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Original scientific paper

DYNAMICS OF PLASTID NUCLEOIDS
CHANGES IN WILD- AND AUREA-TYPE
LEAVES OF *LIGUSTRUM OVALIFOLIUM*
HASSK. VAR. *AUREUM*

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The changes in number, size, distribution and DNA content of plastid nucleoids in wild- and mutant-type leaves of an aurea variety of privet (*Ligustrum ovalifolium* Hassk. var. *aureum*) were studied. Wild-type leaves did not show changes (in nucleoid number, size and distribution) induced by different light intensities. Mutant leaves responded to changes in light conditions by changes in colour. During yellowing of regreened aurea leaves and bleaching of yellow aurea leaves the number of plastid nucleoids decreased with concomitant increase of their volume in such a fashion that the total volume of nucleoids and the DNA content per plastid remained constant. No degradation of chloroplast DNA before and during yellowing and bleaching of the aurea leaves was observed.

Introduction

Chloroplasts possess their own circular, double-stranded DNA which is quite different from nuclear DNA. Electron microscopy as well as restriction mapping (Ohyama et al. 1986) show that chloroplast DNA (cp DNA) is ten to twelve times smaller than the *E. coli* chromosome. Like in procaryotes, regions in chloroplast stroma containing DNA molecules are not enclosed by membranes. However, they are predominantly associated with the thylakoid membranes and, in particular, the grana. Terms such as chloroplast nuclei, nucleoplasm, plastome and genophore have often been used to denote the genome of chloroplasts. In this paper we prefer the term nucleoids, which designates the DNA containing re-

gions within the chloroplasts. The number, size, distribution and the DNA content of nucleoids at different stages of chloroplast and plant development, as influenced by various environmental factors, have already been subjected to extensive study (Jordan and Hopley 1990, Kuroiwa et al. 1989, Miyamura et al. 1986, Nemoto et al. 1988, Sodmergen et al. 1989, 1991).

The extreme sensitivity to changes in light intensity of some conditional mutants makes them very suitable for experimental work. This especially concerns the aurea varieties of many plants. Aurea-type leaves cannot green while exposed to strong illumination, but turn yellow instead, and eventually bleach. Adaptation to different light intensities is possible throughout the active vegetation period, whenever the light conditions are altered.

During this events the ultrastructure of the chloroplasts changes drastically (Kunst and Wrischer 1984).

In the present study we examine changes in number, size, distribution and DNA content of nucleoids in the course of yellowing and bleaching. Possible degradation of cp DNA during these events was of special interest.

Materials and Methods

Plant material

The experiments were carried out during late spring and early autumn on an aurea variety of privet (*Ligustrum ovalifolium* Hassk. var. *aureum*), growing in the garden of the Ruđer Bošković Institute, Zagreb. Young, undamaged green (wild-type), yellow, regreened and bleached (aurea-type) leaves from different branches of the same shrub were studied.

Visualization of plastids and determination of the size, number, and volume of nucleoids

Frequently both wild- and aurea-type areas are present on the same leaf, so only sections of interest were studied. For fluorescence microscopy, hand cut leaf pieces were fixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) and stained with 5 µg/ml DAPI (4,6-diamidino-2-phenylindole) solution for five minutes. Before microscopic observation the cover glass was gently pressed against the sample. All investigations were carried out with an Opton Axiovert 35 epifluorescent microscope equipped with phase contrast and differential interference contrast (DIC) optics. A HBO 50 mercury lamp, G 365, FT 395 and LP 420 filters were used. Number of nucleoids per plastid was determined by direct counting while varying the focal plane of the microscope. Photomicrographs were taken with a Contax 167 MT camera using Fotokemika 35 mm KB-27 film and automatic exposure time. Size of nucleoids was measured from photomicrographs using magnifying lens. The linear dimensions of individual nucleoids were used to compute their volumes using the equation for a sphere. Comparisons of number, size and volume of nucleoids were made by standard statistical methods.

Results

MACROSCOPIC OBSERVATIONS

Both green wild-type and aurea mutant leaves grow on the same shrub. Variegated leaves containing patches of wild- (predominantly organized around the central nerve) and aurea-type tissue can also be found. Regreened aurea leaves are mostly located on the shaded lower and inner branches of the shrub. The green colour of such leaves is never quite as intense as in the wild-type leaves.

Aurea-type leaves located on the outermost branches of the shrub are exposed to intense sunlight. As a result of this their colour is golden yellow. If they are shaded with a transparent paper bag they turn green in the same fashion as leaves from shaded branches (Kunst and Wrischer 1984.). Leaves exposed to strong prolonged sunlight lose their pigments and become white — bleached. They may even fall off during the summer due to extensive damage of the leaf tissue. Seasonal macroscopic observations showed that there are no significant changes in wild-type leaves, while in the aurea-type the number of bleached leaves increases throughout the season.

MICROSCOPIC OBSERVATIONS

Microscopic studies show significant differences in the microstructures of wild-type and aurea-type plastids.

Wild type plastids

Chloroplasts in green wild-type leaves are oval in shape and contain starch in large quantities. Light microscopy did not show significant changes in microstructure throughout the season, although starch quantity did vary. Fluorescence microscopy and the use of DAPI showed, on average, eight to ten nucleoids (Fig. 1c). Their arrangement is influenced by the distribution of starch grains; no uniform pattern was observed. The

Table 1. Number (arithmetic means in parentheses), diameter (arithmetic means with standard errors in parentheses), volume and total volume (arithmetic means) of nucleoids per plastid in wild-type and the three stages of aurea-type plastids.

		Number of nucleoids per plastid	Diameter of nucleoid in μm	Volume of nucleoid in μm^3	Total volume of nucleoids per plastid in μm^3
Wild-type nucleoids		8—10 (9.38)	0.77 (± 0.04)	0.24	2.2
Aurea-type nucleoids	regreened	9—10 (9.64)	0.59 (± 0.01)	0.11	1.0
	yellow	5—6 (6.23)	0.72 (± 0.02)	0.19	1.2
	bleached	1—2 (1.50)	1.3 (± 0.10)	1.21	1.8

nucleoids had an average diameter of $0.77 \mu\text{m}$ which corresponds to a volume of $0.2 \mu\text{m}^3$ of DNA per nucleoid or $2.24 \mu\text{m}^3$ per plastid (Table 1.). No significant seasonal changes were observed.

Aurea-type plastids

Regreened aurea leaves contain plastids with nine to ten oval nucleoids arranged between the starch grains (Fig. 5a-b). Their number remains constant throughout the season, but their arrangement changes with number and size of starch grains. No uniform pattern was observed. The average diameter of the nucleoid was, in this case, $0.59 \mu\text{m}$ which corresponds to a volume of $0.11 \mu\text{m}^3$ of DNA per nucleoid and $1.0 \mu\text{m}^3$ per plastid (Table 1.). The plastids of yellow aurea leaves have randomly scattered nucleoids (Fig. 3a-c). Their number changes from nine to ten in spring to five to six in autumn; their average diameter of $0.72 \mu\text{m}$, did not significantly change. The volume of DNA per nucleoid was $0.19 \mu\text{m}^3$ and $1.2 \mu\text{m}^3$ per plastid (Table 1.). In comparison with green wild-type and regreened aurea-type, yellow leaves have more plastids with a smaller number of somewhat bigger nucleoids. Starch grains are smaller than in green and regreened plastids.

In bleached leaves only a small number of intact plastids was found. They contain one to two large nucleoids (Fig. 4a-c) with an average diameter of $1.3 \mu\text{m}$ and a volume of $1.2 \mu\text{m}^3$. Use of DIC optics revealed that they are situated adjacent to large vacuoles (Fig. 6a-b) which can clearly be seen under the electron microscope (Kunst and Wrischer 1984). The calculated DNA volume is $1.8 \mu\text{m}^3$ per plastid which is slightly more than in regreened and yellow plastids (Table 1.).

During all transformations and in all stages the total nucleoid volume was about the same suggesting that substantial degradation of cp DNA does not occur.

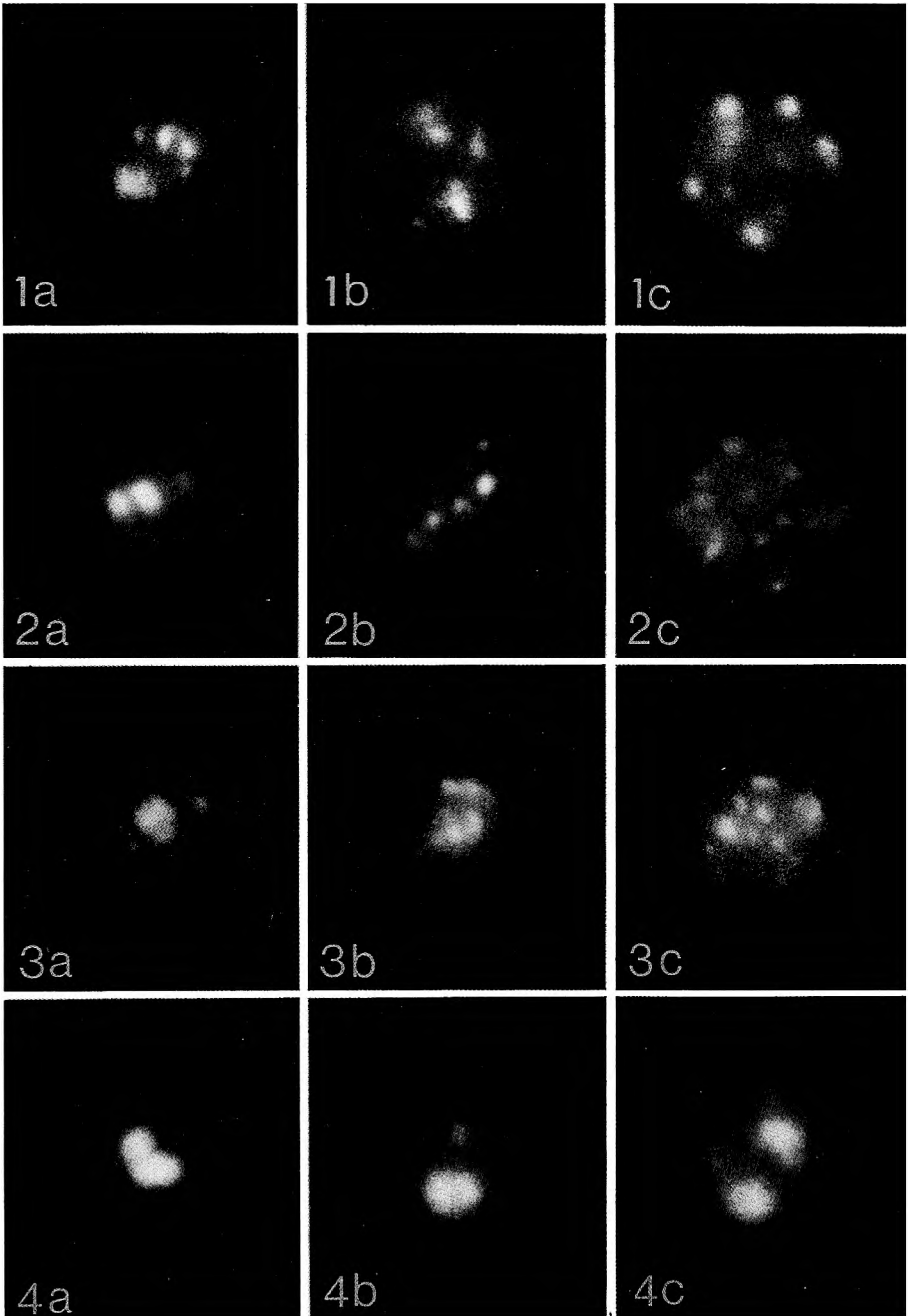
Discussion

Recent studies of chloroplast nucleoids have shown that their number, size and distribution change during differentiation and division of plastids and under the influence of environmental factors (Possingham and Lawrence 1983).

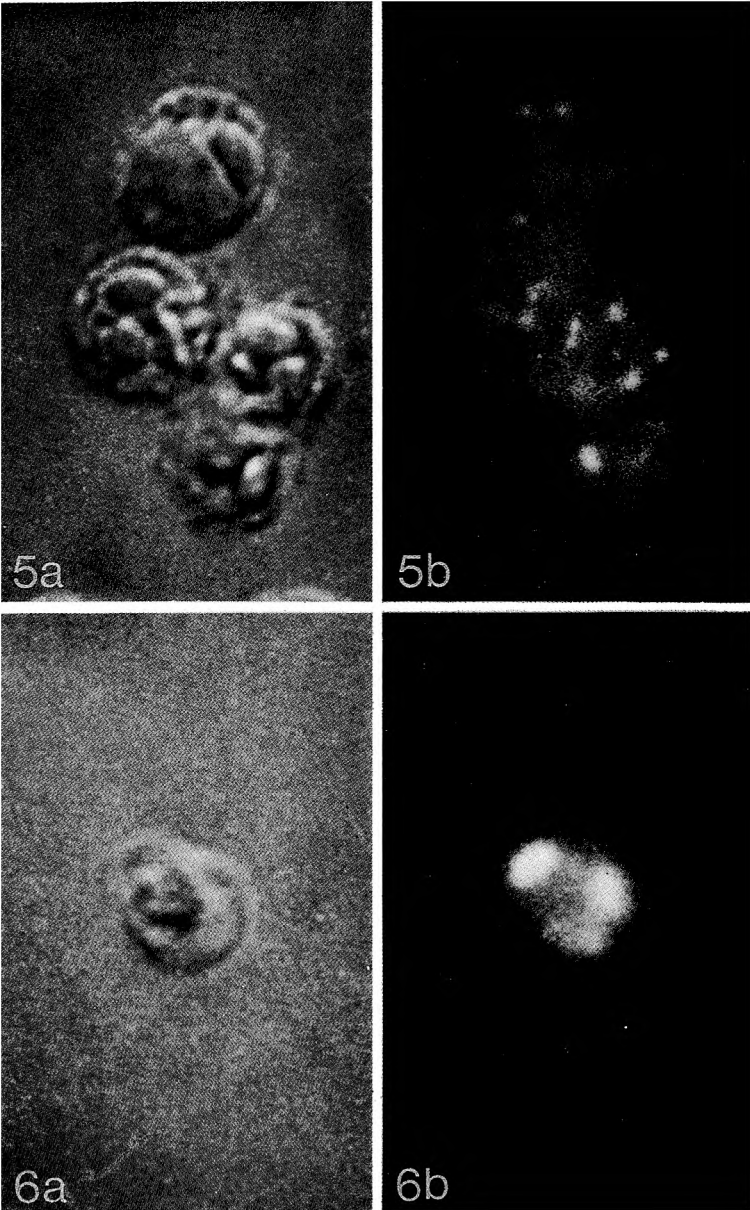
Figs. 1—4. Epifluorescence photomicrographs of plastid nucleoids in green wild-type plastids (1a—1c), and regreened (2a—2c), yellow (3a—3c) and bleached (4a—4c) aurea-type plastids in different stages of plastid development. Young plastids are in lane a and b and mature, fully developed plastids in lane c. Specimens were stained with DAPI. 3,000 : 1.

Fig. 5. Respective DIC (5a) and epifluorescence (5b) photomicrographs of mature bleached aurea-type plastid. Nucleoids are arranged among large starch grains, 2,400 : 1.

Fig. 6. Respective DIC (6a) and epifluorescence (6b) photomicrographs of mature bleached aurea-type plastid. The large nucleoids are located at the periphery of the plastid. The central region is occupied by a large vacuole, 4,000 : 1.



Figs. 1—4.



Figs. 5—6.

Studies of dynamic changes of plastid nucleoids show that their number and DNA content increase during plant development (Miyamura et al. 1986). The amount of cp DNA appears to be positively correlated with plastid volume (Sodmergen et al. 1991). Our results for green, wild-type plastids are in accord with the above dynamics of change in number and size of cp nucleoids. An increase in the number of nucleoids during development (Fig. 1a-c) and a decrease during senescence were observed, but no seasonal changes in number and diameter.

The loss of soluble proteins, chlorophyll, RNA and starch is generally considered a criterion for leaf yellowing and senescence (Kirk and Tilney-Bassett 1978). The studies of Sodmergen et al. (1991) suggest that the degradation of chloroplast DNA begins more than 48 h before the loss of protein and yellowing. This degradation of cp DNA in the mature leaves could be due to enzymatic digestion instead of to dilution of the DNA caused by division of chloroplasts (Sodmergen et al. 1991).

Since aurea mutants possess the ability of transformation from regreened over yellow to bleached leaves and vice versa, we suppose that no DNA degradation is present during these events. Our results show that the number of nucleoids in regreened plastids, which is somewhat larger than in wild-type ones, decreases during yellowing under prolonged sunlight (Fig. 2a-c, 3a-c). Simultaneously the diameter of nucleoids increases, leaving the total volume of DNA per plastid unchanged. As a result of prolonged, strong sunlight irradiation, yellow leaves become bleached. During this event the number of nucleoids decreases to two to three per plastid, but at the same time, their diameter increases drastically (Fig. 4a-c). The ultrastructure of such plastids also undergoes severe changes and degradation of thylakoid membranes; this causes the formation of large vacuoles (Kunst and Wrisher 1984). They, in turn, can gradually squeeze nucleoids into large clusters, thus decreasing their number. At this stage our results suggest a slight increase of the DNA volume per plastid. This is probably a result of a less organised and more spacious arrangement of cp DNA in these structures. Use of fluorimetry with a video-intensified microscope photon-counting system (VIMPCS) (Miyamura et al. 1986) would probably yield more accurate results about the DNA content of these structures.

Since little or no work, regarding leaf senescence, yellowing, bleaching and regreening in aurea mutants of higher plants has as yet been published, our present study may contribute to the efforts to understand these phenomena.

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SAŽETAK

DINAMIČKE PROMJENE PLASTIDNIH NUKLEOIDA U DIVLJEG TIPA I AUREA TIPA LISTOVA KALINE (*Ligustrum ovalifolium* Hassk. var. *aureum*)

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Istraživali smo promjene broja, veličine, razmještaja i sadržaja DNA, plastidnih nukleoida u divljih i mutiranih listova kaline (*Ligustrum ovalifolium* Hassk. var. *aureum*). Listovi divljeg tipa ne pokazuju promjene (broja, veličine i razmještaja nukleoida) uzrokovane različitim intenzitetima svjetlosti. Mutirani listovi mijenjaju boju s obzirom na intenzitet osvjetljenosti. Tijekom žućenja ozelenjelih listova i izbljeđivanja žutih listova aurea, broj nukleoida se smanjuje s istovremenim povećanjem njihova volumena. Stoga, ukupni volumen nukleoida, a time i količina DNA, ostaju konstantni u svim fazama. Razgradnja kloroplastne DNA prije i tijekom žućenja i izbljeđivanja nije opažena.

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