves that were cut into small pieces and incubated for 1 hr in N&M medium (0.4 M sucrose, 1 mM NaH_2PO_4, 6 mM $CaCl_2$, 25 mM KNO_3, 1 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$ and 3 mM NH_4NO_3) before treatment with an enzyme mixture of 1% cellulase and 0.25% macerozyme. After incubation at 24°C for 15 hr, protoplasts were filtered through 150-μm nylon mesh and washed with CPW (0.55 M mannitol, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, and 10 mM $CaCl_2$). The protoplast pellet was stored at −80°C. DNA was extracted from leaves and protoplasts by modification of the C-elyltrimethyl ammonium bromide (CTAB) method (Wagner et al., 1987).

MSAP Assay

The MSAP assay was performed essentially as described (Shaked et al., 2001) by using the methylation isoschizomers *Hpa*II and *Msp*I as frequent cutters, and *Eco*RI as the rare cutter (Vos et al., 1995). The *Hpa*II-*Msp*I (HM) adapter was generated after annealing the HM-sense 5′-GATCATGAGTCCTG with the HM-antisense 5′-CGAGCAGGACTCATGA oligonucleotide primer. Restriction-ligation reactions were set up as follows: genomic DNA (0.5 μg) was digested with *Eco*RI (15 units) and either *Hpa*II or *Msp*I (each 15 units; New England Biolabs) in a DNA ligase buffer (final volume, 10 μl) containing 1 unit of T4-DNA ligase (New England Biolabs), 0.5 pmol *Eco*RI adapter, and 5 pmol HM adapter. Reactions were carried out for 2 hr at 37°C and then diluted by adding 90 μl water for preselective PCR amplification.

Preselective amplification was performed with primers complementary to the core of the adapter sequences, namely, HM primer 5′-ATCATGAGTCCTGCTCGG and E+1 primer with the addition of one selective nucleotide (5′-GACTGCGTACCAATTCA). Preamplification was carried out by using 4 μl of the restriction-ligation diluted reaction with E+1/HM primers in a volume of 20 μl containing 50 ng of each primer, 1 U of Taq DNA polymerase (Promega), 2 μl of 10× Taq DNA polymerase buffer (Promega), 2 μl of 25 mM $MgCl_2$, and 2.5 mM dNTPs. PCR reactions were performed essentially as described (Shaked et al., 2001). The preamplified products were diluted with water (1:20) and stored at −20°C until use.

Selective amplifications were conducted by using two HM-selective primers with four selective nucleotides (HM+4; HM+TCAA; HM+TCAG) and eight E-selective primers with three selective nucleotides (E+3; E+ACA; E+ACT; E+AAC; E+ACC; E+AGC; E+AAG; E+AGG; E+ACG). PCR reactions (11 combinations) were set up, each containing 3 μl of the preamplification product, 30 ng of selective E+3 primer, and 40 ng of selective HM+4 primer end-labeled with (γ-^{32}P)ATP. PCR amplification reactions were performed by using the touch-down cycles as described in the original AFLP protocol (Vos et al., 1995). The PCR products were mixed with 20 μl of formamide dye and separated on 5% standard sequencing gel at 50 watts for 1.5 hr. The gel was then dried and exposed to X-ray film. Fragments showing alterations in cytosine methylation were excised from the gel, re-amplified by PCR, and subcloned into pGEM-T vector (Promega) as described (Shaked et al., 2001).

Total RNA Isolation, RT-PCR, and Northern Analysis

Total RNA was extracted from *Arabidopsis* leaves, freshly prepared protoplasts or *Arabidopsis* cultured cells using the LiCl method. RNA was treated at 37°C for 15 min with RNase-free DNase (Promega) 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 cDNA was used as a template for semiquantitative PCR by using Reddymix PCR master mix (ABgene) and the resultant fragments were resolved in 1% agarose gel containing ethidium bromide. We designed primers to distinguish between a PCR product derived from DNA or from cDNA. All primers were first approved for their ability to direct PCR by using genomic DNA as a template. The following primers were used: VIP1-S, 5′-ATGGAAGGAGGAGGAAGAGGACC-3′, and VIP1-AS, 5′-TCGCCGCAGATTGTCTATTCGC-3′; Athila-S, 5′-GTGATGGCTAATGAGGTGAATGC-3′, and Athila-AS, 5′-GAATCCTACTGCCTCTGTTAGTGCG-3′; Actin-S, 5′-GGTTTTGCTGGGGAT-

GATGC-3′, and Actin-AS, 5′-CATGGCTGGACATTGAATGTCTC-3′. Northern blot analysis was performed by using formaldehyde denaturing gel essentially as described (Maniatis et al., 1982).

Slide Preparation, Probe Labeling, and FISH Assay

Fixed nuclei (5–10 μl, 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× SSC. Slides were washed three times, 5 min each, with 2× SSC and subjected to denaturation solution containing 70% formamide in 1× 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. Probes were labeled directly either with fluorescein-12-dUTP (Roche) or with tetramethylrhodamine-5-dUTP (Roche) using PCR or Nick translation kit (Roche). The centromeric 180-bp repeats were labeled by PCR using rhodamine-deoxynucleotide mixture (2 mM each of dATP, dCTP, and dGTP, 1.5 mM dTTP, and 0.5 mM tetramethylrhodamine-5-dUTP), *Arabidopsis* genomic DNA as template (20 ng), and the following primers: 180-S 5′-GAGAGGATCCCGTAAGAATTGTATCCTTGTTAG-3′ and 180-AS 5′-GAGAGAATTCCCTTTAAGATCCGGTTGTGG-3′. BACs (kindly provided by the *Arabidopsis* Biological Resource Center (ABRC)) F2J6 (108,061 bp), F28H19 (131,692 bp) (Chr 1), and T12J2 (85,463 bp) (Chr 2) were labeled with fluorescein-12-dUTP using the Nick translation kit according to the manufacturer's protocol (Roche). 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′). The 18S PCR fragment was labeled with fluorescein-12-dUTP using the Nick translation kit (Roche). Probes were mixed with a hybridization solution (final volume 100 μl) containing 10% (w/v) sodium dextran sulphate, 50% deionized formamide, and 2× SSPE, denatured at 80°C for 5 min, and cooled on ice. Each probe was added to a slide, covered with a cover glass, and incubated at 37°C in the dark for 16–20 hr followed by washings essentially as described (Fransz et al., 1996). Slides were then stained for 10 min with 10 μg/ml diamidino-phenylindole (DAPI), washed twice, and mounted in Vectashield (Vector Laboratories, CA). Hybridization signals were visualized by a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonics) using Olympus filters U-MNU, U-MWIBA2, and U-MNG to detect DAPI, fluorescein, and rhodamine, respectively. Images were pseudocolored and merged by using TILL Vision version 3.3 software. All images were processed by using Adobe Photoshop software.

Shoot Regeneration

Leaf discs (~1.5 cm in diameter) derived from wild-type (*Nicotiana tabacum* cv. Turk) or transgenic tobacco expressing *VIP1* (Tzfira et al., 2001, 2002) were incubated on MS medium (Murashige and Skoog, 1962) containing 3 μg/ml 1-naphthaleneacetic acid and 1 μg/ml 6-benzylaminopurine to obtain a solid callus for shoot regeneration experiments. Shoot regeneration was studied either with detached leaves (each with four longitudinal cuts) or in solid calli incubated on shoot regeneration medium consisting of MS medium containing 2 μg/ml kinetin and 0.8 μg/ml indoleacetic acid. In some experiments, gibberellic acid and paclobutrazol, an inhibitor of gibberellin biosynthesis, were added. Formation of buds was assessed visually after 3 to 4 weeks. Tissues were photographed and images were processed with Adobe Photoshop software (Adobe systems).

Generation of GFP-VIP1 Transgenic Tobacco Plants and Fluorescence Microscopy Analysis

For expression in plant cells, VIP1 open reading frame was PCR-amplified and fused as *Sal*I–*Bam*HI fragment in pEGFP-C1 (Clontech) to produce pEGFP-C1-VIP1. The GFP-VIP1 DNA fragment was then released as *Nco*I–*Bam*HI and cloned into the same sites of pRTL2-GUS (Citovsky et al., 1992), replacing GUS and producing pRTL2-GFP-VIP1. The 35S-GFP-VIP1 expression cassette was then transferred to the intermediate plasmid pSL301 as a *Sph*I fragment and was subcloned as *Stu*I/*Sma*I fragment into *Sma*I site of the pBIN19 binary plasmid. PCR reaction was performed by using a high fidelity Pfu DNA polymerase (Promega), and the product was verified by dideoxynucleotide sequencing. The binary vector pBIN19/GFP-VIP1 was introduced into the disarmed *Agrobacterium* strain EHA105, which was then used to transform tobacco plants (*Nicotiana tabacum* cv. Turk) as described (Horsch et al., 1985). Transgenic tobacco plants expressing GFP-VIP1 were selected on a kanamycin-containing medium and maintained for 1 month at sterile conditions on an MS basal medium (Murashige and Skoog, 1962) with no exogenous growth regulators. Cells were then examined under a fluorescence microscope (Olympus) equipped with a CCD camera (Imago, Photonics) by using Olympus filters U-MWIBA2 to detect GFP. Images were pseudocolored by using TILL Vision version 3.3 software and processed by Adobe Photoshop software.

ACKNOWLEDGMENTS

We thank E. Tanaka, A. Sanchez-Alvarado, A. Zemach, M. Edelman, and A.A. Levy for critical reading of the manuscript, S. Weissmann for helping with FISH analysis, O. Davydov, Z. Zeira, and R. Ophir for helping with the microarray analysis, and The Arabidopsis Biological Resource Center for providing BAC clones.

REFERENCES

Alleman M, Doctor J. 2000. Genomic imprinting in plants: observations and evolutionary implications. Plant Mol Biol 43:147–161.

Blank T, Trendelenburg M, Kleinschmidt JA. 1992. Reactivation of DNA replication in erythrocyte nuclei by *Xenopus* egg extract involves energy-dependent chromatin decondensation and changes in histone phosphorylation. Exp Cell Res 202:224–232.

Brannan CI, Bartolomei MS. 1999. Mechanisms of genomic imprinting. Curr Opin Genet Dev 9:164–170.

Brockes JP, Kumar A. 2002. Plasticity and reprogramming of differentiated cells in amphibian regeneration. Nat Rev Mol Cell Biol 3:566–574.

Buschhausen G, Wittig B, Graessmann M, Graessmann A. 1987. Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. Proc Natl Acad Sci U S A 84:1177–1181.

Cedar H. 1988. DNA methylation and gene activity. Cell 53:3–4.

Chiabrera A, Hinsenkamp M, Pilla AA, Ryaby J, Ponta D, Belmont A, Beltrame F, Grattarola M, Nicolini C. 1979. Cytofluorometry of electromagnetically controlled cell dedifferentiation. Histochem Cytochem 27:375–381.

Citovsky V, Zupan J, Warnick D, Zambryski P. 1992. Nuclear localization of *Agrobacterium* VirE2 protein in plant cells. Science 256:1802–1805.

Duval M, Hsieh TF, Kim SY, Thomas TL. 2002. Molecular characterization of *AtNAM*: a member of the *Arabidopsis* NAC domain superfamily. Plant Mol Biol 50:237–248.

Echeverri K, Tanaka EM. 2002. Mechanisms of muscle dedifferentiation during regeneration. Semin Cell Dev Biol 13:353–360.

Ezura H, Harberd NP. 1995. Endogenous gibberellin levels influence in-vitro shoot regeneration in *Arabidopsis thaliana* (L.) Heynh. Planta 197:301–305.

Fontes M, Marilley M, Le Parco Y, Thouveny Y. 1980. Variations in accessibility of DNA during traumatic regeneration by *Owenia fusiformis* (polychaete annelid). Cell Differ 9:83–93.

Fransz PF, Alonso-Blanco C, Liharska TB, Peeters AJ, Zabel P, de Jong JH. 1996. High-resolution physical mapping in *Arabidopsis thaliana* and tomato by fluorescence in situ hybridization to extended DNA fibres. Plant J 9:421–430.

Fromont-Racine M, Senger B, Saveanu C, Fasiolo F. 2003. Ribosome assembly in eukaryotes. Gene 313:17–42.

Fukazawa J, Sakai T, Ishida S, Yamaguchi I, Kamiya Y, Takahashi Y. 2000. REPRESSION OF SHOOT GROWTH, a bZIP transcriptional activator, regulates cell elongation by controlling the level of gibberellins. Plant Cell 12:901–915.

Galun E. 1981. Plant protoplasts as physiological tools. Annu Rev Plant Physiol 32:237–266.

Georgel PT, Horowitz-Scherer RA, Adkins N, Woodcock CL, Wade PA, Hansen JC. 2003. Chromatin compaction by human MeCP2: assembly of novel secondary chromatin structures in the absence of DNA methylation. J Biol Chem 278:32181–32188.

Gigot C, Kopp M, Schmitt C, Milne RG. 1975. Subcellular changes during isolation and culture of tobacco mesophyll protoplasts. Protoplasma 84:31–41.

Gonda K, Fowler J, Katoku-Kikyo N, Haroldson J, Wudel J, Kikyo N. 2003. Reversible disassembly of somatic nucleoli by the germ cell proteins FRGY2a and FRGY2b. Nat Cell Biol 5:205–210.

Graf T. 2002. Differentiation plasticity of hematopoietic cells. Blood 99:3089–3101.

Hirochika H, Okamoto H, Kakutani T. 2000. Silencing of retrotransposons in *Arabidopsis* and reactivation by the *ddm1* mutation. Plant Cell 12:357–369.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT. 1985. A simple and general method for transferring genes into plants. Science 227:1229–1231.

Jamet E, Durr A, Parmentier Y, Criqui MC, Fleck J. 1990. Is ubiquitin involved in the dedifferentiation of higher plant cells? Cell Differ Dev 29:37–46.

Jenuwein T, Allis CD. 2001. Translating the histone code. Science 293:1074–1080.

Jones PL, Wolffe AP. 1999. Relationships between chromatin organization and DNA methylation in determining gene expression. Semin Cancer Biol 9:339–347.

Kass SU, Pruss D, Wolffe AP. 1997. How does DNA methylation repress transcription? Trends Genet 13:444–449.

Keshet I, Lieman-Hurwitz J, Cedar H. 1986. DNA methylation affects the formation of active chromatin. Cell 44:535–543.

Kikyo N, Wade PA, Guschin D, Ge H, Wolffe AP. 2000. Active remodeling of somatic nuclei in egg cytoplasm by the nucleosomal ATPase ISWI. Science 289:2360–2362.

Lazar G, Borbely G, Udvardy J, Premecz G, Farkas GL. 1973. Osmotic shock triggers an increase in ribonuclease level in protoplasts isolated from tobacco leaves. Plant Sci Lett 1:53–57.

Leary DJ, Huang S. 2001. Regulation of ribosome biogenesis within the nucleolus. FEBS Lett 509:145–150.

Liu Y, Rao MS. 2003. Transdifferentiation - fact or artifact. J Cell Biochem 88:29–40.

Low RK, Prakash AP, Swarup S, Goh CJ, Kumar PP. 2001. Lambda exonuclease-based subtractive hybridization approach to isolate differentially expressed genes from leaf cultures of *Paulownia kawakamii*. Anal Biochem 295:240–247.

Maniatis T, Fritsch E, Sambrook J. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Martienssen RA, Colot V. 2001. DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science 293:1070–1074.

Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H, Kakutani T. 2001. Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature 411:212–214.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497.

Odelberg SJ. 2002. Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals. Semin Cell Dev Biol 13:335–343.

Pelissier T, Tutois S, Tourmente S, Deragon JM, Picard G. 1996. DNA regions flanking the major *Arabidopsis thaliana* satellite are principally enriched in *Athila* retroelement sequences. Genetica 97:141–151.

Pikaard CS. 1999. Nucleolar dominance and silencing of transcription. Trends Plant Sci 4:478–483.

Reyna-Lopez GE, Simpson J, Ruiz-Herrera J. 1997. Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. Mol Gen Genet 253:703–710.

Sanchez-Alvarado A. 2000. Regeneration in the metazoans: why does it happen? Bioessays 22:578–590.

Sell S. 1993. Cellular origin of cancer: dedifferentiation or stem cell maturation arrest? Environ Health Perspect 5:15–26.

Shaked H, Kashkush K, Ozkan H, Feldman M, Levy AA. 2001. Sequence elimination and cytosine methylation are rapid and reproducible responses of the genome to wide hybridization and allopolyploidy in wheat. Plant Cell 13:1749–1759.

Soppe WJ, Jasencakova Z, Houben A, Kakutani T, Meister A, Huang MS, Jacobsen SE, Schubert I, Fransz PF. 2002. DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. EMBO J 21:6549–6559.

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R. 1996. The *NO APICAL MERISTEM* gene of petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 85:159–170.

Takebe I, Labib G, Melchers G. 1971. Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58:318–320.

Taylor SM, Jones PA. 1979. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell 17:771–779.

Tosh D, Slack JMW. 2002. How cells change their phenotype. Nat Rev Mol Cell Biol 3:187–194.

Tzfira T, Vaidya M, Citovsky V. 2001. VIP1, an Arabidopsis protein that interacts with *Agrobacterium* VirE2, is involved in

VirE2 nuclear import and *Agrobacterium* infectivity. EMBO J 20:3596–3607.

Tzfira T, Vaidya M, Citovsky V. 2002. Increasing plant susceptibility to *Agrobacterium* infection by overexpression of the *Arabidopsis* nuclear protein VIP1. Proc Natl Acad Sci U S A 99:10435–10440.

van Holde KE. 1989. Chromatin. Berlin, Germany: Springer-Verlag.

Vos P, Hogers R, Bleeker M, Reijans M, Vandelee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995. AFLP - a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414.

Wagner DB, Furnier GR, Saghai-Maroof MA, Williams SM, Dancik BP, Allard RW. 1987. Chloroplast DNA polymorphisms in lodgepole and jack pines and their hybrids. Proc Natl Acad Sci U S A 84: 2097–2100.

Williams L, Zhao J, Morozova N, Li Y, Avivi Y, Grafi G. 2003. Chromatin reorganization accompanying cellular dedifferentiation is associated with modifications of histone H3, redistribution of HP1, and activation of E2F-target genes. Dev Dyn 228:113–120.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813.

Wolffe AP. 1992. Chromatin: structure and function. San Diego: Academic Press.

Xiong LZ, Xu CG, Maroof MAS, Zhang QF. 1999. Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation sensitive amplification polymorphism technique. Mol Gen Genet 261:439–446.

Zelcer A, Galun E. 1976. Culture of newly isolated tobacco protoplasts: precursor incorporation into protein, RNA and DNA. Plant Sci Lett 7:331–336.

Zhao J, Morozova N, Williams L, Libs L, Avivi Y, Grafi G. 2001. Two phases of chromatin decondensation during dedifferentiation of plant cells: distinction between competence for cell fate switch and a commitment for S phase. J Biol Chem 276:22772–22778.