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Molecules and Cells

Chromatin Structural Rearrangement during Dedifferentiation of Protoplasts of *Cucumis sativus* L.

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This paper reports on the structural rearrangement of satellite DNA type I repeats and heterochromatin during the dedifferentiation and cell cycling of mesophyll protoplasts of cucumber (*Cucumis sativus*). These repeats were localized in the telomeric heterochromatin of cucumber chromosomes and in the chromocenters of interphase nuclei. The dramatic reduction of heterochromatin involves decondensation of subtelomeric repeats in freshly isolated protoplasts; however, there are not a great many remarkable changes in the expression profile. In spite of that, reformation of the chromocenters, occurring 48 h after protoplast isolation, is accompanied by recondensation of satellite DNA type I; however, only partial reassembly of these repeats was revealed. In this study, FISH and a flow cytometry assay show a correlation between the partial chromocenter and the repeats reassembly, and with the re-entry of cultivated protoplasts into the cell cycle and first cell division. After that, divided cells displayed a higher variability in the expression profile than did leaves' mesophyll cells and protoplasts.

INTRODUCTION

It is well established that differentiated plant cells, in contrast to those of animals, hold developmental potentialities during development, and retain a plasticity that enables dedifferentiation processes and the acquisition of new outcomes. The remarkable example of pluripotency of plant determined cells is the dedifferentiation of isolated plant protoplasts from different tissues. Only upon application of phytohormones, such as auxins and cytokinins, do the protoplasts reenter the cell cycle, proliferate, and undergo regeneration processes; resulting in new plantlets (Cocking, 1960; Debeaujon and Branchard, 1992; Takebe and Otsuki, 1969). Protoplast dedifferentiation is characterized by a new balance between the less-dense portion of the genome that is transcribed (euchromatin), and that which is condensed with repressed transcription (heterochromatin). In plants, except those with high genome size (reviewed in van Driel and Fransz, 2004) highly condensed chromatin is easily recognized by light microscopy, after DAPI staining of the interphase nuclei. These blocks of heterochromatin (chromocenters) lose their compaction during protoplast dedifferentiation, as has been recognized in tobacco (Zhao et al., 2001) and *Arabidopsis* (Tessadori et al., 2007). The chromocenters consist predominantly of centromeric and pericentromeric heterochromatin, enriched with epigenetic markers of silent chromatin, such as the CpG methylation of DNA, and histone H3K9 dimethylation (reviewed in van Driel and Fransz, 2004; Tessadori et al., 2004). The heterochromatin decondensation of *Arabidospsis* protoplasts is accompanied by structural relaxation of centromeric (180 bp), pericentromeric and 5S rDNA repeats, and also transposons localized at chromocenters of leaves (Tessadori et al., 2004). Only subtelomeric 45S rDNA repeat regions, the core constituents of certain chromocenters, remain in their condensed state (Tessadori et al., 2004).

Cucumis sativus (2n = 14) has a genome size about two times larger than *Arabidopsis* (Lebeda et al., 2007), forming chromocenters within the interphase nucleus. The chromosomes of cucumber have not been well characterized cytogenetically. There have been only a few works which used the FISH technique to distinguish all 7 chromosomal pairs, using probes for 5S and 45S rDNA, and pericentric repeats (Koo et al., 2002; 2005). In this study, we localized satellite DNA type I of cucumber, first described by Ganal et al. (1986), in heterochromatic telomere-associated regions. These types of repeats are also a component of chromocenters inside the interphase nuclei and undergo decondensation together with chromocenters, also indicating chromatin rearrangement in telomere-associated domains during dedifferentiation of the cucumber protoplasts. Reassembly of satellite DNA type I repeats, and chromocenters are followed by the re-entry of protoplasts into the cell cycle, and changes in the expression patterns.

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MATERIALS AND METHODS

Protoplast isolation and culture

Seeds of *Cucumis sativus* L. (cv. Marketer; SEMO Ltd., Smržice, Czech Republic) were sown under sterile conditions on half-strength MS medium (Duchefa). After germination, the seedlings were planted on MS medium (supplemented with 20 g/L sucrose, 0.8% agar, 0.049 μmol/L IBA, and 0.044 μmol/L BA) in plastic boxes. The plants were cultivated in a culture room with a 16 h photoperiod (light intensity 32-36 $\mu mol/m^2s$), and a temperature of 22 ± 2°C.

The leaves of the plantlets were chopped and digested in maceration enzyme mixture containing 1% (w/v) Cellulase Onozuka R-10 (Duchefa) and 0.25% (w/v) Macerozyme R-10 (Duchefa), dissolved in PGly washing solution (Debeaujon and Branchard 1992). The pieces of leaves were incubated in the enzyme mixture for 16-17 h in the dark at 25°C. The protoplasts were isolated according to Gajdová et al. (2007) and re-suspended in LCM1 culture medium (Debeaujon and Branchard, 1992).

The relative fluorescence of the nuclei was measured with a CyFlow ML cytometer (Partec GmbH, Germany), equipped with an argon ion laser. The instrument was calibrated before each set of measurements with a standard of pea nuclei (*Pisum sativum* cv. Ctirad; 2C = 9.09 pg). The nuclei were isolated, and re-suspended in buffer, supplemented with DNAse free RNase A (50 μg/ml) and propidium iodide (50 μg/ml). At least 3,000 nuclei were analyzed in each sample.

Fixation, fluorescence *in situ* hybridization (FISH), image acquisition

Freshly isolated protoplasts and cells from protoplast culture 24, 48, and 72 h after protoplast isolation were fixed in ethanol: acetic acid (3:1) (EAA), and then prepared for FISH. Cells from culture were treated with a solution of 1% Pectinase and 2% Cellulase Onozuka R-10 (Duchefa) for 15 min at 37°C, in order to remove newly synthesized cell walls. Young leaves and root tips were also fixed in EAA, macerated in a solution of 1% Pectinase from *Aspergillus niger* (Serva) and 2% Cellulase for 30 min at 37°C, gently squashed in 45% acetic acid, washed in 96% ethanol, dried, and then prepared for FISH.

FISH was carried-out on the interphase nuclei of leaves, protoplasts, protoplast-derived cells, and metaphase chromosomes in order to test the probes, with slight modifications of the hybridization protocol used by Ondřej et al. (2008). The probe for telomere-associated regions was prepared by PCR amplification of satellite DNA type I repeats from genomic DNA using primers: forward 5′-CTGGGTGGCCTCATTTTG-3′ and reverse 5′-GACCTTTGGCACCGTTGT-3′. The PCR products were identified by electrophoresis on 2% agarose gel, purified (Gene Elute PCR Clean-Up Kit, Sigma), and labelled by the DIG-nick translation kit (Roche), according to the manufacturer's protocol. The hybridized probe was detected using mouse anti-digoxin rhodamine red-X-conjugated antibody (Jackson Immunoresearch Laboratories, Inc.). Nuclei were counterstained, using DAPI diluted in Vectashield (Vector Laboratories, USA), in order to reduce photobleaching.

Image acquisition was carried-out with a fluorescent microscope (Olympus BX 60) fitted with a CCD camera (Cool Snap, Photometrics).

cDNA-AFLP analyses

Total RNA was isolated from the young leaves of plantlets cultivated under sterile conditions, from freshly isolated protoplasts and microcalli, derived from protoplast culture using a Spectrum Total RNA isolation Kit (Sigma), and treated by RNAse free DNAse. RNA was reversely transcribed into the cDNA by oligo dT(18) primers and a Transcriptor High Fidelity cDNA Synthesis Sample Kit (Roche). The purified cDNA was subjected to the AFLP procedure, adopted from Vos et al. (1995), with some modifications after Kitner et al. (2008). cDNA (100 ng) was restricted with *Eco*RI (rare cutter) and *Mse*I (frequent cutter); *Eco*RI and *Mse*I adapters were subsequently ligated to digested DNA fragments. The adapter-ligated DNA was pre-amplified with go*Taq* polymerase (Promega), and pre-amplified DNA was used as a template for the selective amplification with five primer combinations. The amplified fragments were denatured, and the samples were uploaded on a 6% polyacrylamide denaturating gel in a vertical electrophoresis unit GibcoBRL MODEL S2 (Life Technologies) (70 W, 60 min., 55-58°C). The resulting banding patterns were visualized by the silver staining method.

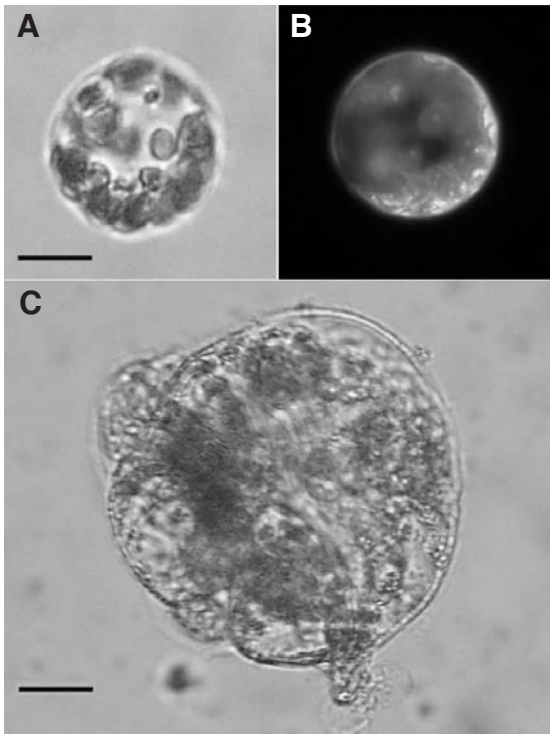

Fig. 1. Isolated protoplast of *Cucumis sativus* (A), and protoplast after FDA staining to test viability of isolated protoplasts (B). The dividing cells were observed (C) between the third and fifth day of culture. The scale bars = 10 μm.

RESULTS

To investigate the chromatin changes during protoplast differentiation, we used established isolation and culture systems for the *Cucumis* species. The protoplasts of cucumber (Fig. 1A) were isolated from mesophyll tissues originating from young leaves of *in vitro* cultured plantlets. The viability of isolated protoplasts was approximately 80%, estimated by FDA staining (Fig. 1B). The protoplasts started to divide between the third and fifth day after isolation (Fig. 1C).

The interphase nuclei of young leaf mesophyll tissues of cucumber have up to 15 heterochromatic domains of similar size (Fig. 2B). In the nuclei of protoplasts fixed immediately after

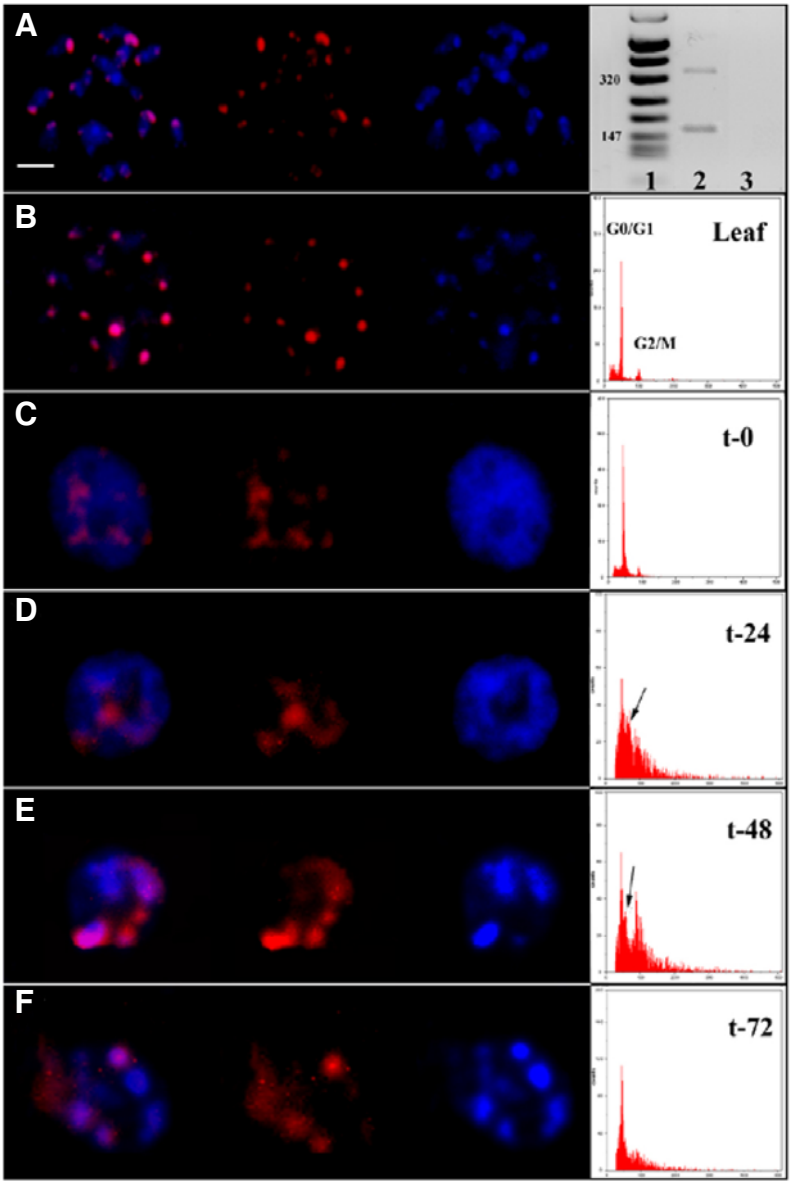

Fig. 2. Structural changes of chromatin and cell cycling during cucumber cell dedifferentiation by FISH and FCM assay. (A) FISH on the metaphase chromosomes of *C. sativus* revealed localization of satellite DNA type I repeats (red signals) in telomeric heterochromatin at the ends of chromosomes. Additionally, the electrophoresis of PCR products of the satellite DNA type I used for probe preparation: lane 1 - ladder, lane 2 - PCR products of 160 and 340 bp, lane 3 - control PCR with water. FISH using satellite DNA type I probe (red signals) on the interphase nuclei, counterstained with DAPI (blue), of leaf (B), freshly isolated protoplast (C) and protoplast-derived cells 24 (D), 48 (E) and 72 h (F) after protoplast isolation, compared with FCM histograms. Arrows in FCM histograms indicate supra-G_1 peak. The scale bar = 2 μm.

isolation, chromocenters were largely decondensed, resulting for the most part in their total disappearance from most of the nuclei (Fig. 2C). DAPI staining of the nuclei showed that within 48 h the chromatin became condensed by reforming of the chromocenters (Fig. 2E). The nuclei showed differences in the number and size of chromocenters across the cells population 72 h after protoplast isolation (Fig. 2F).

The PCR amplification of satellite DNA type I repeats resulted in two products of about 160 and 340 bp (Fig. 2A), which are in agreement with the primers used. With these products, FISH revealed their location in telomeric heterochromatin at the ends of the cucumber chromosomes (Fig. 2A). Within leaf interphase nuclei, the satellite DNA type I probe is entirely localized in DAPI stained chromocenters (Fig. 2B).

In contrast to the mesophyll nuclei, the satellite DNA type I relaxed its structure as a consequence of dramatic heterochromatin decondensation in freshly isolated protoplast nuclei (Figs. 2B and 2C). The scattered FISH signals of satellite DNA type I became more condensed at the time of the chromocenters' reassembly, 48 h after protoplast isolation. Although condensed satellite DNA type I repeats are associated with newly reformed chromocenters, there exists a substantial part of the non-condensed satellite DNA type I repeats in the cell nuclei, 48 and 72 h after protoplast isolation as is demonstrated in Figs. 2E and 2F. These data indicate slower reformation of chromocenters, including a sequential reassembly of chromocenters by subtelomeric repeats, such as satellite DNA type I, rather than in the decondensation process.

FCM analyses showed a clear and dominant G_0/G_1 peak within a population of freshly isolated (t-0) protoplasts, similar to the leaf cells (Figs. 2B and 2C). A small number of protoplasts were found in S and G_2/M phases of the cell cycle (Fig. 2C). Subsequent cultures of protoplasts (up to 72 h) showed shifting from the G_0/G_1 position, by formation of an additional (supra-G_1) peak near the G_0/G_1 peak, in the FCM histogram (Figs. 2D and 2E). The supra-G_1 peak displayed maximal intensity 24 h after protoplast isolation, then decreased until it disappeared 72 h after protoplast isolation. Forty-eight hours after protoplast

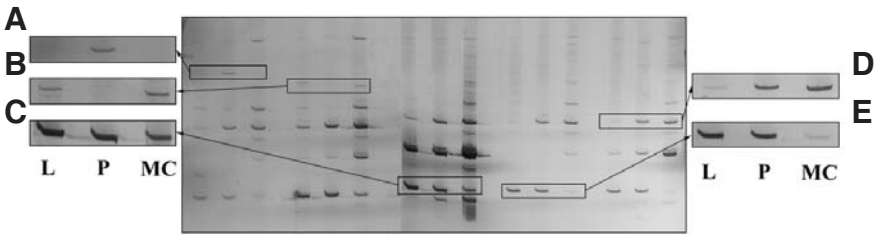

Fig. 3. A picture of a silver stained cDNA-AFLP gel showing differences in the expression of leaves' mesophyl cells (L), freshly isolated protoplasts (P), and microcalli cells derived from protoplasts culture (MC). The close-up view (A) shows a unique transcript-derived fragment of protoplasts and microcalli (B). The view (C) displays an example transcript, transcribed through the whole protoplast culture, differing from the view (D) of up-regulated and (E) down-regulated transcripts.

isolation, the approaching S and G_2/M phases, and reduction of the supra-G_1 peak in the FCM histogram corresponds to the chromocenters reformation and partial satellite DNA repeats reassembly in the population of protoplast-derived cells (Figs. 2E and 2F).

The analyses of expression profiles of mesophyll cells, freshly isolated protoplasts, and proliferated cells of protoplast-derived microcalli, performed by cDNA-AFLP, revealed tissue-specific variability of transcript-derived fragments (Fig. 3). Several up- and down-regulated transcripts in protoplasts' differentiation and also unique transcripts for protoplasts or microcalli have been recognized (Fig. 3). Additionally, the transcript-derived fragments of microcalli cells had a higher variability than protoplasts which differed slightly from leaves of mesophyll cells (Fig. 3).

DISCUSSION

Chromatin is a substrate for several key processes related to the switching on and off of genes within cell differentiation, and development of the organism (Chen and Tian, 2007; Exner and Hennig, 2008). The chromatin, in both animal and plant cells, undergoes epigenetic changes, such as in chromatin condensation (Sablowski, 2007; Tessadori et al., 2004; 2007), covalent modifications in DNA and in histone tails (Bártová et al., 2008; Jenuwein and Allis, 2001; Mathieu et al., 2003; Verbsky and Richards, 2001), as well as the 3D organization of the genome (Cremer and Cremer, 2001; Kozubek et al., 2002; Ondřej et al., 2008; Pečinka et al., 2004).

In *Arabidopsis* protoplasts, Tessadori et al. (2007) demonstrated the decondensation of the heterochromatin, accompanied by relaxation of centromeric and rDNA repetitive sequences. On this point, we also observed a dramatic reduction of heterochromatin in cucumber protoplast nuclei; however, in addition to Tessadori et al. (2007) we firstly demonstrated the relaxation of subtelomeric satellite repeats. The cucumber satellite DNA type I repeats were described by Ganal et al. (1986). We localized these repeats in telomeric heterochromatin, which occurs at both ends of the cucumber chromosomes (2n = 14), except chromosome six (Koo et al., 2002; 2005). Within the interphase nucleus, the satellite DNA type I repeats are localized in the chromocenters. As a consequence of the centromeric and rDNA repeats, which are localized in the chromocentres (Tessadori et al., 2007), the subtelomeric repeats relaxed after protoplast isolation. They started to condense at the time of the chromocenters' reassembly; 48 h after protoplast isolation (but up to 72 h of cultivation) they display only partial condensation. During culture of *Arabidopsis* protoplasts, a partial condensation was also observed by Tessadori et al. (2007) for 180 bp, Athila transposons, and 5S rDNA repeats.

The chromatin changes related to the dedifferentiation have been reported in tobacco protoplast culture, using micrococcal nuclease and flow cytometry assay (Zhao et al., 2001); but this data doesn't compare with FISH image data. Zhao et al. (2001) described two phases of heterochromatin decondensation prior to entry of cells into the S phase, using flow cytometry. The first phase takes place during the course of protoplast isolation, following treatment with cell wall degrading enzymes; whereas, the second occurs only after protoplasts are induced with phytohormones to re-enter the cell cycle. The re-entry into the cell cycle is demonstrated by the occurrence of an extra G_1 peak, referred to as a supra-G_1; evident 72 h after protoplast isolation and phytohormones treatment. In this work, the flow cytometry analyses of cucumber protoplast culture revealed an additional peak near the G_0/G_1 one, 24 h after protoplast isolation. The intensity of the supra-G_1 peak decreased at the time of the chromocenters' reassembly and subtelomeric repeats condensation. At this time, protoplast-derived cells showed evidence of approaching the S and G_2/M phases of the cell cycle. This suggests the necessity of a partial reassembly of the chromocenters, including repeats sequences, to re-enter into the cell cycle and approach the S phase, where DNA replicates. It is well documented that transcriptionally active euchromatic nuclear regions replicate during the first half of the S phase. Conversely, constitutive heterochromatin, containing highly repetitive sequences nearly devoid of genes, replicates very late in the S phase (Goren and Cedar, 2003). These characteristic spatial patterns of replication foci and compartmentalization of the genome are probably disorganized by transient chromatin decondensation; and cells could enter into S phase after partial chromocenters reformation, as a fundamental organizing element of interphase nuclei, owing to these structures.

Although the heterochromatin decondensation is referred to a higher transcriptional activity, the epigenetic indicators for heterochromatin, DNA methylation and H3K9 dimethylation, were not changed upon decondensation, as demonstrated by Tessadori et al. (2007). In this study, the cDNA-AFLP analyses were used to find a correlation between heterochromatin reduction and transcriptional activity in protoplasts. But there were no dramatic changes observed of the expression patterns by cDNA-AFLP analyses, except several unique and up- or down-regulated transcripts. It has been well documented that isolated protoplasts transcribed in addition to genes involved in cell wall regeneration, genes connected with oxidative stress (Milla et al., 2003; Yang et al., 2008). However, a higher variability of transcript-derived fragments was achieved for the expression profile of microcalli cells, than for protoplasts. This indicates that global chromatin decondensation in the protoplast nucleus probably has a different functional meaning, other than support of the transcription during protoplast dedifferentiation. The chromatin decondensation, and also endoreduplication was followed in the root cell nuclei of tomato during mycorrhizal fungal colonization (Berta et al., 2000). Additionally, pathogenic fungi produce cell wall degrading enzymes (reviewed in Annis and Goodwin, 1997); in a similar way as it is used in protoplast isolation procedures. Also the oxidative stress occurring in iso-

lated protoplasts is involved in the plant defense response to fungal pathogens (Papadakis and Roubelakis-Angelakis, 2002; Vera-Estrella et al., 1994). We speculate that dramatic chromatin decondensation is, to a certain extent, a manifestation of the host-pathogen reaction, rather than a process of protoplast dedifferentiation. The real dedifferentiation of protoplasts could, perhaps, then be shifted to the time-point of the chromocenters' partial reassembly.

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