Plant Physiol. (1984) 76, 575–578
0032-0889/84/76/0575/04/$01.00/0

Benzyladenine-Induced Increase in DNA Content per Chloroplast in Intact Bean Leaves

Received for publication January 12, 1984

ISAO KINOSHITA* AND HIDEO TSUJI
Laboratory for Plant Ecological Studies, Faculty of Science, Kyoto University, Kyoto 606, Japan

ABSTRACT

Benzyladenine (BA) treatment was found to induce chloroplast DNA (ctDNA) synthesis after it had stopped in primary leaves of light-grown intact bean plants (*Phaseolus vulgaris* L.). The leaves were treated with BA from 7 days after sowing. Chloroplasts were isolated and the ctDNA content per chloroplast was determined. Chloroplast division occurred until 13 days after sowing in untreated leaves. BA stimulated the division keeping the level of ctDNA content per chloroplast the same as that in the untreated controls. After the division period, the ctDNA content per chloroplast increased in BA-treated leaves, but not in controls. Consequently, ctDNA per leaf (or per cell) increased immediately after the beginning of BA treatment, but remained constant in the control leaves.

Naito *et al.* (10) have shown that BA induces a marked increase in total cellular DNA and chloroplast number per cell in growing primary leaves of bean plants. The present study was undertaken to find to what extent the increase in ctDNA per cell contributes to the increase in total cellular DNA.

The proportion of ctDNA in total cellular DNA was determined by several workers using reassociation kinetics (5, 14–16). To correctly estimate the DNA content per chloroplast, Boffey and Leech (3) measured the DNA content of isolated chloroplasts after DNase treatment. These studies showed that the ctDNA content per chloroplast decreased during the development of leaves in pea (6), spinach (14), wheat (3), and beet (16). However, no information is yet available on the influence of BA on the change in ctDNA content per chloroplast during growth and aging of the leaf. In the present study, we measured the ctDNA content per chloroplast in BA-treated and untreated bean leaves at different ages, and correlated it with the phase of chloroplast division.

MATERIALS AND METHODS

Plant Material. Bean seeds (*Phaseolus vulgaris* L. cv Yamashiro-kurosando-saito) were sterilized with Antiformin (NaOCl solution, 1% Cl) for 10 min, soaked in water for 16 h, then germinated on wet filter paper in plastic trays at 25°C in the dark. After 24 h, 15 germinated seeds were transplanted onto a plastic tray (32 × 23 × 5 cm) containing 3 L vermiculite wetted with 1 L of half-strength Hoagland solution. They were grown in a growth chamber at 25°C under a light regime of 16 h light and 8 h dark. Light intensity was 163 $\mu E/m^2 \cdot s$ at 400 to 700 nm (9.6 klux) on vermiculite, supplied by a mixture (1:1) of white and growth fluorescent lamps (Mitsubishi FL 40S SPG). One liter of half-strength Hoagland solution was added to the tray at 4 d after sowing. Aging of primary leaves of the control plants was delayed 1 d in the present experiments compared with that characterized in a previous paper (11). Primary leaves were unfolded at 7 d after sowing. At this stage, seven to eight seedlings were selected for uniformity. The adaxial surface of the primary leaves was painted with 30 mg/l BA solution containing Tween 80 at 50 mg/l every 4th d beginning at 7 d.

Isolation of Chloroplasts. Chloroplasts were isolated by a modification of the methods of Mourioux and Douce (7) and Mudd and Dezacks (8). Twenty grams of primary leaves was homogenized with 100 ml of extraction medium (330 mM sorbitol, 30 mM Mops[1]-NaOH [pH 7.8], 2 mM EDTA, and 0.15% BSA) in a Waring Blendor. The brei was filtered through two layers of Miracloth (Calbiochem) and the filtrate was centrifuged at 2200*g* for 30 s. Chloroplast pellets thus obtained were further purified on a step gradient of Percoll (Pharmacia). The 3-step gradient contained 2.5 ml of 90% (v/v), 5 ml of 40%, and 5 ml of 20% Percoll mixtures containing 330 mM sorbitol, 50 mM Mops-NaOH (pH 7.4), 2 mM EDTA, and 0.15% BSA. Chloroplasts were resuspended in the extraction medium, and a 1.5-ml aliquot was layered on top of the gradient and centrifuged at 3500*g* for 20 min. Intact chloroplasts banding at 40/90% interface were taken with a Pasteur pipette, diluted with extraction medium, and pelleted at 2200*g* for 30 s. All procedures of isolation and purification were carried out at 0 to 4°C.

The number of chloroplasts in the suspension was counted on a hemocytometer after proper dilution of the suspension. The amount of ctDNA per chloroplast (Fig. 1D) was calculated by dividing the value of DNA/ml of a chloroplast suspension by the number of chloroplasts/ml of the same suspension. In the experiments for Figure 1C, the number of chloroplasts per leaf was calculated from the values of Chl/leaf and Chl/chloroplast (*cf.* "Results").

DNase Treatment of Isolated Chloroplasts and Staining with DAPI. To accurately determine the DNA content per chloroplast, isolated chloroplasts were treated with DNase and then stained with DAPI, a sensitive and specific probe for DNA, according to the procedure of Boffey and Leech (3). Purified chloroplasts were resuspended in 6 ml of a medium containing 330 mM sorbitol, 2.5 mM Mg acetate, and 50 mM Mes (pH 7.0). Next, 300 μl of 2 mg/ml DNase I (Worthington) was added and the mixture was incubated on ice for 10 min to digest DNA from nuclear contamination and broken chloroplasts. After that, a 4-ml aliquot was removed and 1 ml of 40% TCA solution was added to it. From this sample, DNA was extracted and determined. Another aliquot of the DNase-treated chloroplast suspension was used for UV fluorescence microscopy. DAPI was added to obtain a concentration of 20 μg/ml. The remainder was used to determine the chloroplast number and examine the intactness.

DNA and Chl Determination. Nucleic acids were extracted by

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; DAPI, 4′,6-diamidino-2-phenylindole.

Schmidt-Thannhauser-Schneider's method (13) from tissues or chloroplast suspensions. The defatted residue was resuspended in 5% $HClO_4$ and heated at 90°C for 15 min. The suspension was centrifuged at 10,000g for 10 min. The supernatant was assayed for DNA by the diphenylamine method of Giles and Myers (4) using highly polymerized DNA from calf thymus (Sigma) as the standard.

Chl was extracted from tissues or chloroplast suspensions with 80% (v/v) acetone and determined by the method of Arnon (1).

RESULTS

In primary leaves of bean plants grown under light-dark cycles, cell division ceased by 6 d after sowing regardless of whether BA was applied or not (10). Therefore, the data presented here on a per leaf basis would show the same kinetics when expressed on a per cell basis.

Total cellular DNA increased about 50% in BA-treated leaves during the 7- to 21-d period but remained unchanged in the control leaves (Fig. 1A). This agrees with the previous results of our laboratory (9).

The Chl content per leaf increased markedly during the 7- to 13-d period in control leaves (Fig. 1B). BA enhanced this increase. The Chl content began to decrease at 17 d in the controls, while still increasing in BA-treated leaves. The Chl content per chloroplast changed in a way similar to the Chl content per leaf, although BA enhancement was less than in the former case (Fig. 1B).

The chloroplast number per leaf (Fig. 1C) was calculated by dividing each value of Chl content per leaf by that of the Chl content per chloroplast at the same age in Figure 1B. The changes in chloroplast number per leaf coincide with earlier results (10) obtained by methods other than those used in the present study. Chloroplast division terminated at 13 d and thereafter the number of chloroplasts per cell remained unchanged. Using the values of 50 and 40 chloroplasts/cell at the plateau for BA-treated and untreated leaves, respectively, together with the value of 200×10^5 cells/leaf reported previously (10), the number of chloroplasts per leaf can be calculated to be 10^9 and 8×10^8 for the above-mentioned samples, respectively. These values agree well with those presented in Figure 1C, i.e. 1.02 to 1.06×10^9 and 8.3 to 8.6×10^8 chloroplasts/leaf in the 13- to 21-d period for BA-treated and untreated samples, respectively. This coincidence indicates the validity of the present method for determining chloroplast number per leaf.

The ctDNA per chloroplast decreased in both BA-treated and untreated leaves during the 7- to 13-d period (Fig. 1D) when chloroplasts were duplicating (Fig. 1C). After 13 d, it increased markedly in BA-treated leaves, while still decreasing slowly in controls. At 21 d, a chloroplast in BA-treated leaves contained about 1.6 times as much ctDNA as that in control leaves.

Examination by phase-contrast microscopy showed that 70% to 95% of the isolated chloroplasts were intact. Neither nuclei nor nuclear fragments were found in the chloroplast suspensions when they were examined not only with a light microscope but also with a fluorescence microscope after staining with DAPI without DNase treatment. In the latter case, DNA was detected in every chloroplast (data not shown). However, the possibilities could not be excluded that the chloroplast fraction was contaminated with a small amount of nuclear fragments which might have escaped microscopic examination and that some part of the ctDNA was lost from the broken chloroplasts during their preparation. Therefore, we tried to accurately determine the ctDNA per chloroplast using the method of Boffey and Leech (3). Chloroplast suspensions were treated with DNase and then with DAPI. Chloroplasts with distinct margins surrounded by a halo under phase optics (Fig. 2A) were the same ones that showed DAPI fluorescence (Fig. 2B). Here again no contaminants which fluoresce with DAPI were detected (Fig. 2B). Thus, our preparations contained only ctDNA in intact chloroplasts without DNA in broken chloroplasts or in fragments of other organelles. The quantity of ctDNA per chloroplast was calculated by divid-

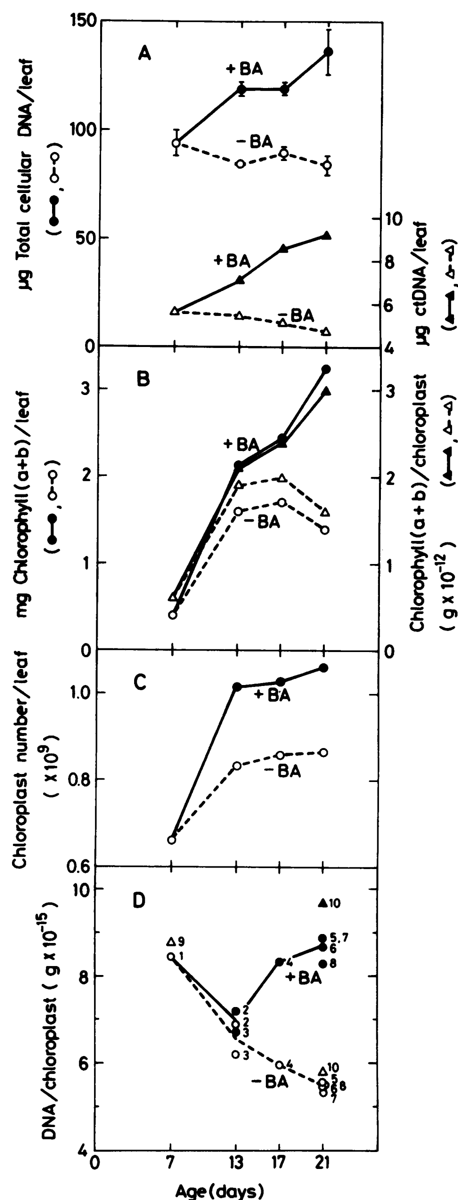

FIG. 1. BA effects on changes in total cellular DNA and ctDNA contents per leaf (A), Chl contents per leaf and per chloroplast (B), chloroplast number per leaf (C), and ctDNA content per chloroplast (D). (●, ▲), BA; (O, Δ), control. (A) Each plot of total cellular DNA/leaf (●——●), (O--O) represents the mean of values obtained from three determinations. Vertical bars through the points indicate the magnitude of the standard deviation. The values of ctDNA/leaf (▲——▲, Δ--Δ) were calculated from the results in C and D. (B) Each plot of Chl/leaf (●——●, O--O) represents the mean of values obtained from two separate experiments. Chl/chloroplast (▲——▲, Δ--Δ) was calculated by dividing the values of Chl/ml of a chloroplast suspension by the number of chloroplasts/ml in the same suspension. Each plot represents the mean of values obtained from two separate experiments. (C) The values were calculated from the results in B. See text for details. (D) DNA in isolated chloroplasts was determined with (▲, Δ) or without (●, O) DNase pretreatment as described in the text. The figures on the symbols indicate experiment numbers.

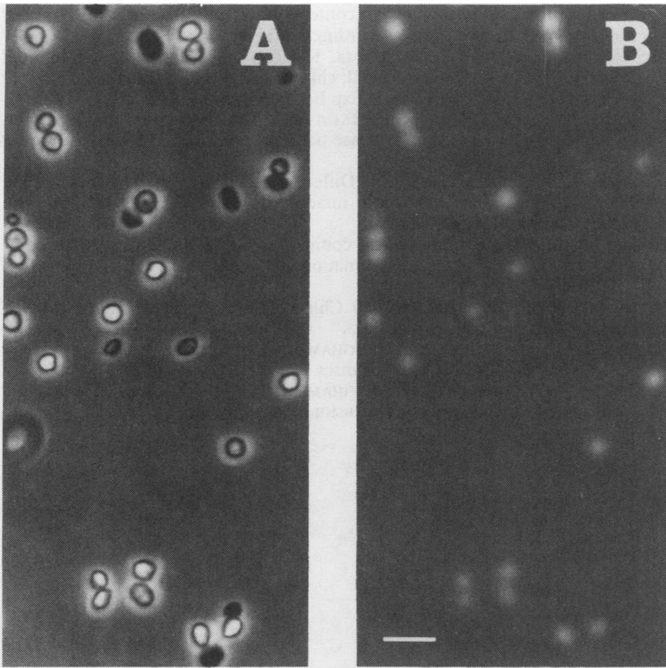

FIG. 2. Isolated chloroplasts of the same field viewed by phase-contrast (A) and UV fluorescence (B) microscopy in the presence of DAPI after DNase treatment. See text for details of the methods. Chloroplasts isolated from primary leaves of 7-d-old plants were used. Bar, 10 μm.

ing the value of DNA/ml of the DNase-treated chloroplast suspension by the number of intact chloroplasts/ml in the same suspension. The ctDNA per chloroplast determined by this method was close to the values obtained by the assay of DNA in chloroplasts without the DNase treatment (Fig. 1D). This indicates that the procedure of chloroplast isolation used in this study was suitable for isolating *Phaseolus* chloroplasts with little loss of ctDNA and no contamination from nuclear DNA.

The ctDNA content per leaf (Fig. 1A) was estimated by multiplying each value of ctDNA/chloroplast in Figure 1D by that of chloroplast number/leaf at the same age in Figure 1C. The ctDNA level per leaf showed little change in controls throughout the experimental period (7–21 d), while it was increasing in BA-treated leaves due to stimulation of chloroplast division with the ctDNA per chloroplast kept at the same level as that of controls during the 7- to 13-d period and due to the increase in DNA content per chloroplast during the 13- to 21-d period.

DISCUSSION

Recently, a number of papers have been published on the changes in chloroplast number per cell and ctDNA content per chloroplast during leaf development (2, 3, 6, 10, 14, 16). To illustrate the modification of the patterns of changes in those parameters by BA treatment, their changes in the absence of BA are schematically shown in Figure 3 according to Boffey and Leech's discussion (3). During the early stages of leaf development, ctDNA is synthesized, keeping pace with the rate of chloroplast division: ctDNA/chloroplast is maintained at a constant level. However, ctDNA synthesis ceases before the end of chloroplast division. During the period between the cessations of ctDNA synthesis and chloroplast division, ctDNA/chloroplast decreased (dilution phase). After this phase, neither ctDNA synthesis nor increase in chloroplast number occurs. ctDNA/chloroplast again remains constant but at a lower level than in the early stages.

At age 7 d, untreated leaves in the present experiments were in the dilution phase, because ctDNA/leaf (or cell) remained

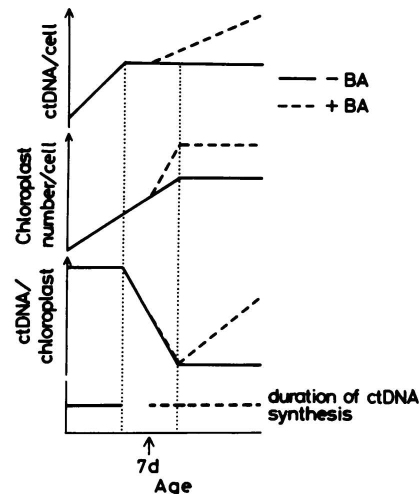

FIG. 3. A schematic illustration of the patterns of changes in ctDNA/cell, chloroplast number/cell, and ctDNA/chloroplast in BA-treated and untreated leaves. The changes in these parameters in leaves receiving no BA treatment (——) are depicted according to Boffey and Leech (3). The present results with BA treatment (-----) were added.

unchanged (Fig. 1A), chloroplast number/leaf (or cell) was increasing (Fig. 1C), and ctDNA/chloroplast was decreasing in the 7- to 13-d period (Fig. 1D). In BA-treated leaves, however, ctDNA/leaf continued increasing even after 7 d (Fig. 1A), suggesting that ctDNA synthesis in the chloroplasts was restored. As for the resumption of ctDNA increase in chloroplasts, although it has been reported to be brought about by irradiation with a low intensity green light in spinach leaf discs (15), the present observation is the first one that a cytokinin induced a ctDNA increase in intact leaves in which the ctDNA increase and chloroplast division had already ended.

Naito *et al.* (10) have shown that BA treatment stimulated chloroplast division during the period when chloroplast division occurred in control leaves and that chloroplast enlargement was induced by BA treatment after the division period (10, 12). The results of the present experiment agree well with those mentioned above both with respect to division (Fig. 1C) and enlargement (data not shown) of chloroplasts. Until 13 d, ctDNA content per chloroplast in BA-treated leaves did not differ from that in controls in spite of the marked acceleration of chloroplast division (Fig. 1D). This indicates that BA treatment stimulated chloroplast division and induced ctDNA synthesis simultaneously during this period. After 13 d, an increase occurred in the ctDNA content per chloroplast together with chloroplast enlargement in BA-treated leaves (Fig. 1D).

Although BA induced an increase in both total cellular DNA and ctDNA, the proportion of ctDNA in total cellular DNA at 21 d did not differ greatly between BA-treated and untreated leaves, being 6.8% and 5.6% respectively. At that stage, ctDNA per leaf was only 4.5 μg higher in BA-treated samples than in controls, while total cellular DNA was about 50 μg higher in the former than in the latter (Fig. 1A). This suggests that BA induced an increase not only in ctDNA but also in nuclear DNA. Studies with Feulgen staining of nuclei in leaf tissues (Kinoshita and Tsuji, in preparation) have provided supporting evidence for this inference.

Acknowledgment—We gratefully acknowledge the use of the fluorescence microscope of the Department of Botany, Faculty of Science, Kyoto University.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15

2. BOFFEY SA, JR ELLIS, G SELLDÉN, RM LEECH 1979 Chloroplast division and DNA synthesis in light-grown wheat leaves. Plant Physiol 64: 502–505
3. BOFFEY SA, RM LEECH 1982 Chloroplast DNA levels and the control of chloroplast division in light-grown wheat leaves. Plant Physiol 69: 1387–1391
4. GILES KW, A MYERS 1965 An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 206: 93
5. LAMPPA GK, AJ BENDICH 1979 Changes in chloroplast DNA levels during development of pea (*Pisum sativum*). Plant Physiol 64: 126–130
6. LAMPPA GK, LV ELLIOT, AJ BENDICH 1980 Changes in chloroplast number during pea leaf development. An analysis of a protoplast population. Planta 148: 437–443
7. MOURIOUX G, R DOUCE 1981 Slow passive diffusion of orthophosphate between intact isolated chloroplasts and suspending medium. Plant Physiol 67: 470–473
8. MUDD JB, R DEZACKS 1981 Synthesis of phosphatidylglycerol by chloroplasts from leaves of *Spinacia oleracea* L. (spinach). Arch Biochem Biophys 209: 584–591
9. NAITO K, H TSUJI, I HATAKEYAMA 1978 Effect of benzyladenine on DNA, RNA, protein, and chlorophyll contents in intact bean leaves: Differential responses to benzyladenine according to leaf age. Physiol Plant 43: 367–371
10. NAITO K, H TSUJI, I HATAKEYAMA, K UEDA 1979 Benzyladenine-induced increase in DNA content per cell, chloroplast size, and chloroplast number per cell in intact bean leaves. J Exp Bot 30: 1145–1151
11. NAITO K, A IIDA, H SUZUKI, H TSUJI 1979 The effect of benzyladenine on changes in nuclease and protease activities in intact bean leaves during ageing. Physiol Plant 46: 50–53
12. NAITO K, K UEDA, H TSUJI 1981 Differential effects of benzyladenine on the ultrastructure of chloroplasts in intact bean leaves according to their age. Protoplasma 105: 293–306
13. SCHNEIDER WC 1946 Phosphorus compounds in animal tissues. III. A comparison of methods for the estimation of nucleic acids. J Biol Chem 164: 747–751
14. SCOTT NS, JV POSSINGHAM 1980 Chloroplast DNA in expanding spinach leaves. J Exp Bot 31: 1081–1092
15. TYMMS MJ, NS SCOTT, JV POSSINGHAM 1982 Chloroplast and nuclear DNA content of cultured spinach leaf discs. J Exp Bot 33: 831–837
16. TYMMS MJ, NS SCOTT, JV POSSINGHAM 1983 DNA content of *Beta vulgaris* chloroplasts during leaf cell expansion. Plant Physiol 71: 785–788