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# Localization of DNA in Mature and Young Wheat Chloroplasts Using the Fluorescent Probe 4′-6-Diamidino-2-phenylindole[1]

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GUN SELLDÉN[2] AND RACHEL M. LEECH
*Department of Biology, University of York, York YO1 5DD, United Kingdom*

## ABSTRACT

**The spatial organization of chloroplast DNA in developing and dividing wheat chloroplasts was studied in the light microscope using the fluorescent probe 4′-6-diamidino-2-phenylindole, which binds specifically to DNA.**

**The DNA of wheat chloroplasts was localized at the periphery of the plastid, frequently in a discrete band. No relocalization of the DNA was observed during plastid replication. This peripheral location of the DNA was shown to differ from the central random location of DNA in tobacco and spinach chloroplasts.**

Chloroplast differentiation in light-grown wheat seedlings is accompanied by a large increase in the number of chloroplasts per cell (7), associated with a decrease in the number of genome copies per plastid (5). Nothing is known about the location of DNA within these developing wheat plastids nor if the DNA becomes redistributed during chloroplast division: indeed there appears to have been no detailed study of the location of DNA within the chloroplast of any monocotyledonous plant. In dicotyledons, electron microscopical and autoradiographic studies have shown that cpDNA[3] is located in multiple, interconnected regions associated with the thylakoid membranes (10–12, 14, 15, 17, 18, 21, 25). Preliminary investigations of the distribution of DNA in young wheat chloroplasts led us to believe that the location of the cpDNA did not follow the pattern found in dicotyledons and merited more detailed investigation.

The DNA-specific fluorescent probe DAPI was introduced by Williamson and Fennell (23) who used it to study mitochondrial DNA in yeast cells. It has also been successfully used as a means of detecting mycoplasmas in tissue cultures (19) and to study kinetoplast DNA in trypanosomes (9). The more recent demonstration by James and Jope (13) that DAPI can be used to localize cpDNA within the mature plastids of several dicotyledons suggested that the stain would be an excellent probe for investigating the spatial distribution of cpDNA in differentiating and dividing wheat chloroplasts. DAPI promotes a far more intense fluorescence of DNA than the acridine orange used in earlier studies of dicotyledons (16, 24) and so enables an easier investigation of the three-dimensional distribution of cpDNA as the focal plane is moved through the plastid. The method also has an advantage compared with autoradiographic methods since the stain visualizes all the cpDNA, not only the portion which has recently been synthesized. As an alternative to electron microscopy the use of DAPI has the advantage that it avoids digestion of the stroma proteins which conceivably could alter the DNA arrangement within the plastid.

This paper reports our observations in young and mature wheat chloroplasts on the nature of the mode of distribution of DNA during chloroplast division. The arrangement of DNA within the chloroplasts of wheat is compared with its arrangement in tobacco and spinach plastids.

## MATERIALS AND METHODS

**Plant Material.** Seeds of wheat, *Triticum aestivum*, var. Maris Dove, were soaked in running tap water at 20 C for 17 h, with surface sterilization in NaOCl solution (13% free chlorine) after the first h. The seeds were sown in Levington Universal Compost (Fisons, United Kingdom) at a depth of 1 cm and were grown using a photoperiod of 16 h light at 20 C, 8 h darkness at 15 C, 70% RH. The light intensity at the level of the seedlings, measured with a solarimeter (Kipp and Zonen), was 4.0 mw·cm$^{-2}$. Seedlings were harvested 7 days after sowing, 2 h after the start of the light period; leaves were cut at their bases, and coleoptiles gently pulled off. Tobacco plants, *Nicotiana tabacum*, were grown in Levington Universal Compost (Fisons, United Kingdom) for 24 days in a photoperiod of 16 h light at 25 C, 8 h darkness at 20 C, 70% RH. The first of four leaves was used for the isolation of mature plastids. The fourth leaf, which had just emerged and was 0.5 mm long, was used to obtain young plastids. Spinach plants, *Spinacia oleracea*, were grown for 44 days as previously described (1). Mature plastids were isolated from the top half of the second leaf (100 mm long). Young plastids were isolated from the bottom halves of leaves 0.30 mm long.

**Plastid Isolation.** Plastids were isolated by chopping leaf sections with a razor blade in an isolation medium containing 0.4 M sorbitol, 0.75 mM $MgCl_2$, 50 mM Hepes (pH 7.6). The slurry was filtered through eight layers of nylon bolting cloth (25 μm mesh) and was layered onto 1 ml isolation medium, containing 0.4 M sucrose instead of sorbitol. After centrifugation for 5 min at $900g_{max}$ the pellet was resuspended in 1 ml medium containing 0.33 M mannitol, 2.5 mM magnesium acetate, 50 mM Mes (pH 7.0), and incubated with 100 μg DNase I for 10 min at 0 C (2). Five ml DAPI solution, 0.5 to 1.0 μg/ml, was then added to this suspension. After 5 min, 6 ml glutaraldehyde (7%) in isolation medium was added, and after a further 10 min, the suspension was centrifuged for 5 min at $900g_{max}$ and the pellet resuspended by gentle swirling.

**Microscopy.** All observations were made with a Zeiss microscope fitted with an epifluorescence attachment. Samples were excited with a 50-w UV lamp, and by moving the focal plane through the plastids, it was possible to localize precisely individual DNA areas. Photographs were taken using Kodak Tri-X pan and

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[2] Present address: University of Göteborg, Botanical Institute, Department of Plant Physiology, Carl Skottsberg gata 22, S-41319 Göteborg, Sweden.

[3] Abbreviations: cpDNA, chloroplast DNA; DAPI, 4′-6-diamidino-2-phenylindole; nDNA, nuclear DNA.

Ilford HP5 film. Both films were boosted to 1600 ASA during development.

## RESULTS

**DNA of Wheat Chloroplasts.** The pattern of development of chloroplast-containing cells in young wheat leaves has been described in detail by Boffey *et al.* (6). The cells are arranged in a linear developmental sequence with the oldest near the top of the leaf and the youngest nearest its base. After 7 days growth, cells at the same height above the leaf base are of the same age. Chloroplasts from cells of comparable age can therefore be isolated on separate occasions from sections of leaf tissue cut from similar positions above the leaf base.

Chloroplasts isolated from the 70- to 80-mm leaf region were almost fully developed; no further increase in size took place beyond 80 mm. Staining with DAPI reveals that the DNA in these plastids (Fig. 1b) was located in several areas, the number of areas per plastid varying from 7 to 16, the average being 11.5 (SE ± 2.1). The DNA-containing areas in these plastids were more or less evenly distributed round the periphery of the stromal compartment. The majority of the areas were associated together in a narrow band around the plastid, but a few additional areas not associated with this band could always be seen. The DNA-containing band was most clearly seen as the chloroplast tumbled over in the field of view under the microscope. No connections between the individual DNA areas could be observed at this stage of development. There was considerable variation in the size of the individual areas within a single plastid, suggesting an uneven distribution of DNA between the different areas. In all cases, the distribution was only recorded after staining was complete.

In younger plastids, from regions 10 to 30 mm from the leaf base, it was more difficult to distinguish individual DNA staining areas (Fig. 1. a, c-$c_1$). In these younger chloroplasts, the DNA was organized in a more or less continuous band around the periphery of the plastid. Many additional areas not associated with the bands could also be distinguished and such areas were a much more common feature in the younger plastids than in the older ones described above. During the development of the chloroplast, it became increasingly possible to distinguish discrete staining areas, in plastids present in cells above 40 mm from the leaf base it was possible to count the number of staining areas easily.

Several unsuccessful attempts were made to determine the location of cpDNA within the chloroplasts of intact wheat leaf cells, but the DAPI fluorochrome did not penetrate either whole cells or isolated protoplasts.

**DNA in Dividing Wheat Chloroplasts.** In the cells of a narrow strip of leaf tissue in the region between 15 and 30 mm from the wheat leaf base, 10 to 20% chloroplasts are in the process of division. This strip of leaf tissue was dissected out from the plant, and the chloroplasts isolated from them treated with DNase and DAPI and examined under the fluorescence microscope. Two typical chloroplast division profiles are shown in Figure 2, a and b. The DNA in these plastids was evenly distributed and organized in the same way as in nondividing plastids of the same age, *i.e.* in a band at the periphery of the stroma.

**Endogenous DNase in Maturer Regions of Wheat Leaf.** Before treatment with DAPI, all the plastids examined were routinely treated with the DNase (see "Materials and Methods") to remove any DNA sticking either to the outside of the envelopes of intact chloroplasts or to the exposed thylakoids of damaged chloroplasts. After this pretreatment, in all the suspensions of plastids isolated from leaf tissue between 0 and 40 mm from the leaf base, only the intact plastids were stained with DAPI. If the pretreatment of these younger chloroplast suspensions with DNase was omitted, then additional fluorescing material, assumed to be DNA, was revealed in the suspension often loosely associated with the intact plastids and clearly visible within the damaged ones. When older plastids were isolated from leaf regions beyond 40 mm from the leaf base and the DNase pretreatment was omitted, only intact plastids showed DNA/DAPI fluorescence, resembling the situation after pretreatment with DNase in younger material (Fig. 2, c and d). This observation suggests that an active endogenous DNase is present in the older leaf tissue but is absent from the younger leaf cells. This suggestion is supported by the finding that when 50 mM EDTA was added to the isolation medium in an attempt to inhibit the endogenous DNase, the broken plastids in the suspensions from older leaves also stained, in addition to the intact plastids (Fig. 2, e and f).

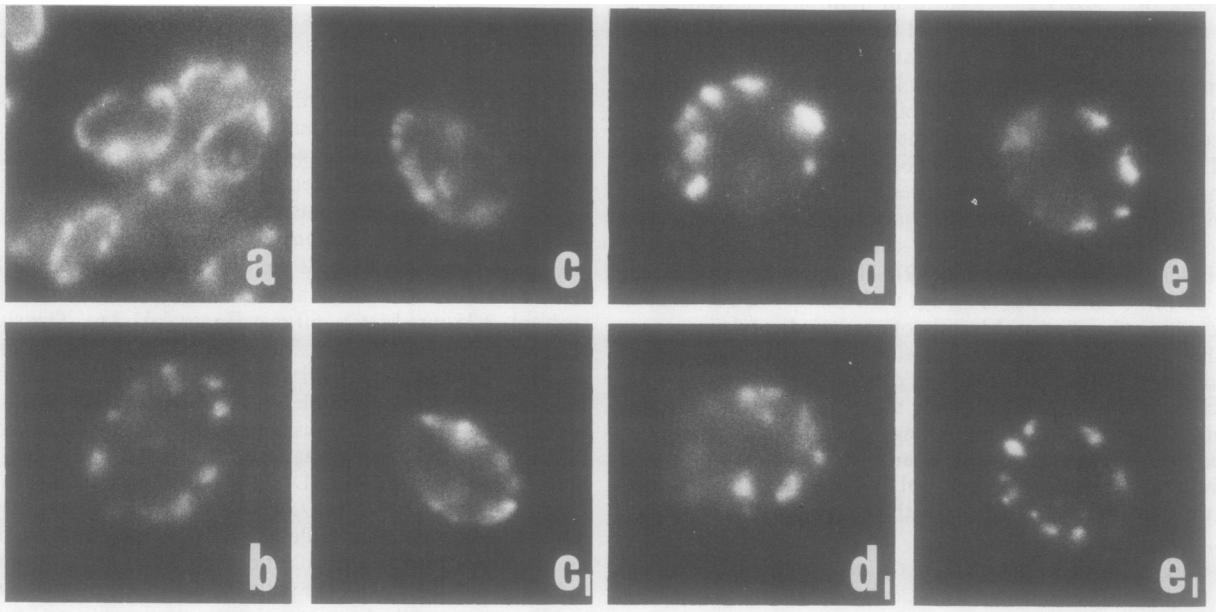


FIG. 1. Fluorescence photomicrographs of wheat chloroplasts stained with DAPI (× 10,000). The chloroplasts were treated with DNase before staining and were shown to be intact when observed by phase contrast microscopy. By moving the focal plane through a plastid all the stained areas within it were revealed. The chloroplasts were isolated from sections cut at the following distances from the leaf base. a, 10 to 20 mm; b, 70 to 80 mm; c-$c_1$, 20 to 30 mm; d-$d_1$, 40 to 50 mm; e-$e_1$, 60 to 70 mm. The pairs c-$c_1$, d-$d_1$, e-$e_1$ are two views of the same plastid at different focal plants.

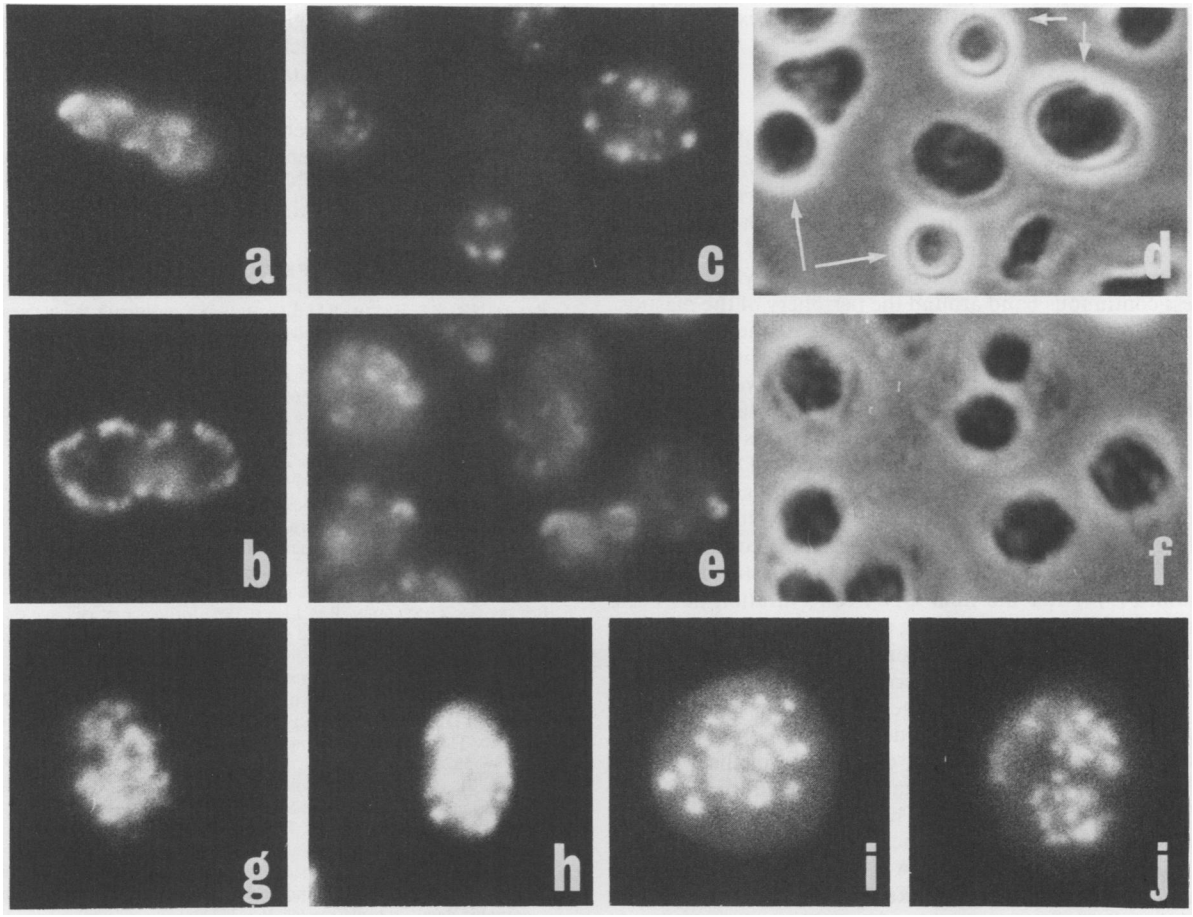


FIG. 2. Fluorescence photomicrographs of wheat chloroplasts stained with DAPI a to b (× 10,000): dividing chloroplasts isolated from sections cut 20 to 30 mm from the leaf base; c to f, (× 4,000); plastids isolated from sections cut 40 to 50 mm from the leaf base. Intact chloroplasts in d are indicated by arrows: none are present in f; c, plastids not treated with DNase before staining with DAPI; d, phase contrast photomicrograph of the same field as c; e, 50 mM EDTA added to the isolation medium, plastids not treated with DNase; f, phase contrast photomicrograph of the same field as e; g, young tobacco chloroplast; h, young spinach chloroplast; i, old tobacco chloroplast; j, old spinach chloroplast.

The standard procedure adopted in this paper for the pretreatment of isolated plastids with exogenous DNase was chosen to provide the shortest period of treatment (10 min at 0 C) which was sufficient to completely remove all DAPI fluorescence from broken plastids. To test whether the DNase might penetrate the intact chloroplast if the pretreatment period was lengthened, plastid suspensions containing added exogenous DNase were left for 2 h at 20 C. Although after this treatment the total number of intact chloroplasts has decreased, plastids still judged to be intact by phase contrast microscopy always showed a normal staining pattern.

**DNA of Tobacco and Spinach Chloroplasts.** Inasmuch as our observations on the location of plastid DNA in wheat chloroplasts differed in several respects from the observations of previous workers who used spinach and tobacco, we decided to carry out parallel experiments using these plants.

Because of the growth pattern of a dicotyledonous leaf, it is more difficult to determine the exact age of the plastids in its cells than in a graminaceous leaf such as wheat. In the spinach leaf, according to Sauer and Possingham (20), the oldest cells are situated at the top of the leaf and the youngest at its base near the petiole. We therefore used the top half of older leaves for the isolation of older plastids from spinach and the bottom central part of the youngest leaves (less than 30 mm in length) for the isolation of younger plastids. Comparable areas of tobacco leaves were used and for the younger plastids a newly emerged leaf was examined.

In the more mature chloroplast, by moving the focal plane through the plastids, it was found that both in tobacco (Fig. 2i) and spinach (Fig. 2j) a large number of discrete DNA regions were randomly scattered in the stroma of the chloroplast. The DAPI staining areas in tobacco and spinach chloroplasts were smaller and the fluorescence less intense than found in wheat chloroplasts. Because the areas containing DNA are not as easy to resolve in spinach as in wheat, it is not possible to obtain an accurate count of their numbers; their aggregate appearance suggested far more areas were present per plastid in spinach than in wheat and an estimate of as many as 40 areas was made for many plastids. The majority of the DNA areas were located in the central regions of the chloroplast and only occasionally was one observed in the periphery of the stroma region. In most of the plastids, the DNA areas were scattered randomly, but on a few occasions, an organization resembling beads on a string was observed. As with the wheat plastids, the standard procedure was to pretreat tobacco and spinach chloroplasts with exogenous DNase for 10 min at 0 C before staining with DAPI. Prolonging this pretreatment to 2 h and increasing the temperature to 20 C as used by James and Jope (13) did not affect the DAPI staining of intact spinach or tobacco chloroplasts. When the DNase treatment was omitted, both intact and broken tobacco and spinach chloroplasts became stained with DAPI, indicating that these plastid suspensions lack an active endogenous DNase. Young chloroplasts of both tobacco and spinach possess a very large number of DNA regions which are impossible to resolve individually (Fig. 2, g and

h). In the youngest chloroplasts, these regions extend to the periphery of the stroma.

## DISCUSSION

By using the fluorochrome DAPI we have been able to follow with precision the changes in distribution of DNA-containing nucleoids inside developing wheat chloroplasts. During all stages of development, the DNA areas are located close to the chloroplast envelope (Figs. 1, 2). During division of young wheat chloroplasts, there appears to be no redistribution of the DNA, which forms a more or less continuous band around the periphery of the plastids. In the young and in the dividing chloroplasts, the number of DAPI staining areas is very high; this is consistent with the results of previous biochemical determinations which showed that there are about 1000 genome copies per plastid at this stage of development (Boffey and Leech, in preparation). This is the stage in the development of the wheat chloroplast when cpDNA replication is more or less complete yet the plastids are still growing, only having reached about one-third of their final volume. As the plastids develop, the DNA content decreases and a constant value of about 300 genome copies is reached before plastids are completely mature (5). After the final DNA concentration has been reached, the number of DAPI staining areas also remains constant.

Before and after chloroplast division in wheat, the DNA areas are also located exclusively on the periphery of the plastids, often confined to a single band. In younger chloroplasts the DNA is much more tightly packed than in older, larger chloroplasts (Fig. 1, a and b). Although the peripheral distribution of the DNA in wheat chloroplasts is much more clearly visualized using DAPI, the same location of the DNA can be observed in the electron micrographs we have prepared of wheat plastids.

The peripheral location of the DNA in wheat chloroplasts of all ages is very distinct and differs markedly from the arrangement previously described for the plastids of several species of dicotyledon (10, 11, 14, 15, 17, 18, 21, 25). Our own observations of the DAPI staining of tobacco and spinach chloroplasts agree with these previous reports and show that the DNA of these species is centrally located. As with young wheat chloroplasts the central region of young tobacco and spinach chloroplasts are packed with DAPI staining areas, in contrast to the older chloroplasts in which the areas are more dispersed. Yet another distinctive arrangement of plastid DNA has been described for some algae (3, 4, 8) in which the cpDNA is associated with the girdle thylakoids and forms a loop around the periphery of the chloroplast. Furthermore, young barley etioplasts have a single polymorphous DNA region located in the centre of the etioplast (22). Clearly, there is no arrangment of DNA which is common for all chloroplasts.

The exogenous addition of DNase effectively removed all nuclear DNA and all cpDNA from damaged wheat, tobacco, and spinach chloroplasts, but it never affected chloroplasts showing a distinct margin in phase contrast microscopy. This characteristic is considered to be diagnostic for morphologically intact chloroplasts, but was not used by James and Jope (13) when they claimed that exogenously added DNase attacked "intact" chloroplasts.

Although it is not possible to quantify the total amount of DNA in the plastids using the DAPI, it is evident from our observations that chloroplast populations isolated from mature cells are heterogeneous and vary considerably in their content of DAPI staining areas. Smaller chloroplasts always have fewer areas than larger chloroplasts. Two extremes are shown in Figure 2c. Furthermore, the nucleoids within a single chloroplast show variation in size. Quantitative biochemical determinations of DNA for populations of isolated plastids cannot take account of individual differences but such differences should be borne in mind when interpreting the mean values of DNA per plastid and DNA/nucleoid obtained by these methods. Heterogeneities should also be considered when discussing the ratio of nDNA/cpDNA of cells, since large cells contain more chloroplasts than smaller ones.

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