Large-scale dissociation and sequential reassembly of pericentric heterochromatin in dedifferentiated *Arabidopsis* cells

Federico Tessadori[1], Marie-Christine Chupeau[2], Yves Chupeau[2], Marijn Knip[1], Sophie Germann[2], Roel van Driel[1], Paul Fransz[1,*] and Valérie Gaudin[2,*]

[1]Nuclear Organization Group, Swammerdam Institute for Life Sciences, University of Amsterdam, BioCentrum Amsterdam, Kruislaan 318, 1098SM Amsterdam, The Netherlands
[2]Laboratoire de Biologie Cellulaire, IJPB, INRA, Route de St Cyr, 78026 Versailles Cedex, France
*Authors for correspondence (e-mail: fransz@science.uva.nl; Valerie.Gaudin@versailles.inra.fr)

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Summary

Chromocenters in *Arabidopsis thaliana* are discrete nuclear domains of mainly pericentric heterochromatin. They are characterized by the presence of repetitive sequences, methylated DNA and dimethylated histone H3K9. Here we show that dedifferentiation of specialized mesophyll cells into undifferentiated protoplasts is accompanied by the disruption of chromocenter structures. The dramatic reduction of heterochromatin involves the decondensation of all major repeat regions, also including the centromeric 180 bp tandem repeats. Only the 45S rDNA repeat remained in a partly compact state in most cells. Remarkably, the epigenetic indicators for heterochromatin, DNA methylation and H3K9 dimethylation, did not change upon decondensation. Furthermore, the decondensation of pericentric heterochromatin did not result in transcriptional reactivation of silent genomic elements. The decondensation process was reversible upon prolonged culturing. Strikingly, recondensation of heterochromatin into chromocenters is a stepwise process. Compaction of the tandemly arranged 45S rDNA regions occurs first, followed by the centromeric 180 bp and the 5S rDNA repeats and finally the dispersed repeats, including transposons. The sequence of reassembly seems to be correlated to the size of the repeat domains. Our results indicate that different types of pericentromeric repeats form different types of heterochromatin, which subsequently merge to form a chromocenter.

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Key words: *Arabidopsis thaliana*, Protoplast, Heterochromatin, Chromocenter

Introduction

The eukaryotic chromosome contains different chromatin regions with varying degrees of compaction. In Arabidopsis thaliana (2*n*=10), highly condensed chromatin is easily discerned by light microscopy in interphase nuclei as conspicuous DAPI-positive domains, chromocenters (Fransz et al., 2002). Chromocenters contain the pericentric regions of all chromosomes. In addition, chromocenters of chromosomes 2 and 4 also contain their subtelomeric 45S ribosomal repeat regions. By contrast, gene-rich chromosome segments are less condensed and organized in loops (Fransz et al., 2002). Molecular and cytogenetic studies in *Arabidopsis* revealed that chromocenters carry epigenetic markers of silent chromatin, such as dimethylated histone H3K9 and methylated DNA, whereas the rest of the nucleus contains chromatin that is enriched in acetylated histone H4 and methylated histone H3K4 (Soppe et al., 2002; Jackson et al., 2004; Naumann et al., 2005). In yeast, *Drosophila* and mammalian cells a clear relationship has been established between epigenetic markers, chromatin organization and gene expression during cell differentiation (Arney and Fisher, 2004). For example, X-chromosome inactivation in mammalian embryonic cells involves successive chromatin-remodeling steps, including histone modifications and DNA methylation, which lead to the formation of facultative heterochromatin (Avner and Heard, 2001; Kohlmaier et al., 2004). Epigenetic changes are crucial for proper cell differentiation. Where animal cells generally lose their pluripotency after cell determination, many specialized plant cells maintain the capability to dedifferentiate and generate de novo organs or even regenerate to complete plants. One of the most spectacular examples of totipotency is the formation of new plantlets from single protoplasts isolated from differentiated tissue (Takebe and Otsuki, 1969; Nitsch and Ohyama, 1971; Damm and Willmitzer, 1988). We have developed an efficient protoplast system in which we demonstrate a dramatic, reversible disassembly of heterochromatin under physiological conditions. In this study we have found a large-scale reorganization of heterochromatin domains, without measurable changes in DNA methylation or histone H3K9me2 content. Strikingly, the reassembly of heterochromatic chromocenters is a stepwise process in which the different repeats recondense at different rates.

Results

Protoplast system

When mesophyll tissue is treated to form protoplasts the cells change their nuclear program and lose their differentiated state. To investigate the nuclear changes during this process, we established a protoplast system that is efficient and reproducible. Mesophyll protoplasts isolated form 18-day-old *Arabidopsis* plantlets are round and green, because of the presence of chloroplasts. Upon culturing, a new cell wall was formed and the cells lost the green color of the chlorophyll. The first cell divisions were observed after 72 hours. After 120 hours in culture up to 70% of the protoplast-derived cells had divided. After 15 days, 50% of the originally plated protoplasts had formed micro-colonies (Fig. 1). Subsequent transfer to propagation and regeneration medium resulted in the formation of plantlets (M.-C. Chupeau, unpublished results). Using this protoplast system, we followed the changes in chromatin compaction at subsequent time intervals up to 120 hours after protoplast isolation. We examined a number of cytological and biochemical features including heterochromatin content, localization of major repeats, methylated DNA and methylated histones and gene activity. The results presented were highly reproducible and collected from over 20 independent protoplast isolations.

Protoplasts contain decondensed chromatin

Mesophyll nuclei have up to 10 distinct heterochromatic domains or chromocenters. During isolation of protoplasts, we observed a dramatic decrease in both size and number of chromocenters (Fig. 2A,B). We determined the mean relative heterochromatin fraction (RHF) from the area and staining intensity of the chromocenters in relation to the entire nucleus (Soppe et al., 2002). The RHF was reduced in protoplasts (RHF=0.03; n=39) to about 27% compared to leaves (RHF=0.11; n=48; 2-tailed type 2 Student t-test, P<0.001). The reduction in heterochromatin was not due to technical artifacts, as the disappearance of chromocenters was also monitored in living protoplasts from a transgenic plant expressing a histone H2B-YFP fusion construct (Fig. 2C). To verify that chromatin decondensation is the result of protoplast formation instead of a response to cutting of the tissue, we repeated the experiment without cell wall-degrading enzymes. This treatment did not result in the formation of protoplasts or in a change in the nuclear phenotype, indicating that the cuts inflicted on the explants are not sufficient to induce heterochromatin reduction.

To examine which chromosomal regions are no longer in chromocenters of protoplast nuclei, we applied FISH with repeat sequences that are known to reside in chromocenters (Fransz et al., 2002; Soppe et al., 2002). The pericentric BAC F28D6 contains a variety of dispersed repeat elements whereas the Athila transposon is one of the most abundant transposable elements (The Arabidopsis Genome Initiative, 2000). FISH signals of both DNA sequences displayed a scattered pattern outside chromocenters (Fig. 3A-D), indicating that these pericentric regions are decondensed. Also the pericentric 5S rDNA regions were largely decondensed showing long fluorescent arrays (Fig. 3E,F). Measurement of the 5S rDNA arrays revealed an average decondensation factor of 5 whereas a maximum decondensation factor of 14 was found for the 5S locus of chromosome 4. A striking observation was the highly dispersed pattern of the centromeric 180 bp repeats throughout

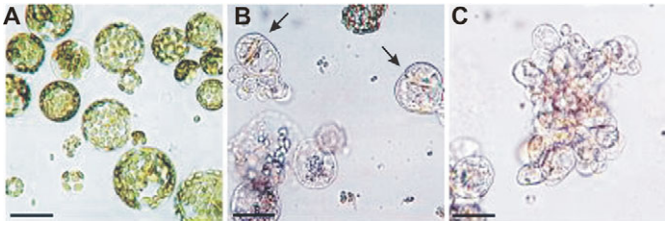


Fig. 1. Micrographs of cultured protoplasts, dividing cells and callus formation. (A) Freshly isolated protoplasts. (B) Protoplasts after 120 hours in culture showing active divisions (arrows). The absence of chloroplasts denotes a dedifferentiated state. (C) Microcallus after 15 days. Bar, 20 μm.

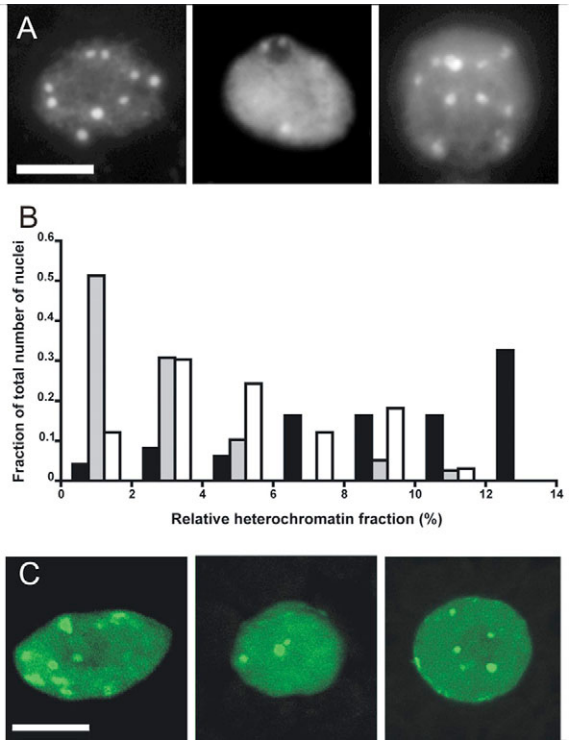


Fig. 2. Protoplast nuclei contain chromocenters that are reduced in number and size. (A) DAPI-stained nuclei show up to three chromocenters in protoplasts (middle), and up to ten chromocenters in leaf (left) and cultured cells (right). Bar, 5 μm. (B) Relative heterochromatin fraction (RHF) in leaves (black bars), protoplasts (gray bars) and cultured cells (white bars). (C) Confocal images of living cells expressing H2B::YFP from leaf (left), protoplasts (middle) and after 72 hours in culture (right). Bar, 5 μm.

the nucleus (Fig. 3G,H). The results indicate that dedifferentiation is accompanied by dramatic changes in the organization of the repeat-rich heterochromatin. None of the pericentric or centromeric repeats showed a clear co-localization with chromocenters in protoplasts. The only major repeat sequence that remained partially condensed was the subtelomeric 45S rDNA. Since FISH signals of the 45S rDNA colocalized with the few chromocenters (Fig. 3E,F) we conclude that chromocenters in protoplasts consist mainly of 45S rDNA chromatin regions.

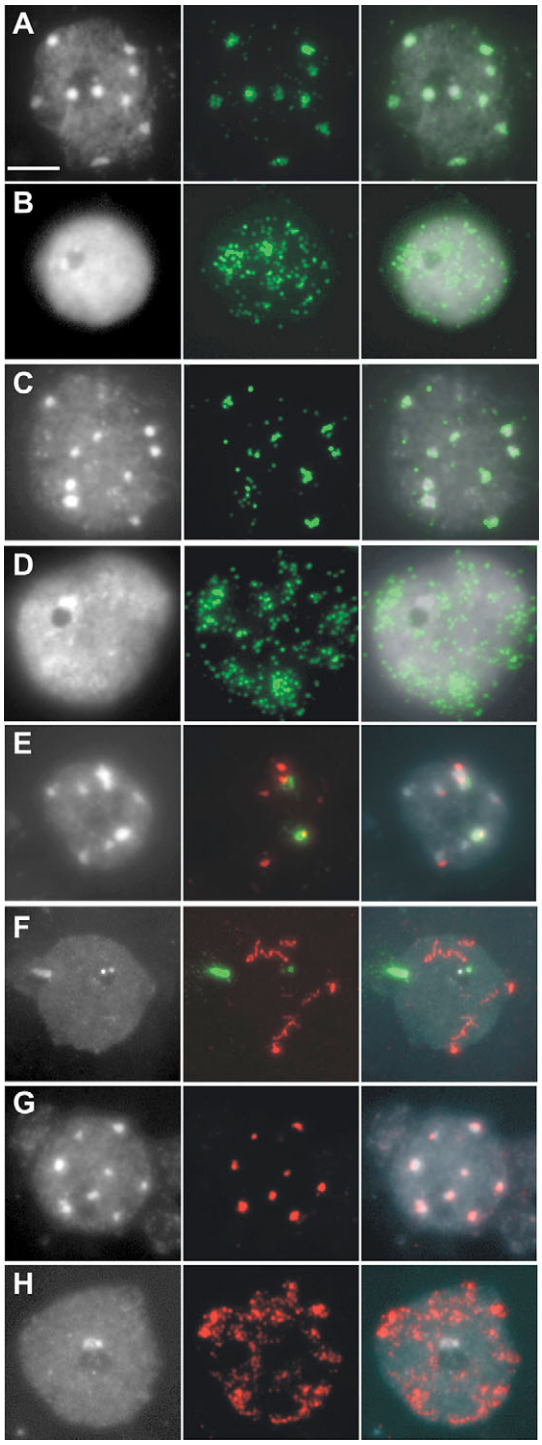


Fig. 3. FISH localization of repetitive sequences in leaf and protoplasts. DAPI staining (left column); FISH signal (middle column); merge (right column). (A,B) The repeats of the pericentric BAC F28D6 localize at chromocenters in leaves (A) but are dispersed in protoplast nuclei (B). (C,D) The Athila transposon localizes at chromocenters in leaves (C) but is dispersed in protoplasts (D). (E) Both 5S (red) and 45S (green) rDNA repeats localize at chromocenters in leaves. (F) In protoplasts, 5S rDNA shows decondensed signals, whereas 45S rDNA signals remain condensed at the chromocenters. (G,H) The 180 bp centromere repeat localizes in chromocenters of leaf nuclei (G), but is dispersed in protoplast nuclei (H). Bar, 5 μm.

Reformation of chromocenters during prolonged culture is established by sequential condensation of repeats

We followed the protoplasts during culturing and recorded the condensation state of chromatin. Already after 24 hours regions with intense DAPI fluorescence, reminiscent of chromocenters, reappeared (Fig. 2A). This was again confirmed in cells expressing H2B-YFP (Fig. 2C). After 120 hours the nuclei showed up to 10 chromocenters with a vague and inconspicuous contour. Apparently, reformation of chromocenters is slower than the decondensation process. We therefore questioned whether heterochromatin had fully recovered. Quantitative analysis of the RHF showed that leaf nuclei had a significantly higher RHF value (RHF=0.11; n=48) than 120-hours cultured cells (RHF=0.05; n=33; P<0.001), suggesting that heterochromatin had not reformed completely. Remarkably, during the first 120 hours of culture the majority of cells have passed mitosis, which is accompanied by chromosome condensation during metaphase. Apparently, this condensation event did not lead to recovery of condensed pericentric heterochromatin to normal levels.

To assess the dynamics of chromocenter condensation we monitored the localization of repeats at different stages using FISH. The 45S rDNA signals remained largely associated with a chromocenter throughout the protoplast culture. After 120 hours, the majority (81%) of the 45S rDNA was condensed (Fig. 4A). The 180 bp repeat and 5S rDNA showed significantly less condensation throughout the culture and only half the signals showed condensation after 120 hours (Fig. 4B-D). At all stages the 180 bp repeat was more condensed than the 5S rDNA sequences, suggesting that the centromeric repeats have a stronger commitment to compaction than 5S rDNA (Fig. 4, Table 1). In addition, we never observed chromocenters with condensed 5S rDNA but without 180 bp repeats. It should be noted that the 5S rDNA repeat arrays are on chromosomes 3, 4 and 5, immediately flanking the 180 bp repeat sequence (Fransz et al., 2000; The Arabidopsis Genome Initiative, 2000). In contrast to the tandem repeats, the interspersed repeats of BAC F28D6 and Athila transposons (Fig. 4E,F) remained largely scattered, even after 5 days in culture. These data indicate a sequential reformation of chromocenters by repetitive elements starting with 45S rDNA genes, followed by the 180 bp repeats and the 5S rDNA genes.

The global level of DNA methylation remains unaltered during protoplast culture

Chromocenters contain heavily methylated DNA (Fransz et al., 2002). Previous studies have shown that DNA hypomethylation affects the organization of pericentric heterochromatin (Fransz et al., 2003; Probst et al., 2003; Soppe et al., 2002; Tariq et al., 2003). To determine whether the heterochromatin reduction in protoplasts is associated with changes in DNA methylation, we investigated the spatial distribution of 5-methylcytosine (5-mC) by immunolabeling. Protoplasts showed a dispersed 5-mC pattern distinct from the clustering at chromocenters in mesophyll cells (Fig. 5A). During prolonged culture some clustering was observed at chromocenters, but the majority of 5-mC signals remained scattered. We observed no large changes in the intensity of the total fluorescent signals per nucleus, suggesting that the overall level of methylation of DNA remained unchanged (Fig. 5A).

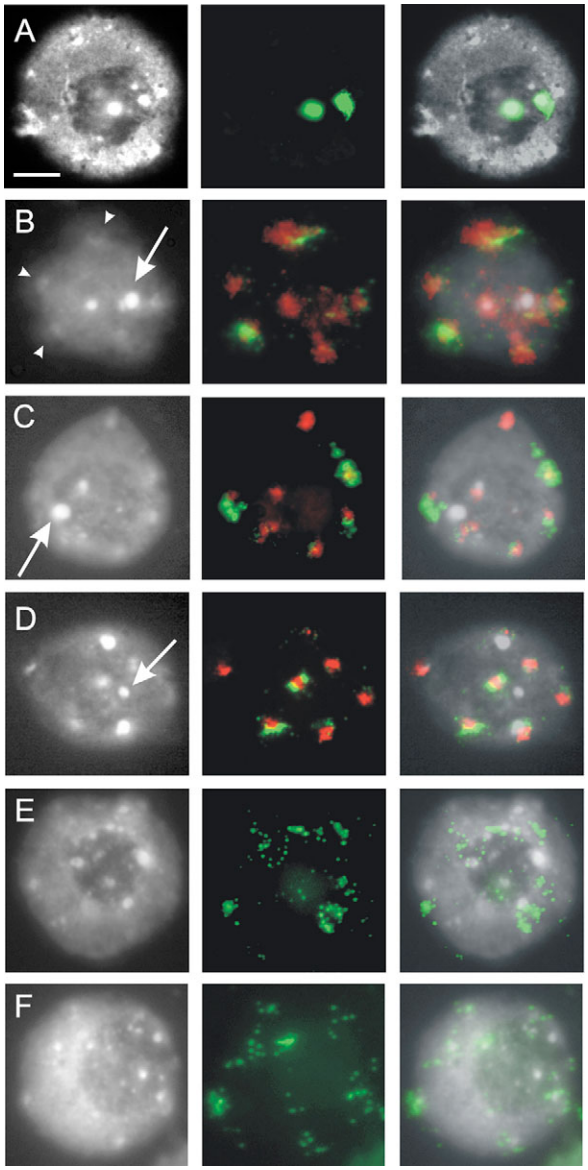


Fig. 4. Localization of repetitive sequences in cells cultured for 120 hours. DAPI staining (left column); FISH signal (middle column); merge (right column). (A) 45S rDNA (green signal) is condensed and located at large chromocenters. (B-D) FISH signals of the 180 bp repeat (red) and 5S rDNA (green) show partial (B,C) to full (D) condensation at chromocenters. Note that partially condensed 180 bp repeats colocalize with faint chromocenters (arrowheads in B). The large chromocenters (arrows) contain 45S repeats. (E,F) BAC F28D6 (E) and the Athila transposon (F) are only partially localized at chromocenters. Bar, 5 μm.

This was verified by Southern blot analysis using *Msp*I and the CpG methylation-sensitive isoschizomer *Hpa*II (Fig. 5B). All repeats, 180 bp, the 45S rDNA and Athila transposon, retained their methylated state irrespective of the nuclear phenotype. These results suggest that DNA methylation changes, at least at CpG sites, are not involved in the large-scale reduction of heterochromatin.

Protoplast isolation and culture does not affect global levels of H3K4me2 and H3K9me2

A large variety of methylation and other histone modifications are known to decorate repeat-rich (H3K9me2, H3K27me1, H3K27me2, H4K20me1) or gene-rich (H3K4me1, H3K4me2, H3K4me3; acetylation) chromosomal regions in *Arabidopsis* (Naumann et al., 2005; Fuchs et al., 2006). Dimethylation of H3K4 and H3K9 are particularly representative of their respective functions, as these are conserved between plants and other organisms. We analyzed whether the reduction of visible heterochromatin is accompanied by changes in histone H3 methylation at lysines K4 and K9. In mesophyll cells, H3K9me2 labeling was essentially at chromocenters. In protoplasts, however, the H3K9me2 signal was in a speckled pattern throughout the nucleus, similar to the pattern of pericentric repeats and methylated DNA (Fig. 6A). Immunolabeling of H3K4me2 in mesophyll cells and in cultured cells gave a strong signal exclusively outside chromocenters and the nucleolus. In protoplast nuclei, H3K4me2 labeling was uniformly distributed but absent from the nucleolus and the remaining 45S rDNA chromocenters (Fig. 6B). We then examined the levels of dimethylated H3K4 and H3K9 by western blot (Fig. 6C) and observed no significant differences during protoplast formation and culture. This unchanged global levels of H3K4me2 and H3K9me2 imply that alterations in chromatin condensation have taken place without significant changes of dimethylation levels of H3K4 and H3K9.

Decondensation of chromatin does not necessarily lead to activation of silent genes

To investigate whether the decondensation of pericentric heterochromatin affected silent genes, we examined the Line A, which carries a silent locus A, containing multiple copies of the hygromycin phosphotransferase (*HPT*) gene (Mittelsten Scheid et al., 1998). Locus A is microscopically visible as a mini chromocenter (Probst et al., 2003). During protoplast formation the *HPT* mini-chromocenters decondense (Fig. 7). However, no increase in transcription of *HPT* in protoplasts or cultured cells was found (Table S1 in supplementary material) suggesting that decondensation of the *HPT* locus is not sufficient to reactivate the *HPT* gene.

Table 1. Percentage of condensed signals at different stages of the protoplast culture for the major repeat sequences

	Leaf		Protoplast		24 hours		120 hours	
Sequence	cond.	(n)	cond.	(n)	cond.	(n)	cond.	(n)
180 bp	90	(700)	28	(251)	41	(158)	57	(210)
5S rDNA	68	(266)	11	(284)	34	(154)	48	(105)
45S rDNA	100	(74)	65	(103)	70	(61)	81	(63)

Condensed (cond.) fluorescent signals colocalize entirely with chromocenters

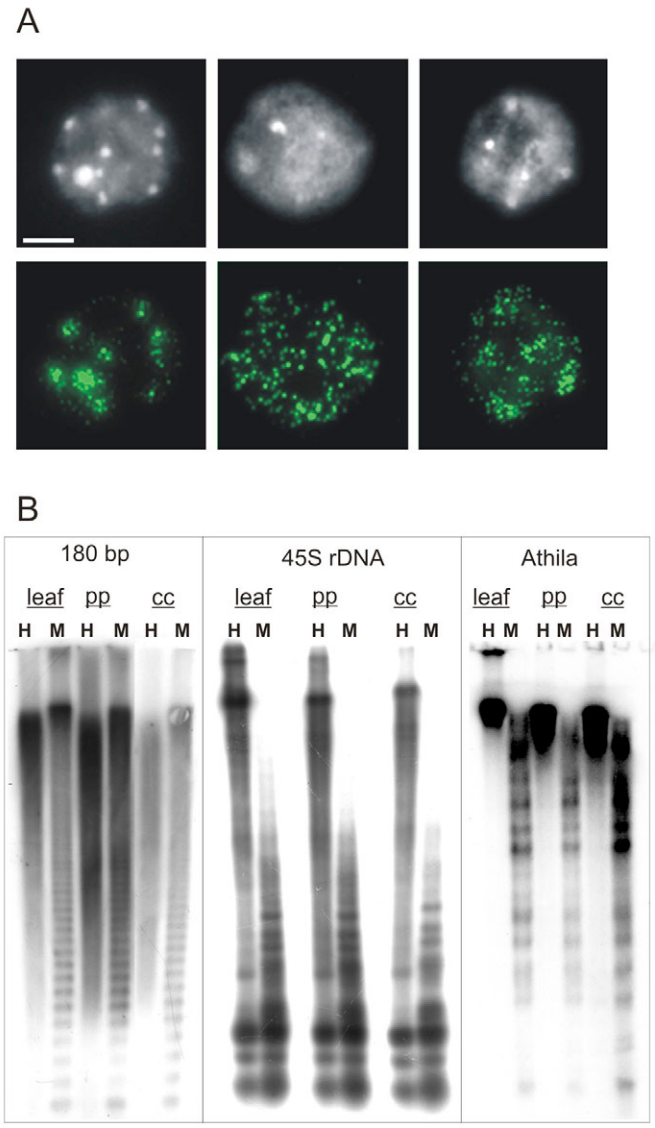


Fig. 5. DNA methylation pattern in nuclei and in repeat regions. (A) DAPI staining (upper panels) and immunolocalization of 5-methylcytosine (lower panels) in leaf (left), protoplast (middle) and cultured cell (right). Bar, 5 μm. (B) Southern blot analysis of leaf, protoplast (pp) and cultured (cc) cells. DNA was digested with *Msp*I (M) and *Hpa*II (H). The blots were hybridized with DNA probes from the 180 bp repeat, 45S rDNA and Athila transposon.

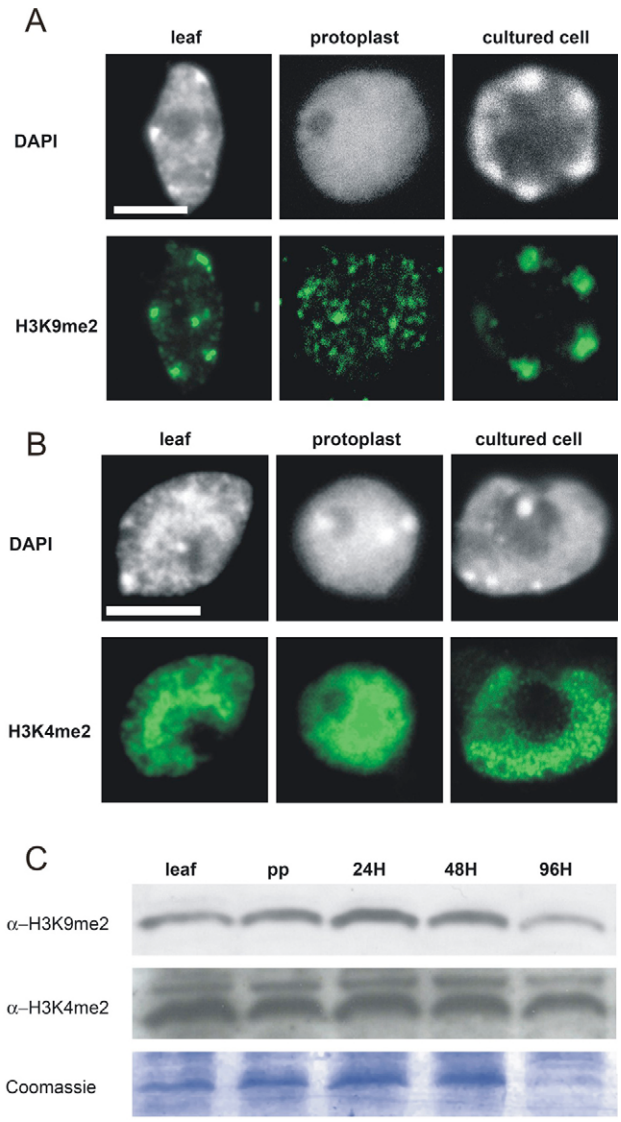


Fig. 6. Distribution pattern and concentration of histone H3K9 and H3K4 dimethylation. (A) DAPI staining (upper panels) and immunolocalization of H3K9me2 (lower panels). (B) DAPI staining (upper panels) and immunolocalization of H3K4me2 (lower panels). Bar, 5 μm. (C) Western blot analysis of histone-enriched protein extracts from leaves, protoplasts and cultured cells (24 hours, 48 hours, 96 hours) using antibodies against H3K9me2 and H3K4me2.

Discussion

Heterochromatin reorganization in protoplasts

The plant protoplast is an attractive experimental system to investigate dedifferentiation and the acquisition of totipotency. Here we show that dedifferentiation of *Arabidopsis* mesophyll cells is accompanied by a dramatic transient decondensation of pericentromeric heterochromatin. This involves all tandem and dispersed pericentromeric repeats and results in the almost complete disassembly of chromocenters in leaf nuclei. Dedifferentiation-related changes in chromatin structure have been reported in tobacco protoplasts, using a micrococcal nuclease assay (Williams et al., 2003; Zhao et al., 2001). However, these studies did not show which chromosomal regions were involved and what the extent of decondensation was. A major problem in tobacco nuclei is the high content of heterochromatin (Libault et al., 2005). By contrast, the *Arabidopsis* nucleus contains a limited number of discrete, well-characterized heterochromatin domains, called chromocenters, that constitute easily detectable DAPI-bright domains during interphase (Fransz et al., 2002). Chromocenters contain large arrays of tandem repeats and dispersed repeats. Chromocenters are rich in dimethylated histone H3K9, an epigenetic marker for heterochromatin in plants (Soppe et al., 2002). By monitoring these features of chromocenters we have followed the disassembly and

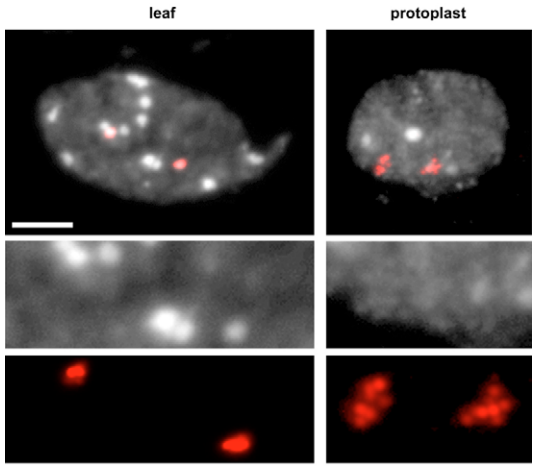


Fig. 7. The heterochromatic transgene Locus A containing a multicopy *HPT* array is decondensed in protoplast nuclei. FISH signals (red; top and bottom panels) for the *HPT* sequence localize at mini-chromocenters (brightly fluorescent areas) in nuclei of *Arabidopsis* Line A. DAPI-stained (top and middle panels) mini-chromocenters are no longer visible in protoplasts and the FISH signal for the *HPT* sequence is decondensed. The bottom four panels are higher magnifications of the HPT regions. Bar, 5 μm.

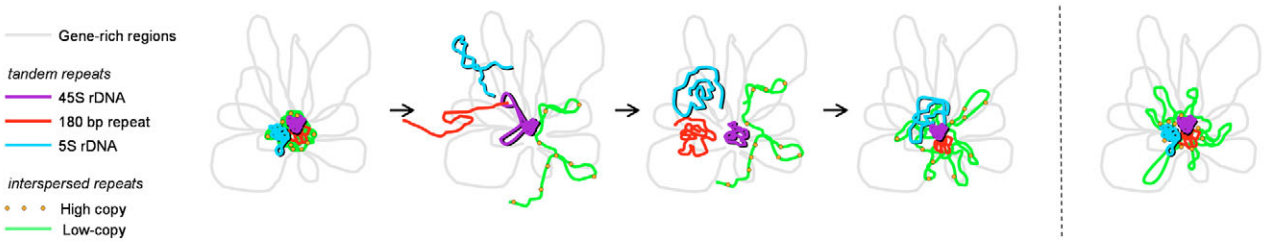
subsequent partial reassembly of heterochromatin domains that was triggered by the formation of protoplast.

Large-scale chromatin decondensation is not accompanied by changes in methylation of DNA and of histone H3K9

The decondensed state of chromatin in *Arabidopsis* protoplasts is unique in its extent and in the fact that it is present in all nuclei (Fig. 2B). The severe DNA hypomethylation mutant *ddm1-5* (Mittelsten Scheid et al., 2002; Probst et al., 2003), which lacks the ATP-dependent DNA helicase DDM1 (Jeddeloh et al., 1999), displays a similar nuclear phenotype but only in a minority (<10%) of the leaf cells (Mittelsten Scheid et al., 2002). The majority of the *ddm1-5* nuclei show a limited decondensation of chromocenter heterochromatin. A similar phenotype was found also in other DNA hypomethylation mutants (Fransz et al., 2003), such as *ddm1-2* (allelic with *ddm1-5*), producing a non-functional DDM1 protein (Jeddeloh et al., 1999), *met1-1*, a DNA methyltransferase for CpG sites, and *hog1*, a *S*-adenosyl-L-homocysteine hydrolase. The mutants *ddm1-2* and *met1* show a 40% reduction in heterochromatin compared to wild type. Other cases of reduced heterochromatin have been reported in mutants defective in CAF1, which is involved in nucleosome assembly (Schonrock et al., 2006), in HDA6, a histone deacetylase (Fransz et al., 2006), and in BRU1, a protein involved in DNA repair (Takeda et al., 2004). However, none of these mutants shows the dramatic level of decondensed pericentric heterochromatin observed in protoplasts. The fact that several DNA methylation mutants show a partial decondensation of heterochromatin suggests that a DNA methylation imprint is required for the assembly of pericentric heterochromatin in chromocenters. This has been confirmed by crossing *ddm1-2* and *met1-1* with the wild-type Col-0 plant (Soppe et al., 2002). However, restoration of DNA methylation activity by back-crossing with wild-type plants did not lead to wild-type levels of DNA methylation or promote heterochromatin assembly (Soppe et al., 2002). Evidently, DNA methylation is not sufficient for wild-type levels of heterochromatin. In the present study we show that the global level of DNA methylation remains unchanged in protoplasts showing extensive heterochromatin decondensation. This underscores the notion that heterochromatin disassembly is not controlled by DNA demethylation.

Our results show that reduction of pericentric heterochromatin is not accompanied by a global loss of histone H3K9 dimethylation, indicating that, like DNA methylation, this histone modification is not sufficient to induce compaction of heterochromatin. The observation is in line with microscopic studies of the *suvh4* mutant, which lacks the methyltransferase SUVH4/KYP (responsible for H3K9 dimethylation). This mutant has normal chromocenters but the level of H3K9me2 is strongly reduced (Jasencakova et al., 2003; Naumann et al., 2005; Zemach et al., 2005). Similarly, the DNA methyltransferase mutant *met1-3* shows normal chromocenters, despite the fact that they lack both CG methylation and H3K9me2 (Tariq et al., 2003). We cannot exclude that H3K9 dimethylation in pericentric heterochromatin has been replaced by H3K9 methylation in gene-rich regions. However, considering the normal phenotypes of nuclei in the mutants *suvh4* and *met1-3* it is not likely that such a redistribution results in decondensation of pericentric heterochromatin. It remains to be resolved if other histone modifications are important for chromocenter decondensation.

Chromatin decondensation is generally associated with upregulation of gene activity (Probst et al., 2003; Mathieu et al., 2003; Chambeyron and Bickmore, 2004; Wegel et al., 2005). For example, gene-silencing mutants, such as *ddm1*, *met1*, *hog1* and *sil1*, show reduced heterochromatin in combination with transcriptional activation of silent genes, including transgenes, pericentric elements and transposons (Steimer et al., 2000; Hirochika et al., 2000; Miura et al., 2001; Soppe et al., 2002; Probst et al., 2003). By contrast, in protoplasts the massive heterochromatin decondensation does not result in activation of silent elements, such as the multicopy transgene *hpt* locus, suggesting that decondensation by itself is not sufficient for transcriptional activation. It is likely that the persistence of DNA methylation and H3K9me2 levels after decondensation inhibits transcription. Evidently, heterochromatin condensation is determined by molecular mechanisms other than the deposition of these two epigenetic marks. Whereas silent repeats remain inactive in *Arabidopsis* protoplasts, microarray studies have shown that gene expression drastically changes upon formation of protoplasts (Avivi et al., 2004). For example, members of the NAC (NAM, ATAF1, 2, CUC2) family of transcription factors, which are involved in developmental processes such as establishment of stem cell lineage and in stress responses, are activated in protoplasts (Olsen et al., 2005). Remarkably, dissociation and reassembly of chromocenter-like heterochromatic structures also accompany nuclear reprogramming and differentiation in mouse and cattle (Martin et al., 2006a; Martin et al., 2006b; Tamada et al., 2006). This suggests that, despite the fact that chromocenters are gene-poor regions of the genome (Fransz et al., 2006), their transient dissociation in protoplasts and



	leaf	protoplast	cultured cell	ddm1[c]
Total size of repeats in chromocenters (Mb)[a]	30.7	7.5	13.2	> 13.2
Relative size of repeats in chromocenters[b]	1.00	0.24	0.43	> 0.43
Mean RHF[b]	1.00	0.27	0.45	0.60

Fig. 8. Chromatin dynamics during protoplast culture. Schematic representation of an interphase chromosome before and after protoplast formation and in the *ddm1* mutant, showing repeat regions (colors) and gene-rich regions (gray). The loop organization of gene-rich euchromatin is based on previous studies (Fransz et al., 2002). For simplicity only chromosome 4 is shown, which has all major tandem repeats (180 bp, 45S rDNA and 5S rDNA). Most repeat regions become decondensed in protoplasts. Only the 45S rDNA domain remains partly condensed. Cultured cells show various levels of chromatin condensation. The sequence of recondensation events is 45S rDNA, 180 bp, 5S rDNA and interspersed repeats, such as transposable elements. [a]The total size of repeat sequences in chromocenters is based on the total genomic size of the repeats shown in Table S2 in supplementary material. [b]Values are normalized to the maximal value of dataset (=leaf). [c]The total size of high copy transposons in *Arabidopsis* is unknown. In the *ddm1* mutant all repeats, except the low copy repeats, are in chromocenters (Soppe et al., 2002). Hence, the fraction of repeats in chromocenters of *ddm1* is higher compared with chromocenters of cultured cells and lower compared with chromocenters in leaf nuclei.

mammalian ES cells is involved in the correct orchestration of the developmental program.

Chromocenter formation involves the sequential condensation of pericentromeric repeats

Chromocenter reorganization in *Arabidopsis* protoplasts has provided us with a system for studying the dynamics and the molecular mechanism of heterochromatin assembly in higher eukaryotes. *Arabidopsis* chromocenters harbor major tandem and dispersed repeats (Fransz et al., 2002). Cytogenetic studies have demonstrated that chromocenters can increase in size at specific developmental stages (Mathieu et al., 2003; Tessadori et al., 2004). Our present study shows that the formation of pericentromeric heterochromatin domains is a stepwise process in which genomic regions carrying different repeat classes condense one after the other. The 45S rDNA repeats of chromosomes 2 and 4 remain largely condensed throughout protoplast formation and subsequent culturing. The 180 bp repeats and 5S rDNA genes recondense into distinct compact domains that later fuse with each other and with the 45S rDNA domains (Figs 4 and 8). By contrast, the majority of interspersed repeat elements, such as transposons, remain largely decondensed even after 5 days of protoplast culturing. We observed an almost perfect agreement between the genomic size of the repeat elements and their relative contribution to chromocenter heterochromatin (Fig. 8). For example, the genomic size of the 45S rDNA repeats is 7.5 Mb (Copenhaver and Pikaard, 1996), which is 24% of the total amount of repeat sequences. In protoplasts the 45S rDNA genes remain almost completely associated with the residual chromocenters, contributing a relative heterochromatin fraction (RHF) of 0.03, which is 27% of the mean RHF value in leaf nuclei (RHF is 0.11). Evidently, the contribution of these repeat-containing genomic domains to the intensity of DAPI fluorescence of heterochromatin is linearly related to their genomic size (Fig. 8).

The sequential assembly of different repeat classes into newly formed chromocenters indicates that these repeat classes define different types of heterochromatin with distinct properties. Similarly, distinct types of heterochromatin have been proposed in mouse cells, based on major and minor satellite domains that recruit different chromatin-associated proteins (Guenatri et al., 2004). We compared the characteristics of the repeat classes in the *Arabidopsis* genome, including size of the repeat unit, copy number, array size and array number (see Table S2A in supplementary material). These data indicate a correlation between the length of the repeat array and the temporal order of chromatin condensation (Table S2B in supplementary material). The 45S rDNA gene repeat arrays are the longest, spanning 3-4 Mb per locus (Copenhaver and Pikaard, 1996). The centromeric 180 bp repeat arrays are up to 1 Mb in size (The Arabidopsis Genome Initiative, 2000). The smallest arrays of the major tandem repeats are from the 5S rDNA, which span 100-300 kb (The Arabidopsis Genome Initiative, 2000; Cloix et al., 2000), separated over three major loci in Col-0 (Fransz et al., 1998). Although they make up the majority of pericentric heterochromatin, interspersed repeats, such as Athila, are not arranged in long tandem arrays (Fransz et al., 2000). It is not clear which molecular mechanism accounts for the sequential assembly of repeats at chromocenters or why the large repeat arrays are more condensed than the shorter repeats arrays. It is likely that the responsible machinery can distinguish closely associated, identical sequences, such as tandemly arranged repeats, from multicopy interspersed repeats. A possible explanation for this discrimination may be found in the relationship between RNAi components and heterochromatin formation. This association involves transcription of pericentric repeats, which has been reported for *S. pombe* (Grewal and Moazed, 2003), *Drosophila*

(Pal-Bhadra et al., 2004), *Arabidopsis* (Gendrel and Colot, 2005; May et al., 2005) and mammals (Kohlmaier et al., 2004). A RNAi-based mechanism has been proposed for heterochromatin formation from tandemly arrayed repeats (Martienssen, 2003; Lippman and Martienssen, 2004). The model describes how the tandem arrangement of the repeat transcripts enables a continuous production of siRNAs by RNA-dependent RNA polymerase (RdRP). Single copy sequences or dispersed repeats would not undergo such a process. It is attractive to speculate that the local high density of repeats results in high local transcript concentration, which in turn provides a scaffold for the binding of transcript-specific heterochromatin proteins. In a recent paper, Bouzinba-Segard et al. (Bouzinba-Segard et al., 2006) report that stress and differentiation can lead to the accumulation of non-coding small centromeric RNAs at chromocenters in murine cells, resulting in loss of centromeric function. The idea that repeat-derived RNA transcripts play an important role in heterochromatin compaction is further supported by the disintegration of mouse chromocenters after RNAse treatment (Maison et al., 2002). The relationship between RNAi and heterochromatin condensation in *Arabidopsis* will be the subject of future work using mutants impaired in RNAi-mediated silencing.

Materials and Methods

Protoplast isolation and culturing

Seeds of *Arabidopsis thaliana* Col-0, Zurich Line A, carrying a multicopy Hpt transgene locus (Mittelsten Scheid et al., 1998), and a C24 transgenic plant, carrying a H2B-YFP construct (Boisnard-Lorig et al., 2001), were sown under sterile conditions on bouturage medium (Duchefa, Haarlem, The Netherlands; pH 5.6) supplemented with Micro 1-3 microelements (21 mM $ZnSO_4$, 24 mM H_3BO_3, 89 mM $MnSO_4$, 180 μM $CuSO_4$, 2.4 mM KI, 1.9 mM Na_2MoO_4, 189 μM $CoCl_2$), 3.5 mM MES and 15 μM Bromocresol Purple (Chupeau et al., 1989). Growth conditions were 14 hours light:10 hours dark, 60 μmol m^{-2} s^{-1} Biolux light, 70% relative humidity and 20°C. Plantlets (18-days old) were chopped and digested in macerating medium (Chupeau et al., 1989) containing cell wall-degrading enzymes [0.01% (w/v) Macerozyme (Duchefa), 0.1% (w/v) Cellulase Onozuka R10 (Yakult, Tokyo, Japan), 0.02% (w/v) Driselase® (Fluka, Buchs SG, Switzerland)] for 16 hours, at 22°C. After filtration through 80 μm sieves, protoplasts were centrifuged (70 ***g*** for 6 minutes) three times and resuspended in washing medium (Bourgin, 1978). They were cultured in the dark, at 20°C in culture medium at a final concentration of 70,000 protoplasts/ml (Chupeau et al., 1993).

Fluorescence in situ hybridization (FISH) and signal measurement

The following DNA was labeled with either Biotin- or Digoxigenin-Nick Translation kits (Roche, Mannheim, Germany): plasmid pAL1 (Martinez-Zapater et al., 1986); 5S rDNA (Campell et al., 1992), 45S rDNA (Gerlach and Bedbrook, 1979), BAC F28D6 (DDBJ/EMBL/GenBank accession no. AF147262), Athila transposon and hygromycin phosphotransferase (HPT) fragment. For the detection of Athila, a PCR fragment was used, based on nucleotides 293-2704 of BAC T1J24 (accession no. AF147263; primers and BAC kindly provided by W. J. Soppe, MPIZ, Cologne, Germany). The HPT fragment was detected using a PCR-generated probe based on nucleotides 320-602 of the HPT gene. FISH experiments were carried out as described by Soppe et al. (Soppe et al., 2002).

The length of the FISH signals were measured using ImageJ software. The average decondensation factor was calculated from the FISH signals as follows: average length from all 5S signals in mesophyll cells divided by the average length from all 5S signals in protoplasts.

5-Methylcytosine detection

5-Methylcytosine detection was carried out on cells fixed in ethanol:acetic acid (3:1) essentially as described previously (Fransz et al., 2002). Incubation with the mouse antibody against 5-methylcytosine (Eurogentec, Seraing, Belgium) was performed at 37°C for 1 hour.

Immunolabeling of histones

Protoplast nuclei were isolated by gently grinding the cells in a 1.5-ml reaction tube and were then fixed in 4% formaldehyde for 20 minutes. The nuclei were then centrifuged twice at 500 ***g*** for 5 minutes and resuspended in PBS. Slides with nuclei were subsequently prepared as described previously (Fransz et al., 2002). Immunolabeling of modified histones was carried out as described by Soppe et al. (Soppe et al., 2002) with primary antibodies against H3K9me2 (Upstate, Charlottesville, VA; Ref. 07-212) and H3K4me2 (Upstate; Ref. 07-030). After overnight incubation at room temperature, detection was carried out with goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) and donkey anti-goat FITC (Jackson ImmunoResearch Laboratories, Soham, UK), each step at 37°C for 30 minutes. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/ml in Vectashield, Vector Laboratories).

Image acquisition and processing

In vivo imaging of H2B-YFP nuclei was carried out on a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany). The images were pseudocolored with the Zeiss LSM ImageViewer program. Image acquisition for FISH and immunolabeling were carried out on a Leitz Aristoplan (Leica Microsystems AG, Wetzlar, Germany) microscope with filters for DAPI, Texas Red and FITC. Images were captured with a charge-coupled device (CCD) camera (Apogee, Logan, UT) using ImageProPlus Software (Media Cybernetics, Silver Spring, MD). The FISH images were digitally processed using Adobe Photoshop (Adobe, San Jose, CA).

Quantification of the relative heterochromatin fraction (RHF)

Nuclei were isolated from ethanol:acetic acid (3:1)-fixed material as described previously (Soppe et al., 2002) and stained with DAPI. Image acquisition was carried out with a Leitz Aristoplan. The raw images were analyzed with ObjectImage freeware written by N. Vischer (University of Amsterdam, Amsterdam, The Netherlands) (http://simon.bio.uva.nl/object-image.html). The relative heterochromatin fraction (RHF) was calculated from the area and DAPI-fluorescence intensity of chromocenters in relation to the entire nucleus, as described previously (Soppe et al., 2002).

Southern and western blot analyses

Standard procedures were followed for Southern hybridizations. Ribosomal and centromeric probes were derived from a 3.7 kb *Eco*RI fragment of the pARR17 plasmid and a 180 bp *Hin*dIII fragment of the pARR20-1 plasmid, respectively. Preparation of the Athila probe is described above.

Western blot analysis was carried out on protein samples extracted in ice-cold lysis buffer (20 mM Hepes pH 7.9, 400 mM sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100, 0.02% NaN_3, 1.5 mM PMSF, 1 mM DTT) as described (Houben et al., 2003). The transfer membrane was incubated with the same primary antibodies as used for immunolabeling and detected using an ECL Plus® western detection kit (Amersham Biosciences, Piscataway, NJ).

Quantitative RT-PCR analysis

Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA) and reverse transcribed using Superscript II reverse transcriptase according to the supplier's instructions. Level of *HPT* expression was estimated by real-time RT-PCR on a LightCycler instrument (Roche Molecular Biochemicals), using a ready-to-use Hot-Start reaction mix (LightCycler FastStart DNA Master SYBR Green I, Roche) according to the manufacturer's recommendations and compared to the elongation factor 1-alpha expression (*EF1α* At5g60390). The following primers with a PCR efficiency of 80-120% were used: EF1F 5′-CTGGAGGTTTTGAGGCTGGTAT-3′ and EF1R 5′-CCAAGGGTGAAAGCAAGAAGA3′ (Baud et al., 2003), as well as HPT1 5′-CGCCCGGAGTCGTGGCGATCC-3′ and HPT2 5′-TACGAGGTCGCCA ACATCTTCTTC-3′ to amplify the *EF1α* and *HPT* cDNAs, respectively. All amplification plots were analyzed using the Second Derivative Maximum method to obtain Ct (threshold cycle). Primers were tested with a standard dilution series to obtain the slope and the intercept of the curve for quantification.

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