Circular DNA in Isolated Chromoplasts

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Flower chromoplasts of *Narcissus pseudonarcissus* have been isolated in high purity and integrity by a new procedure. From these chromoplasts circular DNA molecules in supercoiled form have been isolated and demonstrated by electron microscopy to have a contour length of about 44 μm (molecular weight about 92×10^6 daltons).

Plastid DNA has been isolated from many different sources and characterized in terms of bouyant density, base composition, renaturation kinetics, and sedimentation velocity (for references, *cf.* [1, 2]). For many years, however, it was not possible to isolate plastid DNA in a form sufficiently intact to determine its molecular weight by length measurements in electron micrographs. Only recently the isolation of circular, partly supercoiled DNA molecules and their demonstration in the electron microscope (EM) have been accomplished from chloroplasts of algae and higher plants (*Euglena gracilis* [3], pea [4], bean [4], lettuce [4], spinach [5], and corn [5]). In all cases a contour length of about 40 μm was found for circular DNA from chloroplasts.

We now report the electron microscopic demonstration of supercoiled DNA rings, obtained from isolated chromoplasts of the flowers of Daffodil. The yellow corona chromoplasts of this monocotyledonous plant have already been investigated by electron microscopy *in situ* [6–8] and after isolation by different procedures [9], and have been characterized in terms of the chemical composition of their pigment-carrying internal membranes [10]. Isolation and biochemical characterization of DNA from *Narcissus* chromoplasts, and from chloroplasts, mitochondria, and nuclei of the same species as well, is reported by Herrmann [11].

Material and Methods

Isolation of chromoplasts

Flowers of Daffodil (*Narcissus pseudonarcissus* L., wild type) were collected near Gérardmer (Vosges, France). For preparation of chromoplasts only the fully developed, yellow to orange colored coronae of the flowers were used. Chromoplasts are the only plastid type present in all the tissues of the corona as shown by EM examination. (Petals contain some chloroplasts in their basal veins. They have, therefore, not been used in the present investigation.)

The coronae were cut into small pieces with razor blades in small amounts of ice cold isolation medium, containing 0.47 M sucrose, 5 mM $MgCl_2$, 0.2 per cent (w/v) polyvinylpyrrolidone K 90, mol. weight ca. 360 000 (Roth, Karlsruhe, Germany), and 0.067 M phosphate buffer (pH 7.5, Soerensen). The material (tissue : medium = 1 : 3, w/v) was then homogenized with a knife homogenizer (Bühler, Tübingen, Germany) for 4×5 sec at half maximum speed. The homogenate was filtered through four layers of nylon cloth, and cellular debris removed by low-speed centrifugation (15 min, $1000 \times g$, 4 °C). Chromoplasts were sedimented from the supernatant in an 8×38 ml fixed angle rotor at $16\,500 \times g$ for 20 min (Weinkauf P50K ultracentrifuge, Brandau, Germany). The pellets were gently resuspended with the aid of a loose-fitting Potter-Elvehjem glass homogenizer in 0.067 M phosphate buffer, pH 7.5, containing 50% sucrose (w/v) and 5 mM $MgCl_2$, and overlaid with equal volumes of 40%, 30%, and 15% sucrose (w/v) in the same buffer. This discontinuous gradient was centrifuged for 1 h in a 3×34 ml swinging bucket rotor at $50\,000 \times g$. The chromoplasts, banded by flotation on two interphases, between 40% and 30%, and between 30% and 15% sucrose respectively, were carefully removed with a Pasteur pipette. These fractions were diluted with 5 mM $MgCl_2$, 0.067 M phosphate buffer, pH 7.5, to a final concentration of 15% sucrose. A chromoplast pellet was obtained by final centrifugation for 20 min in an 8×38 ml fixed angle rotor at $16\,500 \times g$. All steps of isolation were carried out in the cold (4 °C).

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The purity of the chromoplast fraction was checked by electron microscopy. For this purpose, yellow chromoplast bands of the sucrose gradient were removed and fixed by addition of 25% unbuffered aqueous glutaraldehyde to a final concentration of 3% glutaraldehyde. Fixation was carried out for 1 h at 4 °C. After several washings with 50 mM cacodylate buffer, pH 7.2, the material was postfixed in 2% OsO_4 in the same buffer for 1 h (4 °C). Following dehydration by a graded ethanol series the chromoplasts were embedded in Epon 812. Ultrathin sections were stained in the usual manner with uranyl acetate and lead citrate.

Isolation and electron microscopy of DNA

DNA was extracted from the isolated chromoplasts following exactly the procedure of Manning *et al.*[5] in order to avoid destructive shearing forces. The isolated DNA fraction was prepared for EM observation by the protein monolayer technique[12]. The spreading solution consisted of ammonium acetate (1.0 M), DNA (approx. 0.5 μg/ml), and cytochrome c (100 μg/ml; Boehringer, Mannheim, Germany). The hypophase contained 0.15 M ammonium acetate. The DNA-protein film was picked up on 300 mesh copper grids covered with a thin carbon film obtained by evaporation of carbon onto freshly cleaved mica. After staining the grids with ethanolic phosphotungstic acid[13], and rotational shadowing (Pt : Pd = 75 : 25, 6°), DNA was examined in a Siemens Elmiskop 101 EM operating at 60 kV (30 μm objective aperture), using the anti-contamination device. Electron micrographs where usually taken at magnifications of 6000 to 10 000 (calibrated with a grating replica; Zeiss, Oberkochen, Germany). As a length marker, coliphage T3 DNA (kindly provided by Drs. E. Bautz and H. Küppers, Institute for Genetics, University of Heidelberg) as well as lambda dv1 (from Dr. G. Hobom, Institute of Biology III, University of Freiburg, Germany), was spread under the same conditions. Contour lengths of DNA molecules have been determined by means of a map measurer on drawings, made by projection of the negatives, at a final magnification of approx. 300 000.

Results and Discussion

The isolation procedure as described above resulted in a clean fraction, rich in unbroken chromoplasts (Figs 1 – 3 *). Electron microscopy of DNA, isolated from this fraction, yielded numerous linear strands of different length up to 44.5 μm, and some tightly twisted DNA circles (Figs 4 – 6 **). 'Open' circles, resulting from supercoiled DNA by single-strand breaks, have not been found in our specimens. Such circles have been observed, however, in chloroplast DNA preparations by other authors[3–5]. Contour length measurements on 8 supertwisted DNA molecules led to a mean value of 42.6 ± 1.2 μm. The conformity of all the individual values strongly indicates that there is no significant heterodispersity.

For calculation of the molecular weight of the chromoplast DNA, lambda dv1 and T3 DNAs have been taken as linear density markers. The small circular DNA of lambda dv1 has been shown to have a molecular weight of 4.4×10^6 daltons by Hobom and Hogness[14]. A contour length of 2.12 μm, as measured in our spreadings, leads to a linear density of 2.08×10^6 daltons per μm. The mean contour length of T3 DNA was, in our preparations, 12.05 μm. Unfortunately, no recent data are available regarding the molecular weight of T3 DNA. But as T3 DNA is known to be a very close relative of T7 DNA, matching its molecular dimensions, the molecular weight of T7 DNA, which has been accurately determined by several independent techniques, has been used for calculating the mass per unit length of T3 DNA. The mean value of T7 DNA mol. weight is 25.3×10^6 daltons(for references, see Table 2 of Misra *et al.*[15]). From these data, a linear density of 2.10×10^6 daltons per μm can be calculated. This value agrees well with that of lambda dv1 DNA. Therefore, a linear density of 2.1×10^6 daltons/μm was used for calculations of the mol. weight of Daffodil flower chromoplast DNA.

For comparison with the DNA circles isolated from chloroplasts by others, it is to be kept in mind that only tightly coiled DNA circles have been examined in *Narcissus*. For obvious reasons this form gives length values a little lower than the real length of an untwisted DNA duplex. Manning *et al.*[5] have found a length difference of 3.7% between supertwisted and open circle forms of spinach chloroplast DNA. Therefore, a correction of the *Narcissus* chromoplast DNA mean contour length to a value of approx. 44 μm seems advisable as the value one should expect for open circles of this DNA. Taken together with a linear density of

* Figs 1 — see Table on page 544 a.

** Figs 4—6 see Table on page 544 b.

2.1×10^6 daltons/μm, a molecular weigth of about 92×10^6 daltons results for Daffodil flower chromoplast DNA.

This value lies well above the molecular weight of about 70×10^6 daltons reported for higher plant circular mitochondrial DNA with a contour length of 30 μm [16]. It is, on the other hand, far below the mol. weight values of bacterial DNA (*e. g., E. coli:* 2.8×10^9 daltons). Therefore, any confusion between isolated chromoplast DNA and other DNA species that might be contaminating the fraction used for this investigation (Table I) can be ruled out.

Table I. Characterization of *Narcissus pseudonarcissus* corona chromoplast fraction by particle counting on ultrathin sections (*cf.* Figs 1–3).

Particle type	Number	Percentage
chromoplasts *	784	92.34
mitochondria	5	0.59
bacterial cells	5	0.59
microbodies	4	0.47
small plasma droplets with inclusions	7	0.82
nuclei, and nuclear envelope fragments	none	—
other particles **, not identifiable	44	5.18
Total	849	99.99

* Only chromoplasts the stroma 'core' of which was visible in the sections have been counted. Empty membrane convolutes or tangential sections ('caps') have been ignored.

** None of these particles are either mitochondria or bacteria, which are easily identified in sections. Part of this category is likely to be degraded chromoplasts.

On the other hand, the value of 92×10^6 daltons, as reported here for *chromo*plast DNA of *Narcissus*, is in good agreement with data given so far in the literature for higher plant and even algal *chloro*plast DNA, with the possible exception of *Acetabularia* [17–19] (Table II).

The functional significance of apparently undegraded chloroplast genomes in chromoplasts of the Daffodil flower remains unknown. However it bears, in our opinion, on Schimper's postulate [24] of reversibility of the developmental stages of plastids, including the chromoplast stage which is in several respects an extreme one. This postulate has been challenged for the chromoplast stage in particular, mainly on the basis of an apparently irreversible structural breakdown during chromoplast development [25]. It is interesting to note, therefore, that flower chromoplasts (which under normal conditions will never revert to the chloroplast state) still contain complete and undegraded copies of a normal plastid's genome. This would mean that chromoplasts still possess the entire genetic message that is carried by plastids. Chromoplast formation would appear, at least in this respect, not to be an irreversible or pathological process.

Table II. Molecular weight of chloroplast DNAs.

Plant species	Mol. weight [10^6 daltons]	Method of estimation	References
Chlamydomonas reinhardtii	99	RK *	[20], corr. by [4]
Euglena gracilis	83	L **	[3]
Lettuce	98	RK, L	[21], corr. by [4; 22]
Tobacco	93	RK	[23], corr. by [4]
Pea, Bean	91	L	[4, 22]
Spinach, Corn	89	L	[5, 22]
Daffodil, *chromo*pl.	92	L	this investigation

* RK, renaturation kinetics.

** L, contour length in EM preparations.

However, as the present communication deals with but one case, and as at present no further data on chromoplast DNA are available, it remains to be shown whether similar or different situations are to be encountered in other cases and for other chromoplast structural types [26].

An equally intriguing question concerns the number of identical genomes present in a single chromoplast, *i. e.*, its degree of polyploidy. It would be interesting to know whether chromoplasts are polyploid at all, and if so, whether they are polyploid to the same or to a lesser extent than reported for chloroplasts (*cf., e. g.* [1, 3, 4, 27, 28]). This problem can now be tackled, and work is underway in our laboratory to solve it.

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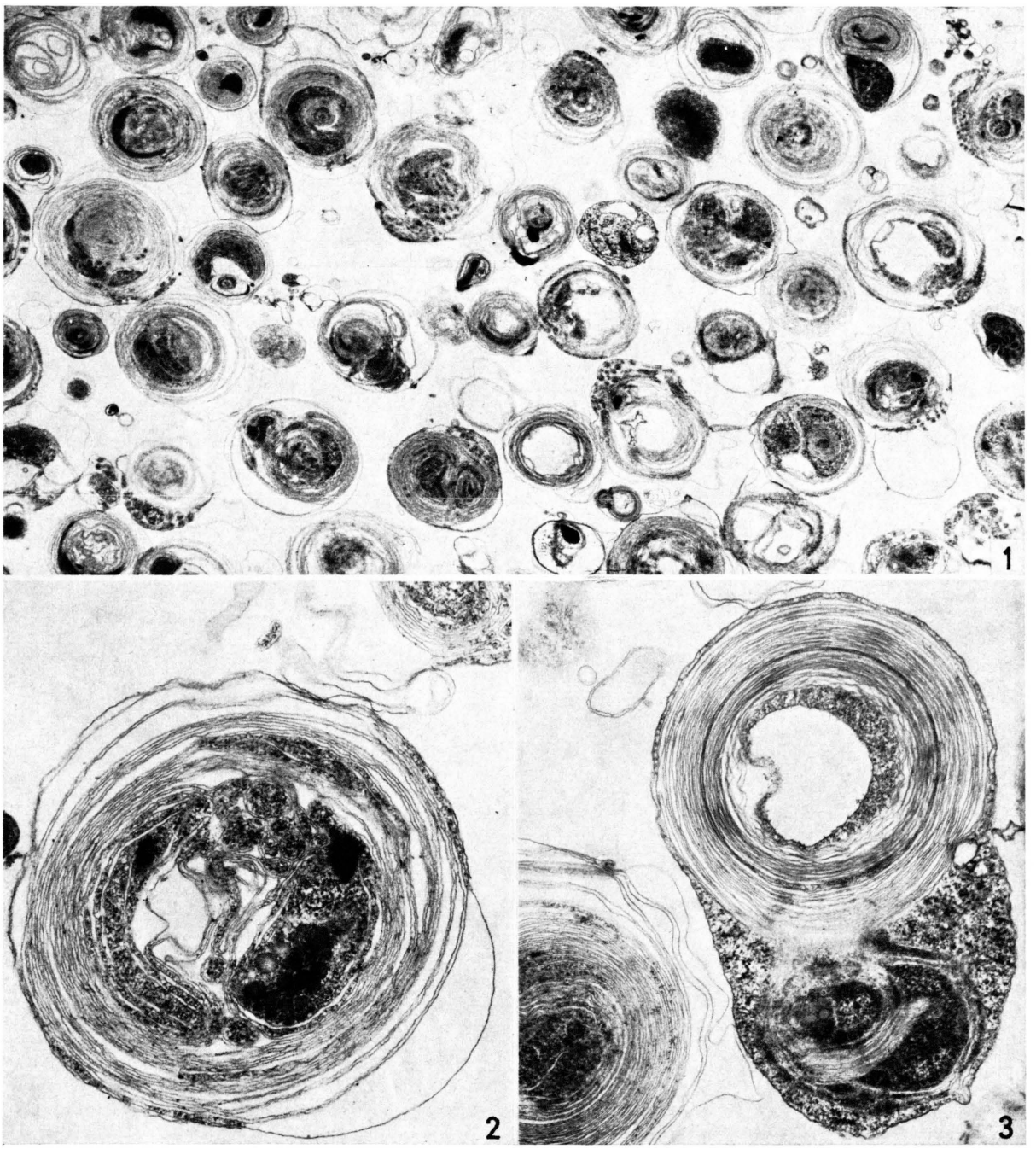
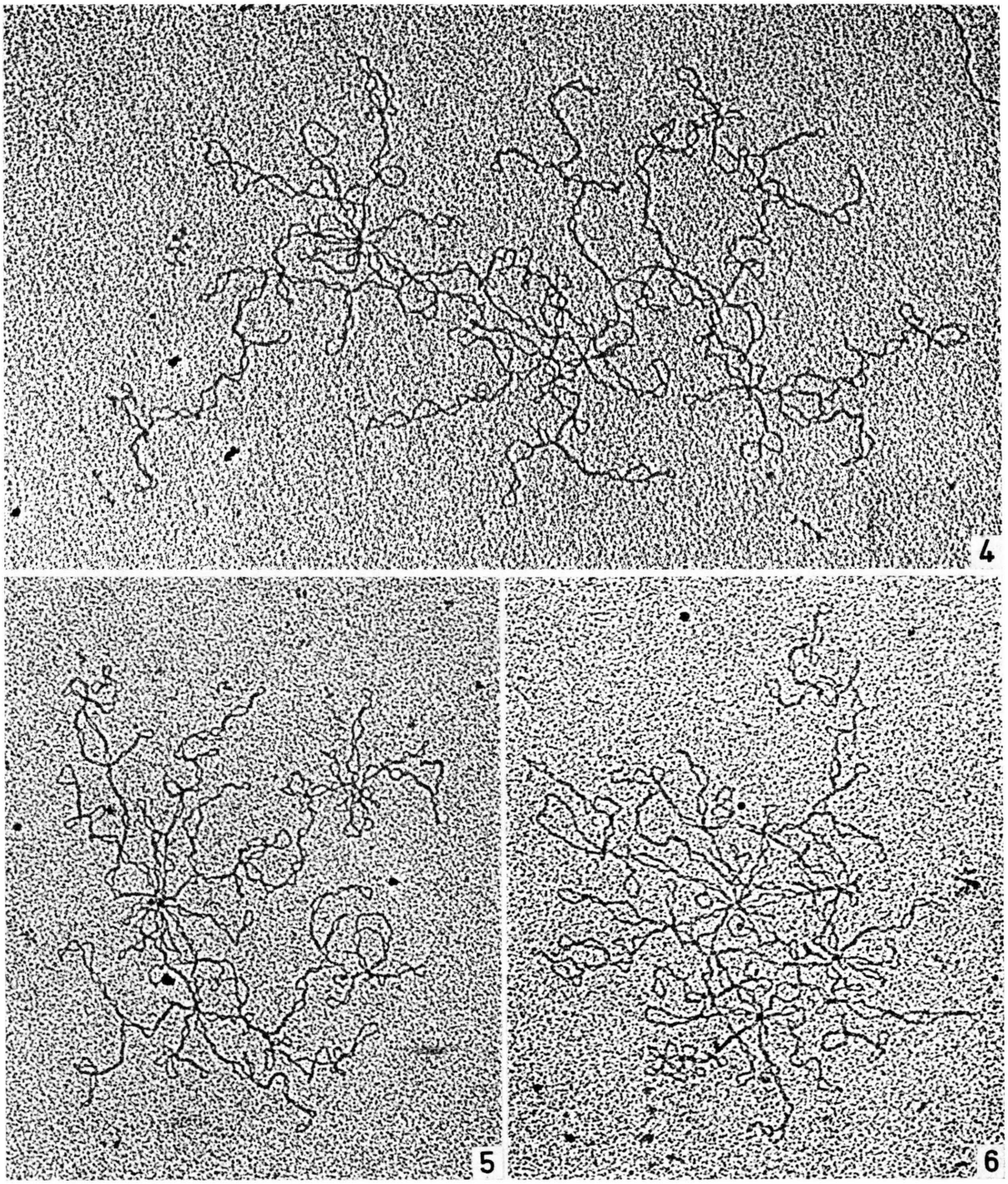


Figs 1—3. Ultrathin sections of isolated chromoplasts of *Narcissus*. Fig. 1: Survey, 6350 ×. — Figs 2, 3: At higher magnification, showing structural integrity of the organelles, 26000 ×, and 20500 ×, respectively.



Figs 4–6. Supercoiled DNA rings from isolated *Narcissus* chromoplasts. Fig. 4: 48000 ×; contour length 43.22 μm. Fig. 5: 36000 ×; contour length 42.96 μm. Fig. 6: 42000 ×; contour length 41.66 μm.