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Dedicated to Prof. dr. MERCEDES WRISCHER
on the occasion of her 70th birthday.

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MOLECULAR STRUCTURE OF SPINACH CHLOROPLAST NUCLEOIDS

HRVOJE FULGOSI*, NIKOLA LJUBEŠIĆ

Ruđer Bošković Institute, Department of Molecular Genetics, POB 1016, HR-10001 Zagreb, Croatia

The ultrastructure and molecular composition of spinach chloroplast nucleoids were studied. Isolated nucleoids retained their three-dimensionally folded structure observed *in situ*. Fluorescence microscopy and staining with DAPI reaffirmed the mostly DNA-containing nature of these complexes. The presence of chloroplast DNA was confirmed by restriction fragment analysis. Electron microscopy revealed a highly condensed, chromatin-like, beaded substructure. Naked DNA supercoils or loops were not observed. Integral nucleoid proteins were isolated and analysed on SDS-polyacrylamide gels. A distinct pattern of the nucleoid proteins, covering the entire range of molecular masses from 5 kDa to >100 kDa revealed that the nucleoid fraction was highly depleted of major thylakoid and stromal constituents.

Key words: spinach, chloroplast nucleoides, cpDNA, proteins, microscopy

Introduction

The genetic material of higher plant chloroplasts is a circular, double-stranded DNA molecule. The nucleotide sequences of the entire chloroplast genomes of at least a dozen evolutionary different species have so far been determined (see MARTIN and HERRMANN 1998, MARTIN et al. 1998). In general, chloroplast

* Corresponding author: Phone: +385-1-4680-238, Fax: +385-1-4561-177, E-mail: fulgosi@yahoo.com

DNA (cpDNA) has the size of about 120 kbp (SUGIURA 1992) and is thus thirty to thirty-five times smaller than the *E. coli* chromosome.

The physical organization and condensation of cpDNA is apparently quite different from that of the nuclear chromatin. Multiple copies of cpDNA molecules, contained within the chloroplast stroma, are not engulfed by membranes and resemble prokaryotic nucleoids (KELLENBERGER 1990). The forces which cause condensation of the cpDNA into nucleoids are poorly understood. Data from electron microscopy studies (HERRMANN et al. 1974, KOWALLIK and HERRMANN 1974) and the identification of the histone-like proteins associated with the cpDNA (BRIAT et al. 1984) suggested nucleosome-like (SALGANIK et al. 1991) higher-order structures within the nucleoids. The number, size, distribution and DNA content of nucleoids at different stages of chloroplast and plant development (KOWALLIK and HERRMANN 1974, MIYAMURA et al. 1986, BAUMGARTNER and MULLET 1991), and also under the influence of various environmental factors (FULGOSI and LJUBEŠIĆ 1992), changes considerably. Although nucleoid rearrangements have been studied extensively in algae (CHIANG et al. 1981, KUROIWA et al. 1989, ZACHLEDER et al. 1996) and in higher plants (HASHIMOTO 1985, MIYAMURA et al. 1986, MODRUŠAN et al. 1989, FUJIE et al. 1994) the molecular mechanisms involved and the biological implications of observed changes remain elusive.

The structure and molecular composition of plastid nucleoids have previously been studied in cultured tobacco cells (NEMOTO et al. 1988, NEMOTO et al. 1989, NEMOTO et al. 1990, NEMOTO et al. 1991, NAKANO et al. 1993) and in *Narcissus pseudonarcissus* (HANSMANN et al. 1985). The distribution patterns (KOWALLIK and HERRMANN 1974, KUROIWA et al. 1981) of nucleoids in plastids differentiating into chromoplasts, etioplasts and proplastids suggest that the form of DNA packaging in the nucleoids may be coupled with the transcriptional control of plastid gene expression (MULLET 1988). Recently, CND41, a chloroplast nucleoid nonspecific DNA-binding protein, has been identified and shown to be the negative regulator of plastid psbA and rbcL genes (NAKANO et al. 1997). The regulatory role of the CND41 has been assigned to its possible architectural transcription factor properties (WOLFFE 1994), mainly because of its ability to alter the condensation state of the cpDNA.

In this study we preliminarily characterize the molecular composition and ultrastructural properties of spinach (*Spinacea oleracea*) chloroplast nucleoids and establish procedures necessary for future identification of other auxiliary components involved in cpDNA structuring and nucleoid condensation.

Materials and methods

Isolation of chloroplasts

Up to 20 g of young and fresh spinach leaves, purchased at the local market, were used. Intact chloroplasts were isolated using a discontinous Percoll (Pharmacia, Sweden) gradient and a procedure modified from BARTLETT et al. (1982). The isolated chloroplasts were washed twice with 10 volumes of homogenization medium (2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM HEPES/KOH

pH 7.6, 0.33 M sorbitol, 5 mM Na-ascorbate) and sedimented by 5 sec. centrifugation at 4 000 g.

Isolation of nucleoids

The procedure for nucleoid isolation was essentially the same as described by HANSMANN et al. (1985). The Percoll purified chloroplasts, corresponding to 0.7–0.8 mg chlorophyll, were pelleted as desribed above and lysed by resuspending the pellets in lysis buffer (0.9 M metrizamide, 20 mM triethanolamine--HCl pH 7.5, 0.1 mM EDTA, 0.4 mM $CaCl_2$, 2 % (w/v) Triton X-100, 10 % (w/v) glycerol, 0.5 mM PMSF) at 22 °C for 5 min. This solution (approx. 0.8 M metrizamide) was overlaid with a discontinuous metrizamide gradient (0, 0.3 and 0.6 metrizamide in the gradient buffer (10 % (w/v) glycerol, 20 mM triethanolamine-HCl pH 7.5, 0.1 mM EDTA, 0.4 mM $CaCl_2$, 1 % (w/v) Triton X-100). After centrifugation for 16 h at 90000 g, the white nucleoid band (in the interphase between the 0.3/0.6 M metrizamide step) was removed, diluted with gradient buffer, and nucleoids were sedimented by a 15 min. centrifugation at 15000 g. The pellet was washed twice by resuspension in the gradient buffer. Ocasionally, the pellet was hard to disolve and was resuspended by several passages through a fine gauge needle followed by a centrifugation step.

Isolation of cpDNA from nucleoids

To the isolated nucleoids, 1/10 vol. 20 % Sarcosyl and 1/50 vol. of Proteinase K (10 mg/mL) were added. The sample was incubated for 2–3 h at 37 °C under light agitation. An equal volume of saturated aq. phenol pH 7.5 was added, mixed by gentle flipping of the tube, and the phases were separated by a 10 min. centrifugation at 4000 g at room temperature. The water phase was transferred to a clean tube and re-extracted with 1 vol. of chloroform. Centrifugation was repeated, the water phase containing cpDNA collected into a clean tube, and 1/10 vol. 3 M sodium acetate pH 7.5 and 0.8 vol. isopropanol were added. Nucleic acids were precipitated by 3 h incubation at room temperature followed by high speed centrifugation (15 min., 15000 g). The pellet was washed with 70 % ethanol, air dried and resuspended in a small volume of redistilled water. RNA was removed by 1 h incubation with RNase A (final conc. 0.1 mg/mL) at 37 °C; the phenolisation step was repeated, and the cpDNA was precipitated by the addition of 1/2 vol. 7.5 M ammonium acetate and 2.5 vol. of absolute ethanol. The precipitation was carried out over-night at –20 °C. The cpDNA was pelleted, washed as described, and resuspended in 100 μL of redistilled water.

Restriction and gel electrophoresis of DNA

The isolated cpDNA was digested with *Eco* RI (Boehringer, Mannheim) endonuclease according to the manufacturer's specifications. The generated fragments were resolved in 0.7 % agarose gel and visualized under UV illumination by staining with ethidium bromide.

Isolation of chloroplast subfractions

Chloroplasts were lysed in 500 μL of Tricine/NaOH, pH 8.0, for 15 min., on ice. The stroma and thylakoid fractions were separated by a 10 min. centrifuga-

tion at 5 000 g. The soluble proteins of the stroma were precipitated from the supernatant by the addition of 4 vol. of ice-cold acetone. The pellet containing the thylakoids was washed by resuspending in washing solution (10 mM Tricine/NaOH pH 8.0, 0.1 M sucrose). The thylakoids were sedimented (2 min. at 13 500 g) and dissolved in the gel sample buffer. The precipitated stroma proteins were also sedimented (5 min. at 13 000 g) and resuspended in the same buffer.

Gel electrophoresis of proteins

Different protein fractions were resuspendend in gel loading buffer (LAEMMLI 1970) and boiled for 2 min. prior to loading onto discontinuous SDS-containing 12.5 % polyacrylamide gels (LAEMMLI 1970). Upon completion of the run, gels were stained either with silver nitrate or with Coomassie Brilliant Blue R250.

Fluorescence microscopy

Nucleoids were stained *in situ* as described previously (FULGOSI and LJUBEŠIĆ 1992). The isolated nucleoids were stained with 5 μg/mL DAPI solution for five minutes prior to microscopy. The observations were made using an Zeiss (Oberkochen, Germany) Axiovert 35 epifluorescence microscope equipped with the phase contrast and Nomarski (DIC) optics. An HBO50 mercury lamp in combination with a G365, FT 395 and LP420 filter set were used.

Electron microscopy

For the direct-mounting technique the samples were placed on carbon-coated formvar film grids. After adsorption of nucleoids for 5 min, the grids were washed in double-distilled water. After removing most of the water with filter paper, the grids were air dried. Specimens were shadowed with palladium at an angle of 30°. All micrographs were taken on a Zeiss EM 10 electron at 60 kV microscope.

Results

In situ, spinach chloroplasts appear to contain SN-type (scattered nucleoid) (KUROIWA et al. 1981) nucleoids. No unusual nucleoid condensation (FULGOSI and LJUBEŠIĆ 1992) or restructuring (LJUBEŠIĆ et al. 1997) was observed in the chloroplasts of green, fully developed leaves exposed to normal growth conditions (Fig. 1a). Furthermore, isolated plastids showed no change in the form of nucleoids. The number, size and distribution of nucleoids changed according to the size and age of chloroplasts, with younger plastids containing fewer and smaller nucleoids (not shown).

Under phase contrast or Nomarski microscopy, isolated nucleoids appeared as compact, colorless conglomerates. Epifluorescent microscopy, following staining with DAPI, supported the mostly DNA-containing nature of these particles (Fig. 1b). The bright blue-white fluorescence of the DAPI-DNA complex indicated large amounts of tightly packed DNA molecules. Although suspended

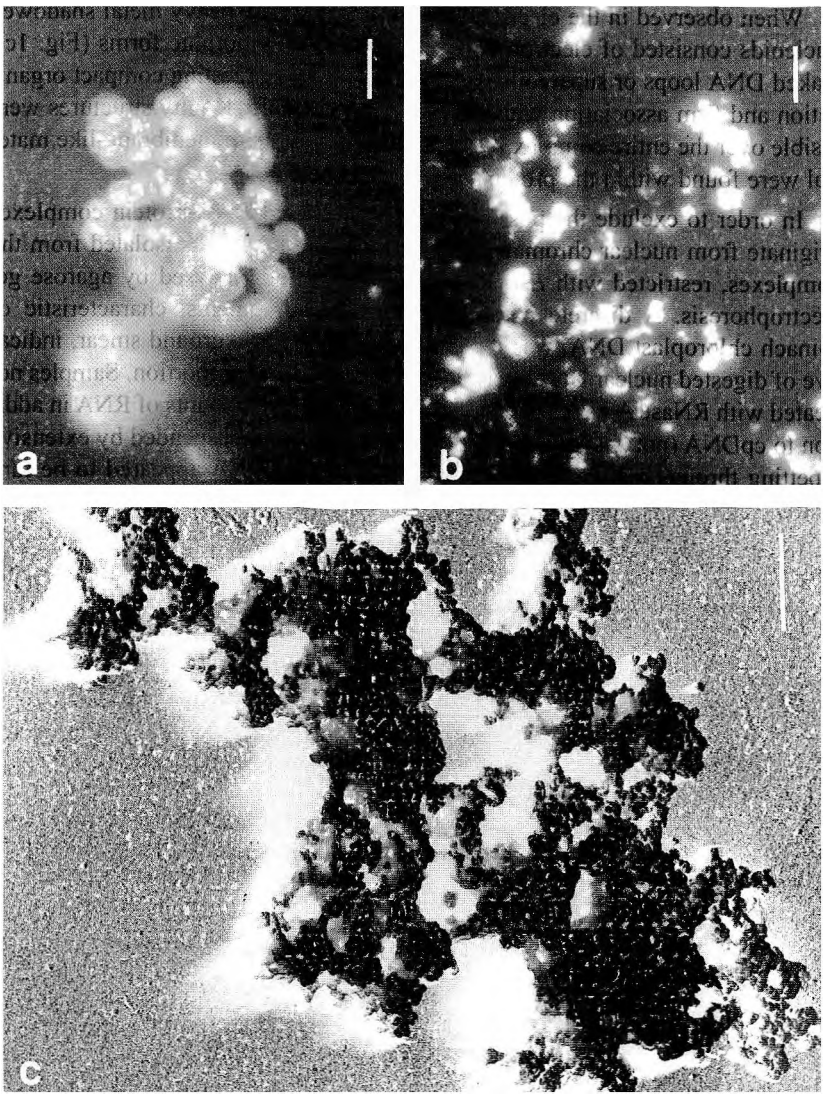

Fig. 1. Panel a: Epifluorescent micrography of *in situ* DAPI-stained chloroplast nucleoids. Bar = 10 μm.

Panel b: Epifluorescent micrography of isolated chloroplast nucleoids stained with DAPI. Bar = 10 μm.

Panel c: Electron micrography of isolated chloroplast nucleoids shaded with palladium. Bar = 1 μm

in water solution, these structures appeared insoluble. The primary red fluorescence of chlorophyll-containing apoproteins of thylakoid membranes, or other pigment-associated structures was not detected. These findings strongly indicate that the isolated particles represent genuine chloroplast nucleoids and not the membrane vesicles generated during the isolation procedure.

When observed in the electron microscope, isolated, heavy metal shadowed nucleoids consisted of electron-dense material in polymorphic forms (Fig. 1c). Naked DNA loops or supercoils were not observed, suggesting compact organization and firm association with the proteinaceous core. Knotty structures were visible over the entire complex. Occasionally, remnants of membrane-like material were found within the proteinaceous core structure.

In order to exclude the possibilty that the isolated DNA-protein complexes originate from nuclear chromatin contaminants the DNA was isolated from the complexes, restricted with *Eco* RI endonuclease, and analysed by agarose gel electrophoresis. A discrete pattern of restriction fragments, characteristic of spinach chloroplast DNA, was detected (Fig. 2a). A background smear, indicative of digested nuclear DNA, was present in a very low proportion. Samples not treated with RNase A were found to contain substantial amounts of RNA in addition to cpDNA (not shown). When the nucleoids were resuspended by extensive pipetting through a fine gauge needle the isolated cpDNA appeared to be randomly fragmented (Fig. 2a).

Integral nucleoid proteins cover the entire range of molecular masses from 5 kDa (data not shown) to >100 kDa (Fig. 2b). For polypeptide pattern comparisons both thylakoid and stroma fractions were analysed simultaneously. The

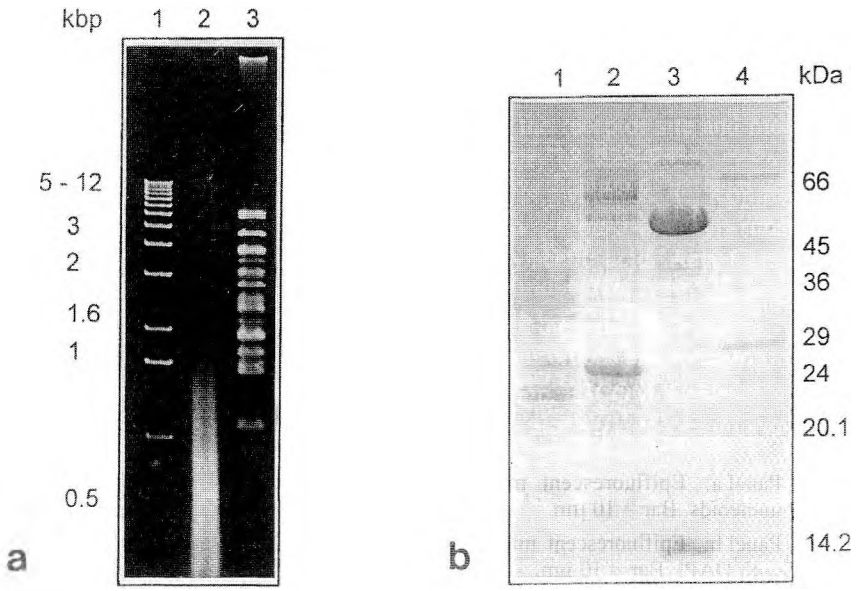

Fig. 2. Panel a: cpDNA from isolated nucleoids. Lane 1: DNA molecular length standard; left: length in kpb. Lane 2: cp DNA fragments isolated from nucleoids that were resuspended using fine gauge needle. Lane 3: cpDNA fragments generated by Eco RI digest.

Panel b: Polypeptide composition of various chloroplast subfractions: Lane 1: isolated nucleoids. Lane 2: thylakoid membranes. Lane 3: stromal proteins. Lane 4: molecular weight standard; right: values in kDa. Proteins were resolved in 12.5 % SDS/polyacrylamide gel and stained with Coomassie BB.

nucleoid fraction is highly depleted of major thylakoid and stromal proteins (Fig. 2b). The most conspicuously enriched nucleoid proteins are in the molecular mass range of 38–40 kDa, 28 kDa, 21–23 kDa and 17 kDa (Fig. 2b). We assumed that these proteins are the major constituents of the proteinaceous core structure. When leaves of the same size and age were taken as the starting material for the chloroplast isolation, the nucleoid polypeptide pattern remained unchanged. However, minor quantitative and qualitative differences were observed in the protein pattern of nucleoids isolated from younger plastids.

Discussion

In this study, we establish a procedure for purifying chloroplast nucleoids from spinach leaves. Although the isolation of cpDNA-protein complexes from leaves has been reported previously (BRIAT et al. 1982; BÜLOW et al. 1987), the resulting preparations mostly contained a transcriptionally active core complex which does not entirely resemble the compact nucleoid arrangement observed *in vivo*. Such preparations usually included only the firmly bound proteins of the 'central body' structure which remained attached to the cpDNA even after a drastic deproteinization step (HERRMANN et al. 1974).

In our preparations isolated nucleoids retained their three-dimensionally folded structure observed *in vivo*. These preparations contained a large number of additional minor polypeptides which could be involved in enzymatic functions such as transcription and replication, or could participate in the condensation of the cpDNA. When observed under the electron microscope, the nucleoids have a highly condensed, chromatin-like, beaded substructure. A similar organization has been observed in proplastid nucleoids isolated from cultured cells of tobacco (SAKAI et al. 1991). Unlike TACs (transcriptionally active chromosome) (BRIAT et al. 1982, BÜLOW et al. 1987), our preparations more closely resemble the organization of the genome in the plastids of living cells. In addition, isolated nucleoids contained substantial amounts of bound RNA. It was reported in the past that the nucleoid preparations appear to transcribe not only rDNA but also protein-coding genes, implying the coisolation of a complete set of RNA polymerases bound to DNA templates (SAKAI et al 1991). It is believed that this transcriptional activity can primarily be ascribed to the chain elongation of nascent transcripts.

The most abundant polypeptides of isolated spinach nucleoids (this work) are well correlated with major protein constituents of spinach TACs (BRIAT et al. 1982). These transcriptionally active preparations are also highly enriched in the proteins in molecular mass range 14–21 kDa and 30–46 kDa. It is reasonable to speculate that exactly those components represent the major elements of the proteinaceous nucleoid core. Previously (HERRMANN et al. 1974), it was reported that nucleoids are bound to the stromal side of thylakoid lamellae. Our observations, made using fluorescence microscopy, exclude, however, the possibility that the chlorophyll-containing antenna apoproteins participate in this binding. This finding was further strenghtened by the results of polypeptide pattern comparisons made between nucleoid and thylakoid fractions. It is likely that other, yet unassigned, thylakoid membrane proteins may be involved in the tethering of

cpDNA molecules. Recently (SATO et al. 1998), PEND, a 130 kDa chloroplast inner envelope membrane protein, has been characterized and shown to be capable of cpDNA binding. The exact role of PEND in the nucleoid structuring remains elusive, but binding of cpDNA to the envelope membrane may play an important role in replication or in certain steps of chloroplast mRNA translation (SATO et al., 1998). Membrane-like structures that we have observed in the electron microscope could therefore, at least partially, originate from remnants of the inner envelope membrane.

Because it is engulfed in photosynthetic organelles, cpDNA is exposed to extensive photooxidative, heat, and UV damage. Rearrangements of nucleoids have been reported in chloroplasts exposed to such light and heat shock conditions. In the pea plant, the chloroplast nucleoids aggregate upon light stress (ADAMSKA and FULGOSI unpublished). Similar condensation and restructuring has also been observed in plastids of light-sensitive aurea mutants of *Ligustrum ovalifolium* (FULGOSI and LJUBEŠIĆ 1992) and *Acer negundo* (FULGOSI unpublished).

In our future studies we will try to characterize nucleoid rearrangement mechanisms on the molecular level. The findings we have made and the procedures that we have established in this work will play an important role in these efforts.

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Sažetak

MOLEKULARNA STRUKTURA KLOROPLASTNIH NUKLEOIDA ŠPINATA (*Spinacea oleracea*)

HRVOJE FULGOSI, NIKOLA LJUBEŠIĆ

Institut "Ruđer Bošković", Laboratorij za molekularnu genetiku, pp. 1016, 10000 Zagreb, Hrvatska

Istraživali smo ultrastrukturu i molekularnu građu kloroplastnih nukleoida špinata (*Spinacea oleracea*). Izolirani nukleoidi zadržavaju svoju trodimenzionalnu građu vidljivu in situ. Fluorescencijskom mikroskopijom i bojenjem s fluorokromom DAPI potvrdili smo visok sadržaj DNA u ovim kompleksima. Prisustvo kloroplastne DNA dokazali smo restrikcijskom analizom. Pod elektronskim mikroskopom vidljiva je kondenzirana (gusta), kromatinu slična, kugličasta građa. Gole DNA petlje ili superzavojnice nisu primjećene. Proteini nukleoida pokrivaju čitav raspon molekulskih masa, od 5 do više od 100 kDa. Glavni proteini tilakoidnih membrana i strome kloroplasta nisu prisutni u nukleoidnoj frakciji.