BUOYANT DENSITY DETERMINATIONS ON CHLOROPLAST DNA IN A VARIEGATED CYTOPLASMIC MUTANT OF GOSSYPIUM HIRSUTUM L.[1]

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Received April 6,1977

SUMMARY

Chloroplast DNA[2] isolated from normal green tissue of Gossypium hirsutum L. was shown to have buoyant density components of 1.697 and 1.706 gm/cc in neutral CsCl gradients. In cDNA of cytoplasmic mutant yellow and white tissue there was an increase in the 1.706 gm/cc component. In addition, the white tissue cDNA exhibited a unique high buoyant density band of 1.719 gm/cc. The nuclear DNA exhibited a significantly lighter buoyant density (1.692 gm/cc). Ultrastructural studies demonstrated that green tissue chloroplasts had normal grana and thylakoid development, yellow tissue chloroplasts showed only thylakoid development and white tissue chloroplasts appeared as empty vesicles.

INTRODUCTION

Recently, a study of cDNA from a tobacco cytoplasmic mutant (1) reported differences in the buoyant density patterns of two DNA components from the cDNA of both the mutant yellow and white and normal green leaves. Another portion of this same study (2) indicated that little to no change in the amount of chloroplast ribosomes occurred between the green, yellow and white leaves of the variegated mutant plants.

Investigations on a similar type of variegated cytoplasmic mutant in cotton, however, did show a lack of chloroplast ribosomes in the white leaves (3). It was concluded that such a loss could have arisen either from a direct photochemical damage to the ribosomes, due to a lack of protective pigments in the white sectors, or to a mutational

[1] Journal Paper No. 2466 of the Arizona Agricultural Experiment Station.

[2] cDNA.

ISSN 0006-291X

alteration of some sort in the cDNA of the white chloroplasts that would affect the production of ribosomal RNA. If the latter alternative were true, it might be anticipated that the buoyant density patterns of the cDNA components in cotton may differ from those of the tobacco mutant and thus form a basis for future characterization and comparative studies of several cytoplasmic variegated mutants in cotton (4-6).

MATERIALS AND METHODS

Green, yellow, and white leaves 3 to 6 weeks old were taken from a variegated greenhouse grown strain of Gossypium hirsutum L. (6). Leaf samples were washed in distilled water and placed in Whitfeld and Spencer's buffer (7), in a ratio of 1 gm leaf tissue to 3 mls of buffer. The leaf tissue was then chopped to a honogeneous pulp at 5° C by means of an electric knife. The brei filtrate (filtered through a double layer of Mira cloth) was pelleted at 1000 x g for 10 min at 5° C and resuspended in 15 ml of Honda's medium (7). Five mls of the resuspended pellet were layered on a discontinuous sucrose gradient in Honda's medium consisting of 10 ml of 60% sucrose, 10 ml of 45% sucrose, and 5 ml of 20% sucrose. The gradients were then centrifuged in a SW 25.1 rotor at 23,000 rpm and 5° C for two hours.

After centrifugation the tubes contained two bands of chloroplasts. The top band (20-45% interface) was used for isolation of cDNA since this band contained a greater proportion of unbroken chloroplasts than that present at the lower 45-60% interface. The presence of broken nuclear fragments were insignificant at this portion of the gradient. The isolated band was diluted with 2 volumes of Whitfeld and Spencer's medium and the chloroplasts were pelleted for 10 min at 5000 x g before the extraction of cDNA.

Chloroplast pellets were resuspended in 2 ml of 0.2M EDTA, 0.05 M Tris (pH 8.0) and 5% Triton X-100. Sodium perchlorate was added to this suspension to a final molarity of 1.5. The mixture was incubated at 50° C for 15 min and then shaken gently with an equal volume of chloroform-isoamyl alcohol (25:1) for 30 min at 25° C on a wrist shaker. After pelleting the solid residue, the aqueous phase was dialyzed for several hours against 0.1 x standard saline citrate (8). The purified DNA sample was then centrifuged for 14 hours in a CsCl step gradient with ethidium bromide (9). At the end of the centrifugation the gradients were examined under far UV light. A bright white-fluorescent band, cDNA, visible near the middle of the centrifugation tube, was extracted with a 22-gauge needle and syringe, and mixed with an equal volume of water-saturated isoamyl alcohol. The aqueous phase was then dialyzed overnight (5° C) against a standard saline citrate solution.

Cotton DNA samples (2 μg) and Micrococcus lysodeikticus DNA (2 μg), as a marker, were centrifuged to equilibrium in analytical CsCl gradients. Photographs were taken and then traced with a densiotometer. Buoyant densities were calculated according to the method of Sueoka (10).

Leaf sections from white, yellow, and green leaf tissue were fixed with 5% gluteraldehyde in 0.1 M phosphate buffer (Sorenson's buffer pH 7.0) for 7 hrs by a previously described method (11). The leaves were

then washed overnight in Sorenson's buffer and treated with 2% OsO_4 in the latter buffer for 4 hrs. Samples were then dehydrated in cold absolute ethanol, followed by three 5 min changes in propylene oxide. The leaf sections were embedded in epon, stained with Reynolds lead citrate (11) and sectioned with a diamond knife on a Porter Blume microtome. The sections were examined in a Phillips-200 electron microscope.

RESULTS

Preliminary attempts to isolate cDNA from the three types of chloroplasts indicated that only the normal green chloroplasts were resistant to the extraction methods routinely used on the chloroplasts of other plants. The procedure described above, which is a modification and combination of several of the standard methods (1, 7), produced yields of cDNA from the green chloroplasts that were comparable to that of the mutant chloroplasts.

Nuclear DNA isolated as previously described (12) from both the mutant and green cotton leaf tissues exhibited a buoyant density of 1.692 + 0.003 gm/cc. The cDNA, however, yielded multiple buoyant density peaks which varied between the three types of leaf tissues (Fig. 1). Chloroplast DNA from both the green and yellow leaf tissues yielded a major component at a buoyant density of 1.697 $\pm$ 0.004 gm/cc and a minor component at 1.706 $\pm$ 0.003 gm/cc. The DNA from the yellow chloroplasts, however, had a larger amount of the 1.706 gm/cc component than that of the green chloroplasts. A major portion of the DNA from the white chloroplasts exhibited a buoyant density of 1.706 gm/cc while two minor buoyant density components appeared at 1.697 and 1.719 gm/cc. With the exception of the high buoyant density component of the white cDNA, these results were similar to those of the variegated mutant of tobacco (1).

Electron micrographs of chloroplasts from green, yellow and white leaf tissue are shown in Fig. 2. The green leaf tissue chloroplasts have normal grana and thylakoid development, while the yellow leaf chloroplast shows only thylakoid development. White leaf tissues

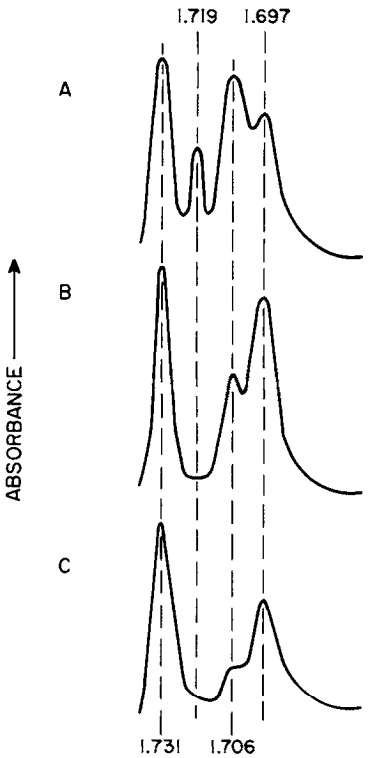
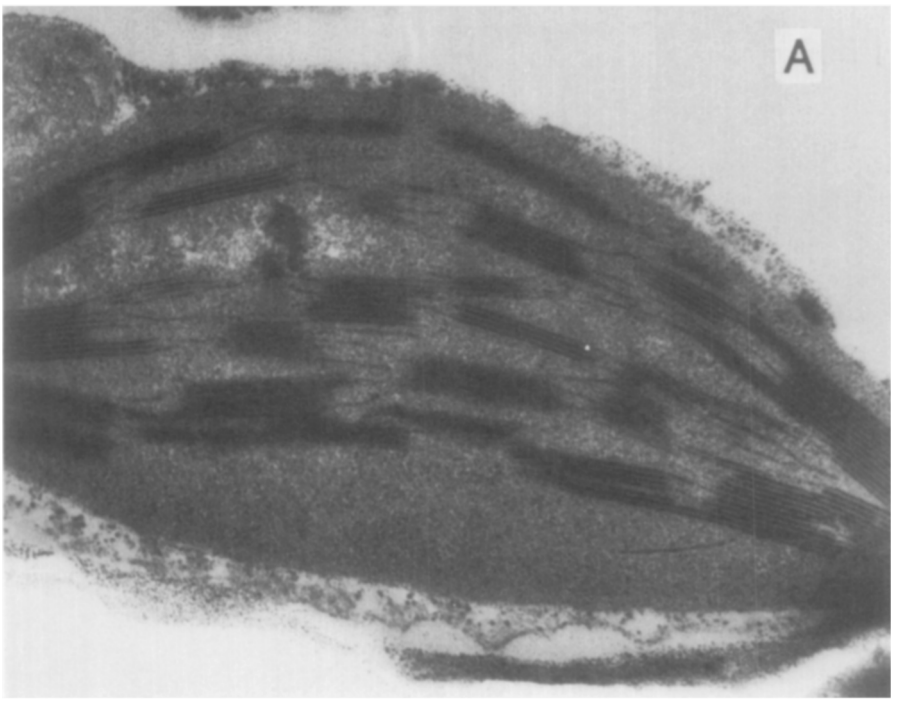


Figure 1. Microdensitometer tracings of CsCl buoyant density analysis for normal and mutant chloroplast DNA's: mixtures of 2 ug each of the corresponding cotton and marker DNA's were centrifuged to equilibrium in CsCl gradients before scanning by means of a Beckmann Model E analytical centrifuge with an AN-D rotor at 44,000 rpm for 20 hours at 20° C. The band at p = 1.731 corresponds to the marker DNA from M. lysodeikticus. (A) Chloroplast DNA from mutant white leaf tissue; (B) Chloroplast DNA from mutant yellow leaf tissues; (C) Chloroplast DNA from normal green leaf tissues.

contain chloroplasts with only empty vesicles. The latter observation is consistent with other studies of white leaf tissue chloroplasts (5, 13).

DISCUSSION

cDNA from the green leaves of cotton contains a main buoyant density component consistent with that of cDNA's from other plants (14). The minor component with a buoyant density of 1.706 has recently been attributed to mitochondrial contamination during the extraction proce-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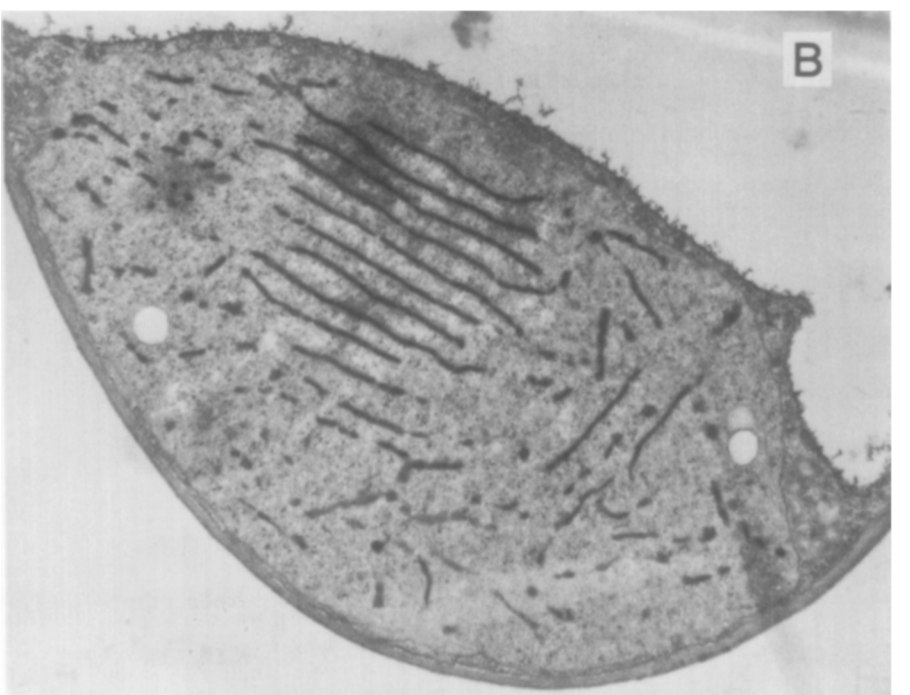
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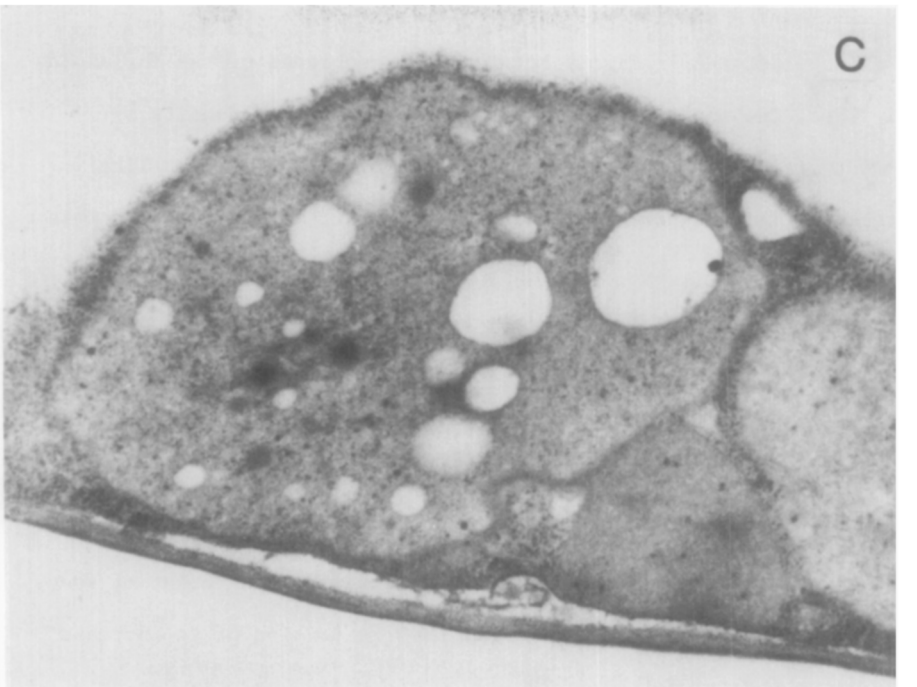

Figure 2. Electron micrographs from normal and mutant chloroplasts: (A) Green leaf tissue chloroplast (45,100X); (B) Yellow leaf tissue chloroplast (38,700X); (C) White leaf tissue chloroplast (40,200X). Specimens were prepared as described in Methods and Materials and examined in a Phillips EM 200 electron microscope.

dure. Such a contamination seems unavoidable in some cases even under carefully controlled conditions because of the interaction which occurs between mitochondria and the mobile phase of both normal and defective chloroplasts (1, 2).

A shift in the relative ratios of the two DNA components between the normal and mutant chloroplasts as noted in the tobacco investigation was also observed for cotton. The apparent decrease in the main buoyant density component of cDNA in the white as compared to the green and yellow chloroplasts (Fig. 1) was not due to a smaller amount of the white chloroplasts per cell since these mutant organelles were present in approximately and same number as the green chloroplasts (3). Electron micrographs (Fig. 2) show a progressive deterioration of the

organized thylakoids within the mutant chloroplasts. It has been shown that chloroplast DNA is firmly bound to the membranes of the thylakoids (15). Thus, the observed decrease of the main buoyant density component of cDNA in the white chloroplasts of cotton is most likely due to a partial loss or disruption of the thylakoid membranes in this organelle.

Although exact DNA measurements were not conducted, it appears (relative area increase under the 1.706 buoyant density band in the mutant chloroplasts of Fig. 1) as if the alleged mitochondrial DNA component is gradually increasing in the mutant yellow and white chloroplasts as compared to the normal green organelles. Such an increase was definitely noted in the tobacco variegated mutant of Wong-Staal and Wildman (1) and could either be a reflection of an increase in the mitochondria of the mutant yellow and white leaf tissues or a result of a greater interaction between the increased mobile phase of the defective chloroplasts and mitochondria (2).

With regard to the 1.719 gm/cc buoyant density peak of DNA observed in the white chloroplast mutants, Chun, et al. (16) discovered a similar high buoyant density component associated with spinach and beet cDNA. This component was later refuted as being of chloroplast origin on the basis that the researchers used commercial sources of bacterially contaminated leaf material (17). The yellow, white, and green leaves in the present investigation, however, were consistently taken from the same plants. Examination of the organelle preparations from each type of leaf sample under a high power light microscope did not reveal any significant bacterial contamination associated with the white chloroplast mutants. Therefore, it is unlikely that only the white leaf tissues contained enough bacterial components to elicit the high density DNA fraction in exactly the same proportions to the other buoyant density bands for five determinations.

Since cotton mitochondrial DNA has been shown to have a value of 1.706 gm/cc (18), the minor 1.719 component found only in the white leaf tissues does not seem to be a suitable candidate for mitochondrial DNA contamination and could be significant as a DNA specifically associated with the mutant white chloroplast.

In conclusion, results of this study agree essentially with those found for a variegated cytoplasmic mutant of tobacco with one notable exception, namely that of a high buoyant density component associated with the white mutant chloroplasts. It is yet too early to assign this DNA satellite to the mutant chloroplast. Work is now in progress to clarify this point.

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