VISUALIZATION BY FLUORESCENCE OF CHLOROPLAST DNA IN HIGHER PLANTS BY MEANS OF THE DNA-SPECIFIC PROBE 4'6-DIAMIDINO-2-PHENYLINDOLE

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ABSTRACT
The DNA in isolated chloroplasts was visualized by the fluorescent probe 4'6-diamidino-2-phenylindole (DAPI). When excited with light of 360 nm, the DNA-DAPI complex fluoresces brilliantly at 450 nm. Nuclei also fluoresce but their nucleoli do not. RNase and Pronase treatment of chloroplasts did not affect the fluorescence but both pre- and posttreatment of DAPI-stained chloroplasts with DNase specifically destroyed the fluorescence. DNA-DAPI complexes in the chloroplasts show up as bright dots. These are distributed uniformly within the chloroplast except for the outer margins. The fluorescent dots can be seen at different focal levels. The number of DNA dots is roughly proportional to chloroplast area which, in turn, is a function of leaf size. The number of fluorescent dots also gave the impression that large leaves with large chloroplasts contain more chloroplast DNA than nuclear DNA.

KEY WORDS: higher plant chloroplast DNA, DAPI-DNA complex, fluorescence microscopy, grana

The existence of DNA in cell organelles other than nuclei evokes questions such as: how much extranuclear DNA is present per cell; how much is present per mitochondrion or chloroplast; how is it duplicated; and how is it arranged relative to the molecular architecture of the organelle? For chloroplasts, an answer to the last question would be greatly facilitated by a methodology whereby DNA could be traced from the level of light microscopy to the details of construction which have been sought by electron microscopy (11, 13, 20, 21). A discovery by Williamson and Fennell (19) has made possible the optical localization of organelle DNA. They showed that a trypanocide, 4'6-diamidino-2-phenylindole (DAPI), synthesized by Dann et al. (2), is a probe capable of revealing mitochondrial DNA in yeast by fluorescence microscopy. Subsequent studies have employed DAPI as a probe to visualize mycoplasmas in tissue culture cells (14) and kinetoplast DNA in trypanosomes (3). Previous studies employed acridine orange as a fluorescence probe for DNA in plastids (13) and chloroplasts (20) of algal forms. In this report, we show that DAPI is both more intense and more specific as a fluorescence probe for DNA in nuclei and chloroplasts isolated from leaves of higher plants than acridine orange. The specificity of DAPI binding to DNA has provided a means to see hitherto unknown details of the organization, character, and relative amounts of DNA per chloroplast.

MATERIALS AND METHODS

Plant Material
Tobacco (Nicotiana tabacum), spinach (Spinacea oleracea), and sugar beet (Beta vulgaris alba) were grown.
The main observations were made on chloroplasts previously provided by Prof. O. Dann of Erlangen, Germany.

RESULTS

Calbiochem (San Diego, Calif.). The DAPI was generated by Promega Corp. (St. Louis, Mo.). Pronase was obtained from Sigma Chemical Co. (St. Louis, Mo.). Acridine orange was purchased from Caltbiochem (San Diego, Calif.). The DAPI was generously provided by Prof. O. Dann of Erlangen, Germany.

Isolation and Staining of Chloroplasts

Detached leaves had their midribs and all major veins removed. The strips of laminar tissue were immersed in liquid containing 0.5 M sucrose to maintain chloroplast structure and 5 mM magnesium acetate to maintain the structure of nuclei (16). Cell-free homogenates were obtained by a chopping technique which is described in more detail in the succeeding paper. Monitoring suspensions with a light microscope shows that a minimum breakage of chloroplasts and nuclei can be achieved by this hand chopping method (16). The cell-free homogenate was then spun at 700 g in a clinical centrifuge for 5 min, and the soft green pellet was gently resuspended in 0.5 ml of homogenization medium. To this suspension was added 1 ml of an aqueous solution of 20 µg/ml DAPI and 5 mM magnesium acetate. The stained preparation was allowed to sit on ice for 2 h. Excess stain was removed by pelleting the organelles in the clinical centrifuge for 5 min. The supernate was discarded and the green pellet was gently resuspended in 0.5 ml of 0.25 M sucrose and 5 mM magnesium acetate. The stained chloroplasts maintain their structure and integrity of the DNA for many days when suspensions are kept cold.

Microscopy

All observations were made with a Zeiss microscope equipped with a V Z condenser and Planachromat and Neofluar phase objectives. Fluorescence of DAPI, as well as autofluorescence of chlorophyll, was excited by light from an HBO 200-W Hg lamp in combination with an F-1 type vertical illuminator. Observation of the DAPI fluorescence was made with Neofluar objectives, using a UG-1 excitation filter in combination with a 410-nm suppression filter. In the case of chlorophyll autofluorescence, a BG-12 excitation filter was used in combination with a 530-nm suppression filter. A well-defined nucleolus surrounded by a halo can be seen in the center of the nucleus. The fluorescence image of the same nucleus is shown in Fig. 1B in which the nucleolus, as well as the main body of the nucleus, shows fluorescence. The intensity of the nuclear staining is so high that chloroplast fluorescence was not revealed photographically at the same exposure. It is evident that the nucleolus is accessible to stains. The nucleus shown in Fig. 1D has been stained with DAPI. Seen by phase-contrast microscopy (similar to Fig. 1A), it contained a well-defined nucleolus in its southern hemisphere. Fig. 1D shows that every portion of the nucleus, except the nucleolus, is fluorescent when stained with DAPI. By this test, we conclude that DAPI has a high degree of specificity for DNA but shows little or no fluorescence with nucleolar RNA.

According to Hajduk (3), DAPI is specific for duplex DNA. However, preliminary fluorometric studies (1) have shown that single-stranded DNA produces about one-quarter the fluorescence intensity found with duplex DNA, while RNA-DAPI solutions give little or no fluorescence, the latter result being consistent with these microscopic findings. Fig. 1C is a photomicrograph of DAPI-stained T4 phage which will be referred to later for comparison.

Fig. 2 contains photomicrographs of different microscopic fields of N. excelsior chloroplasts examined by different techniques. The chloroplasts in Figs. 2A, D, G, and J are phase-contrast images and clearly exhibit defined grana as an intrinsic part of the chloroplast architecture. The location

Isolated from Nicotiana excelsior leaves. The chloroplasts of this species are highly favorable for such studies because they are large (5-25 μm in diameter) and stable in aqueous solutions at very low osmotic pressure. As prepared in sucrose and magnesium, they are freely permeable to the dye and the enzyme DNase. They also contain grana which are readily visible by both phase-contrast and fluorescence microscopy. The specific observations reported are for N. excelsior chloroplasts. However, chloroplasts isolated from N. glutinosa, N. tabacum, and Beta vulgaris alba leaves gave results so similar to those with N. excelsior that it is unnecessary to detail them in this context.

An indication of the in situ specificity of DAPI is shown in Fig. 1A, B, and C. Fig. 1A is a phase-contrast photograph of an N. excelsior nucleus and several chloroplasts after staining with acridine orange. A well-defined nucleolus surrounded by a halo can be seen in the center of the nucleus. The fluorescence image of the same nucleus is shown in Fig. 1B in which the nucleolus, as well as the main body of the nucleus, shows fluorescence. The intensity of the nuclear staining is so high that chloroplast fluorescence was not revealed photographically at the same exposure. It is evident that the nucleolus is accessible to stains. The nucleus shown in Fig. 1D has been stained with DAPI. Seen by phase-contrast microscopy (similar to Fig. 1A), it contained a well-defined nucleolus in its southern hemisphere. Fig. 1D shows that every portion of the nucleus, except the nucleolus, is fluorescent when stained with DAPI. By this test, we conclude that DAPI has a high degree of specificity for DNA but shows little or no fluorescence with nucleolar RNA.

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of DNA in these chloroplasts is shown in Fig. 2, E, H, and K, in which the fluorescence of the DAPI-DNA complex is photographed. The same fields of chloroplasts were also photographed by the long wavelength red light emitted as chlorophyll autofluorescence, with the latter showing no interference with fluorescent images of chloroplast DNA stained with DAPI.

To confirm that the material within chloroplasts stained by DAPI is, in fact, DNA, DNase digestion was used. Either before or after staining with DAPI, the chloroplasts suspended in 0.25 M sucrose and 5 mM magnesium acetate were incubated with 100 μg/ml DNase for 2 h at 25°C. This treatment did not affect the structure of the chloroplasts as checked by phase-contrast microscopy. The autofluorescence of chlorophyll was also unaffected. However, the DNase treatment de-
destroyed the fluorescence of the type shown in Fig. 2B, E, H, and K produced by prestaining the chloroplasts with DAPI. Pretreatment with DNase followed by DAPI staining also abolished DAPI fluorescence within chloroplasts. RNase at 200 µg/ml for 2 h at 36°C did not affect DAPI fluorescence within chloroplasts, nor did Pronase at 1 mg/ml for 4 h at 50°C. By contrast, neither DNase nor RNase completely destroyed the fluorescence within chloroplasts produced by staining
with acridine orange or ethidium bromide, an indication that these intercalating reagents are less specific than DAPI since they appear to stain materials other than DNA.

Fig. 3 contains a series of photomicrographs of DAPI-stained chloroplasts, using both phase-contrast and fluorescence microscopy. Each photomicrograph on the left is a phase-contrast image of the chloroplasts shown to the right, which are fluorescence photographs of the same field but at different focal planes. To produce as much flattening of the chloroplasts as possible without also disrupting them, a 1-μl drop of chloroplast suspension was placed in the center of a 22 × 22 mm coverglass which was inverted onto the slide. Since the fluid volume was limited relative to area, the surface tension forces tended to flatten the normally concave chloroplasts and at the same time to stretch the distance between grana. The photograph in Fig. 3A contains such a well-flattened chloroplast and several smaller ones, and Fig. 3B and C are fluorescence micrographs at different focal levels. These two sets of three photographs show several distinct aspects of chloroplast architecture. First, they show the in-depth distribution of the fluorescing elements if the region under the arrow on Fig. 3B is compared to that in Fig. 3C. Similar vertical localization of elements can be traced in Fig. 3E and F and in Fig. 3H and I. Second, they show that the amount of fluorescing DNA is variable per chloroplast. This can be appreciated if attention is directed to the brightest and largest fluorescing unit just to the left of the arrow in Fig. 3F, a lower image of it in Fig. 3E, and its phase image in Fig. 3D. In the same set of photographs, the two chloroplasts to the far right are smaller in phase contrast and show low fluorescence intensity. Third, the photographs also show that the DNA units are not present in all regions of the chloroplasts. This is seen most clearly in Fig. 3G, H, and I, in which the chloroplast closest to the right edge of the photograph shows this difference. The diameter of the phase-contrast image is 10 μm, while the fluorescence is 6.7 μm. It should be noted that the intensity of the fluorescence also varies with the orientation of the chloroplast. Since quantitative microspectrofluorimetry of the chloroplast DNA aggregates is not feasible due to differences introduced by orientation, focal plane differences, and gradual fading of the DAPI-DNA complex, estimates of the exact amount of DNA per chloroplast could not be made. However, a crude appreciation of the amount of chloroplast DNA in a fluorescing dot can be gained by visual comparison with the fluorescent images of DAPI-stained T4 phage shown in Fig. 1C. A T4 phage contains 120 × 10^6 daltons of DNA (10). Comparison shows the T4 phage fluorescent images to be smaller than the fluorescent dots in chloroplasts, so it is reasonable to assume that the latter contain not less than 120 × 10^6 daltons of DNA per dot.

**FIGURE 2** Phase and fluorescence photomicrographs of DAPI-stained *N. excelsior* chloroplasts. (A, D, G, J) Phase photomicrographs of isolated, DAPI-stained *N. excelsior* chloroplasts. These photomicrographs illustrate the variation in area of the grana-containing region of chloroplasts. In G, one partially folded chloroplast is viewed on edge. In G and J, some of the chloroplasts in the upper right-hand corners have been disrupted. (B, E, H, K) Fluorescence photomicrographs of the same chloroplasts as in A, B, G, J. DAPI fluorescence excited by epi-illumination utilizing an HBO 200-W Hg lamp and photographed by combination of a UG 1 barrier filter and a 410-nm suppression filter. These photomicrographs show that the area of DAPI fluorescence appears to be proportional to the area of the grana, especially in B and E. In a single plane of focus, the fluorescence is observed as discrete spots. However, careful focusing in the light microscope shows that the dots are continuous with one another, being connected by fine threads of DNA. In B, the largest chloroplast has what appears to be very large regions of high DNA concentration. In fact, these are actually composed of many smaller discrete spots whose proximity and intensity of fluorescence have resulted in a fused image. (C, F, I, L) Fluorescence photomicrographs of the same chloroplasts as in A, B, G, J. Autofluorescence of chlorophyll photographed with the use of a BG 12 barrier filter combined with a 530-nm suppression filter. These photomicrographs demonstrate that the area of DAPI-DNA fluorescence is not identical with the sites of chlorophyll fluorescence (the grana), which are arranged with a much higher degree of regularity. In some cases (H vs. I or K vs. L), damaged chloroplasts have lost chlorophyll fluorescence due to disruption of the grana. However, they still retain DAPI-DNA fluorescence. In E, the leftmost chloroplast has a piece of DNA (top right) which lacks chlorophyll fluorescence when examined in E. All photographs 100 X Neofluar phase objective, 8 X ocular. Bar, 10 μm.
FIGURE 3 Phase and fluorescence photomicrographs showing changes in the pattern of DAPI-DNA fluorescence at different depths in the chloroplast. (A, D, G) Phase photomicrographs of isolated N. excelsior chloroplasts suspended in 1 μl of medium to produce maximal flattening of the chloroplasts. The photograph in A depicts one well-flattened chloroplast and several folded and flattened chloroplasts. D and G show flattened chloroplasts with some overlap of adjacent chloroplasts. (B, C) Fluorescence photomicrographs of two planes of focus of the chloroplasts in A. B is a plane of focus as close to the objective as possible, while C is a plane approximately equidistant between the upper and lowermost planes of focus. (E, F) Upper and median planes of focus of chloroplasts in D. (H, I) Upper and median planes of focus of chloroplasts in G. (B, C, E, F, H, I) Illustrate distribution of dense DAPI-DNA areas throughout the thickness of the entire chloroplast. In addition, the arrows in B, F, and H specifically indicate narrowed fluorescent strands between dense regions. All photomicrographs 100× Neofluar phase objective, 8× ocular. Bar, 10 μm.

DISCUSSION

From these numerous direct observations of the organization of the DAPI-stained material in chloroplasts, we are persuaded that the chloroplast DNA is a complex structure that varies with the size of the chloroplast in which it is contained. There are regions which stain intensely with DAPI.
and hints of interconnections by less dense thread-like regions similar in appearance to those observed in yeast mitochondria by Williamson and Fennell (19). Fluorescence of the threads traverses several planes and is too faint to record satisfactorily in photographs. It can be seen that the fluorescent spots or domains become successively visible at several different levels as indicated by the arrows in Fig. 3 F and H.

Previous reports (7, 18) have shown that the chloroplasts used in this study are concavo-convex saucers whose thickness does not exceed 1.5 μm. The dots made visible by DAPI fluorescence are dispersed in depth vis-à-vis the convex and concave surfaces. As conceived from estimating the number of fluorescent dots, the DNA and hence the genome of any particular chloroplast will vary as a function of the area of the chloroplast. Chloroplast areas range from 5 to 60 μm² in the same mesophyll cell of spinach or N. excelsior type leaves (6).

Whitfeld et al. (17) investigated the organization of the chloroplast DNA genome in isolated spinach chloroplasts. They found that two-thirds of the entire genome was available to transcription by an E. coli RNA polymerase and that 90% of the genome was accessible to depolymerization by exogenous DNase. The organization of the DNA as seen by light microscopy together with destruction of its fluorescence by DNase accords with the Whitfeld et al. findings.

Kung (12) has recently reviewed the latest literature on chloroplast DNA of higher plants. The current view is that the DNA is a double-stranded, circular, covalently closed molecule with a contour length of 43-55 μm and a molecular weight of 85-110 × 10⁶ daltons. The genome size determined by renaturation kinetics calculates to 100-200 × 10⁶ daltons (8, 9). Since a single chloroplast fluorescent dot appears to contain at least as much DNA as the 120 × 10⁶ daltons of DNA contained in a T₄ phage, it is evident that chloroplasts contain more than one DNA molecule.

Herrmann and collaborators (4, 5, 11) have made extensive studies of the higher plant chloroplast DNA genome in terms of amount per chloroplast and the manner of its structural organization as revealed by electron microscopic analysis of serial sections of in situ chloroplasts. It was necessary to selectively remove the matrix and ribosomes of chloroplasts by protease treatment before the leaf tissue was sectioned to recognize the chloroplast DNA fibrils by electron microscopy.

They showed that the DNA was distributed into regions which were spatially separated from each other. The number of these regions was a function of size of the chloroplast. The individual region of DNA appeared to contain from 4 to 8 DNA molecules. In an entire chloroplast, from 10 to 90 copies of the chloroplast genome could have been present depending on the size of the chloroplast. If it is valid to assume that one fluorescent dot is a chloroplast DNA molecule, then the number of these dots appears to greatly exceed the amount of DNA per chloroplast found by Herrmann et al. The size, frequency, and spatial arrangement of the DNA regions shown in Fig. 5 (Kowallik and Herrmann, reference 11) and Fig. 2 (Herrmann and Kowallik, reference 5) closely correspond to what we see by fluorescence microscopy.

Because of these suggestions of higher than previously reported DNA content, experiments were performed to determine the total amount of chloroplast DNA vs. the total amount of nuclear DNA in leaves of different sizes, using a technique different from those previously reported. These findings are the subject of the following communication.

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