MEMOIRS OF THE FACULTY OF SCIENCE, KYOTO UNIVERSITY, SERIES OF BIOLOGY
Vol. VIII, pp. 157–172, March 1983

Effect of Benzyladenine on RNA Metabolism during the Life Cycle of Intact Bean Leaves

Kunihiko NAITO

Institute of Biological Sciences, University of Tsukuba,
Sakura-Mura, Ibaraki 305, Japan

(Received December 27, 1981)

Abstract

The effect of benzyladenine (BA) on RNA metabolism during ageing of intact bean (Phaseolus vulgaris) leaves was studied. BA was applied to intact primary leaves at different stages of their growth. When BA was applied from an early stage, it enhanced the increase in RNA content, rate of RNA synthesis, and RNase activity. When it was given from a late stage, it also raised the former two parameters and alleviated the decrease in the latter, while all three values decreased in untreated controls at the late stage. The ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA was much higher in throughout or late BA-treated leaves than in untreated controls. Electron microscopic examination revealed that polysomes in the cytoplasm greatly increased when BA treatment was begun at the late stage. These results suggest that BA might affect chloroplast metabolism through its action on the nucleus and cytoplasm.

Introduction

Most investigations on the effect of cytokinins on senescence of leaf tissues have been made on excised leaves or leaf discs (e.g., Atkin and Srivastava 1969). Very little information is available about the control of senescence of intact leaves by this hormone (e.g., Fletcher 1969).

During senescence of leaf discs and excised leaves, DNA, RNA, and protein content decreased accompanied by an increase in hydrolase activities, and cytokinins depressed these changes (Srivastava and Ware 1965, see Kende 1971 and Dove 1973). With leaves attached to the plant, however, it has also been reported that RNase and protease activities were high in young leaves and declined with ageing (see Beevers 1976).

In attached leaves, as well as those detached, RNA content markedly decreased with age (e.g., Smillie and Krotkov 1961, Spencer and Titus 1972). Cytokinins prevented this decrease in excised leaves or leaf discs (e.g., Osborne 1962, see Thimann 1978). In intact leaves, however, we have shown that BA raised the

RNA content rather than prevented its decrease, and that RNA content responded more markedly to application or deprival of BA treatment than protein and chlorophyll contents (Naito et al. 1978).

The present report is focused on the effect of BA on the change in RNA metabolism in intact bean leaves during ageing. The effect of BA on the rate of RNA synthesis and RNase activity was examined. An attempt was also made to determine whether cytoplasmic or chloroplastic ribosomes are preferentially affected by cytokinin.

Abbreviation: BA, benzyladenine.

Materials and Methods

Growth conditions

Phaseolus vulgaris L. cv. Yamashiro-kurosando-saito was used. Seeds were sterilized with NaOCl solution (1% Cl), soaked in water for 8 h, then placed on wet perlite. After 2 days, germinated seeds were transferred onto vermiculite watered with Hoagland solution and grown under illumination (16 h light and 8 h dark) at 22 klux from a mixture of white and growth fluorescent lamps at 28°/23°C day/night temperatures, unless otherwise indicated. BA solution (30 mg/l) containing Tween 80 (50 mg/l) was painted on the adaxial surfaces of primary leaves as in a previous study (Naito et al. 1978) according to the schedule indicated at the bottom of Figure 1: "early" or "throughout" treatment was begun at 6 days and "late" treatment was begun at 22 days unless otherwise indicated.

Sampling

At least 15 pairs of primary leaves were harvested at different stages. The stages of leaves in control samples were characterized in previous papers (Naito et al. 1978, 1979a). After the petioles were discarded, the blades were measured for fresh weight and used for analysis. After removal of the mid-ribs, the laminae were cut into 0.5×0.5 cm sections (Naito et al. 1978). The sections were randomized, divided into groups of 0.5 g and 3.0 g fresh weights for the determination of RNA content and RNase activity, and for the nucleic acid analysis by electrophoresis, respectively. The grouped sections were frozen with liquid nitrogen and stored at −80°C until use.

RNA content

RNA was extracted according to the Schmidt-Thannhauser-Schneider's method (Schneider 1946). After alkali hydrolysis, RNA was estimated by measuring A 260 nm in 5% (w/v) perchloric acid solution.

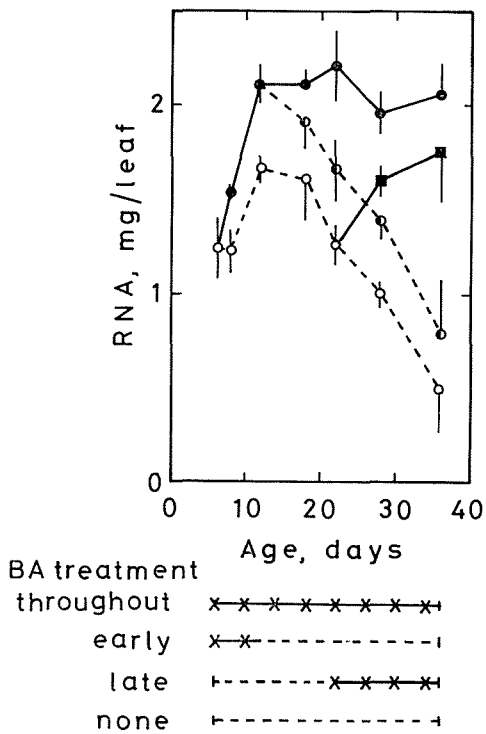

Figure 1. BA effects on RNA content per single leaf during ageing. The schedule of BA treatment is shown at the bottom. Throughout (●): Eight consecutive BA paintings were given every 4th day beginning at 6 days. Early (○→◐): Only the first two consecutive paintings were given. Late (○→■): Only the last four consecutive paintings were given beginning at 22 days. None (○): No BA painting was given. Crosses indicate the days of BA painting. —, Period of BA treatment; - - -, period without BA treatment. Each plot is the mean of five replicated samples. Vertical bars through the points indicate the magnitude of the standard deviation.

In vivo RNA synthesis

A primary leaf in intact plants was allowed to absorb radioactive material for 10 hours through two pairs of cotton threads which passed across the petiol and whose ends were dipped in [5-^{3}H]-uridine solution (10 μCi/ml, specific activity, 0.5 Ci/mM) in a small vial. Blades were excised from plants, rinsed with deionized water, and blotted. These samples were weighted and cut into small sections and RNA was extracted as described above. An aliquot of the combined trichloroacetic acid extract was used for the determination of total uptake of radioactivity. After alkali

hydrolysis, radioactivity of the RNA fraction was determined in a liquid-scintillation counter using toluene/Nissan Nonion NH210 (3 : 1, v/v) solvent containing 4 g of PPO and 0.1 mg of dimethyl POPOP per liter of toluene.

Enzyme assays

Samples of leaf sections were homogenized with ice cold 0.1 M phosphate buffer (pH 6.5). The homogenate was diluted with the same buffer to a final volume of 8 ml and centrifuged at 20,000×g for 30 min at 0 to 2°C. The supernatant fluid (leaf extract) was assayed for enzyme activity.

The increase in acid-soluble nucleotides due to enzyme activity was measured according to the method of Pitt and Coombes (1969) with slight modifications. A 0.2 ml of leaf extract was incubated with 1.0 ml of 1.5 mg/ml yeast RNA in 0.05 M phosphate buffer (pH 6.5) and 0.8 ml of the same buffer at 37°C for 2 h. The reaction was stopped by adding 0.5 ml of chilled 25% (w/v) $HClO_4$ containing 0.75% (w/v) uranyl acetate. After cooling, samples were centrifuged at 2,000×g for 15 min and the supernatant was diluted fifteen times before reading A_{260} against the zero-time blank. One unit of RNase was defined as an increase of one A_{260} unit per hour.

Extraction and polyacrylamide gel electrophoresis of nucleic acid

Leaves were harvested at 28 days. Nucleic acid was extracted in 140 mM Tris buffer (pH 9.0) containing 5 mM $MgCl_2$, 0.5% sodium lauryl sulphate, 0.14 M NaCl, and 0.5% (v/v) dietyl pyrocarbonate. Dietyl pyrocarbonate was added in the extraction medium as a nuclease inhibitor (Solmosy et al. 1968). Nucleic acid was purified by the phenol method (Muramatsu and Fujisawa 1968). After dissolving ethanol-precipitated RNA into saline buffer, starch was removed from the solution by washing it with 2-methoxyethanol (Kirby 1956).

RNA preparations were fractionated on 2.4% polyacrylamide cylindrical gels according to Loening (1967) for 10 h at 5 mA/gel. The gels were scanned at 254 nm with an ISCO UA-2 chromoscan.

Electron microscopy

In both halves of a leaf, epidermis was stripped in the area just below its middle. From this area a 0.8×0.8 cm section (depending on leaf age) was excised. The sections were fixed in 4% glutaraldehyde in 0.05 M cacodylate-HCl buffer (pH 6.8) at 5°C for 1 h, post-fixed in 2% OsO_4 in the same buffer at 5°C for 3 h, and dehydrated through a graded acetone series at 0°C. At 50% acetone step, they were treated with 2% uranyl acetate for 30 min and embedded in Epon 812. The material was cut on an LKB Ultrome, stained with 1% aqueous uranyl acetate followed by lead citrate (Reynolds 1963), and examined by a JEM-7 electron microscope.

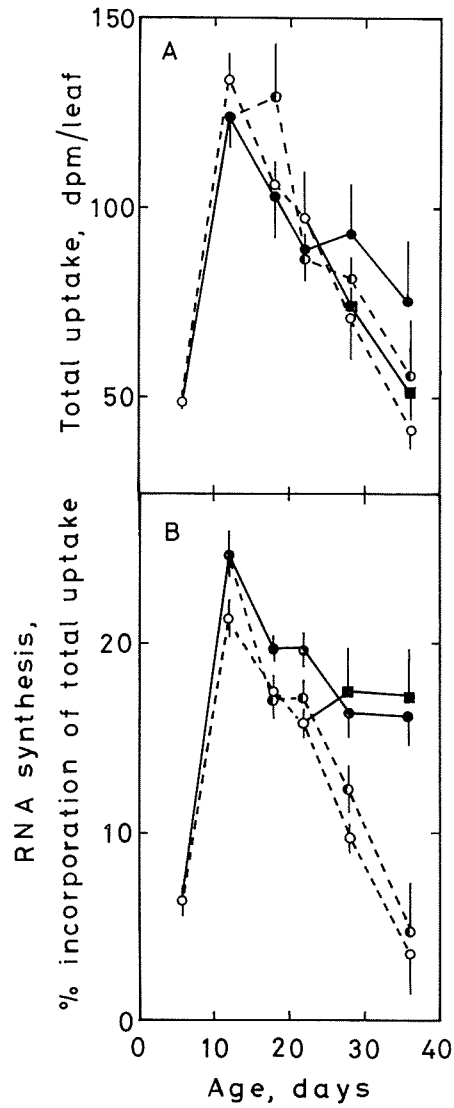

Figure 2. BA effects on the total uptake of (^{3}H)-uridine into leaf (A) and incorporation of the precursor into RNA (B) during ageing. The ratio of RNA synthesis (B) is expressed as (dpm incorporation into RNA/dpm total uptake into leaf) $\times 100$. Each plot is the mean of five replicated samples. Vertical bars through the points indicate the magnitude of the standard deviation.

Effect of BA on RNA content

RNA content per single leaf increased during the 8–12 day period, remained unchanged for a while, and decreased rapidly (Figure 1). BA treatment greatly enhanced the increase in RNA content at the early stage and maintained the same level higher than controls over the experimental period. When BA treatment was discontinued at an early stage, RNA content decreased promptly parallel with untreated controls. BA treatment at a late stage increased the RNA content without a lag period in contrast to the rapid decrease with untreated leaves.

Effect of BA on RNA synthesis

Rate of total uptake of ^{3}H-uridine into the tissue increased rapidly during the early stage to reach a peak at 12 days and decreased thereafter (Figure 2A). In leaves with any type of BA treatment, uptake followed the same pattern of change as untreated controls, except in throughout BA-treated samples after 28 days where decrease in uptake was prevented by BA.

The ratio of dpm incorporated into RNA to dpm of the total uptake into the leaf was employed to express the rate of RNA synthesis. The change in the rate of RNA synthesis for any type of BA treatment was parallel with that of total uptake of ^{3}H-uridine, except the response to the late BA treatment. In untreated controls, RNA synthesis showed initially a sharp rise but began to fall rapidly as early as 12 days when leaves were still expanding. RNA synthesis was greater in throughout BA-treated leaves than in untreated controls over the experimental period especially at the late stage where it decreased rapidly in the latter samples. Stopping BA treatment at an early stage (12 days) resulted in a prompt decrease in RNA synthesis, and late BA treatment which began at 22 days efficiently prevented the decrease.

RNase activity

RNase activity per single leaf increased at early stages of leaf growth, and began to decline at 20 days, when leaf expansion was almost completed (Naito et al. 1978,

Table 1. Effect of various BA treatments on the ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA.

	Untreated	BA-treated		
		throughout	early	late
Ratio cytoplasmic/chloroplastic	1.2	2.2	1.4	1.8

See legend to Figure 1 for schedule of the BA treatments. Samples were harvested at 23 days. The ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA at 6 days was 1.6. Each value represents the mean of values obtained from duplicate gel columns loaded with the same RNA preparation.

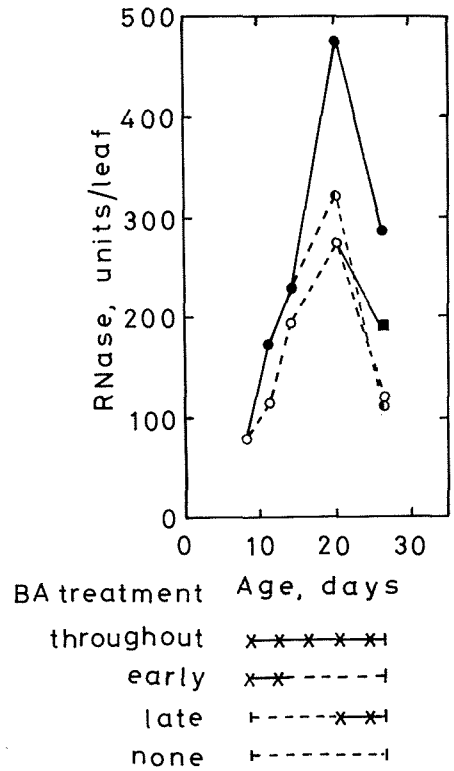

Figure 3. BA effects on RNase activity per single leaf during ageing. Plants were grown under an illumination regime of 12 h hours light and 12 hours dark. Ageing of primary leaves were delayed 2 days under this regime compared with those used for other experiments (See, Naito et al. 1979a Explanation for Table 1 in). BA treatments were carried out as described in legend for Figure 1, except the followings; Throughout: Six consecutive paintings were given at 8 days. Late: Only the last two consecutive paintings were given beginning at 20 days. Notations as in Figure 1. Each plot is the mean of duplicate samples.

Figure 3). BA treatment stimulated the increase in RNase activity during early stages and kept the activity at higher levels than those in untreated controls throughout the experimental period. When BA treatment was discontinued at an early stage (12 days), RNase activity still showed an increase over the untreated controls. After that it decreased rapidly. Beginning BA treatment at a late stage (20 days) alleviated the decrease in RNase activity.

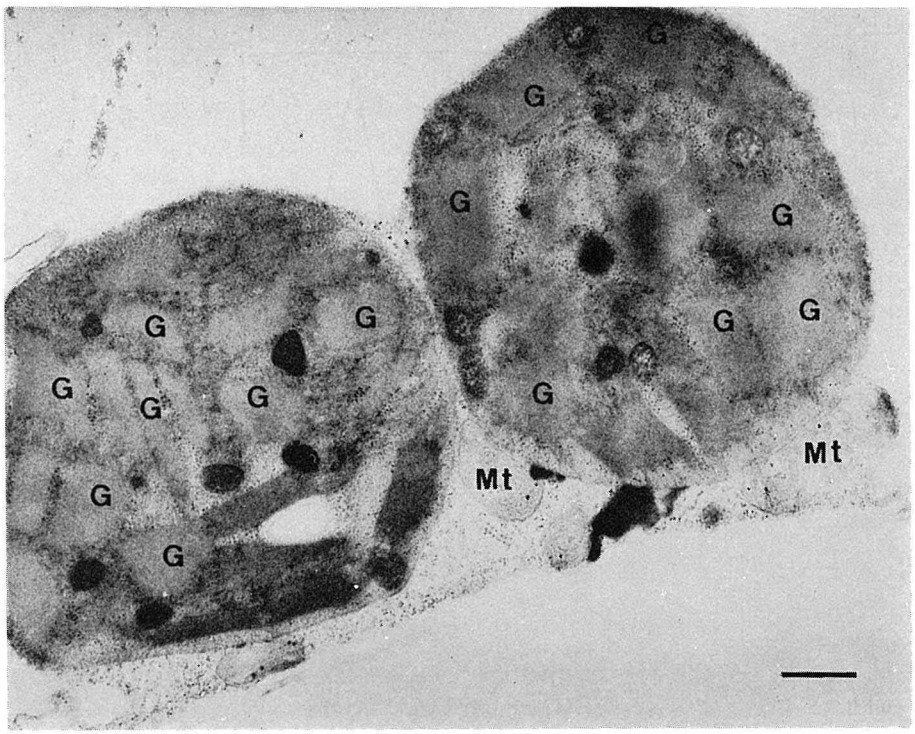

Fig. 4 A

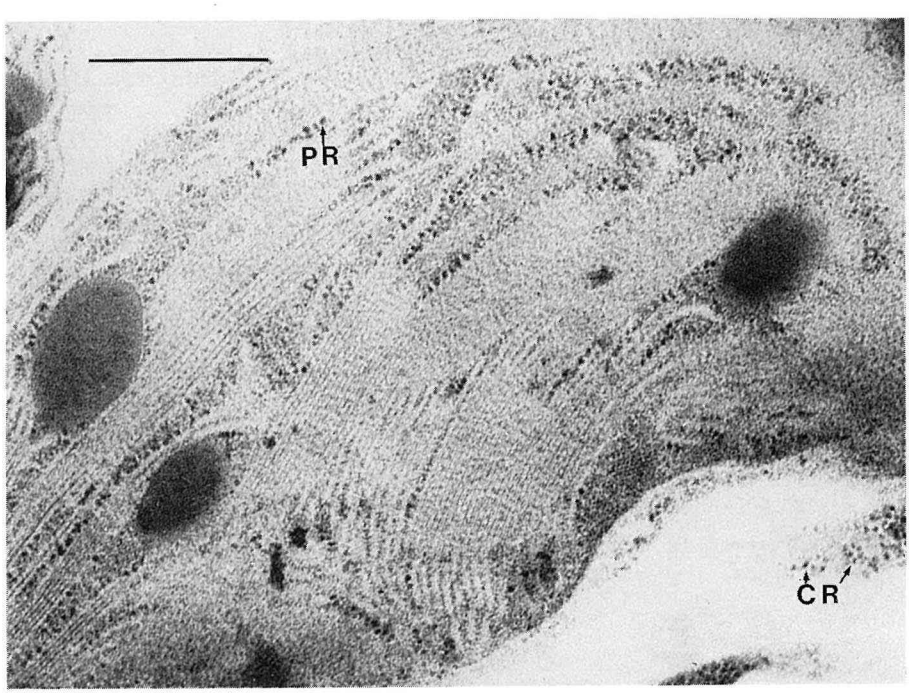

Fig. 4 B

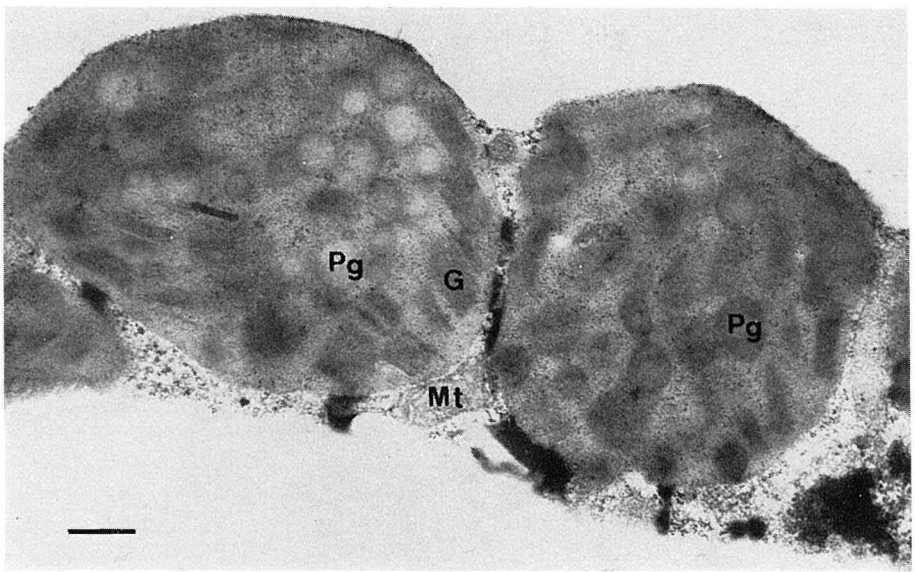

Fig. 5 A

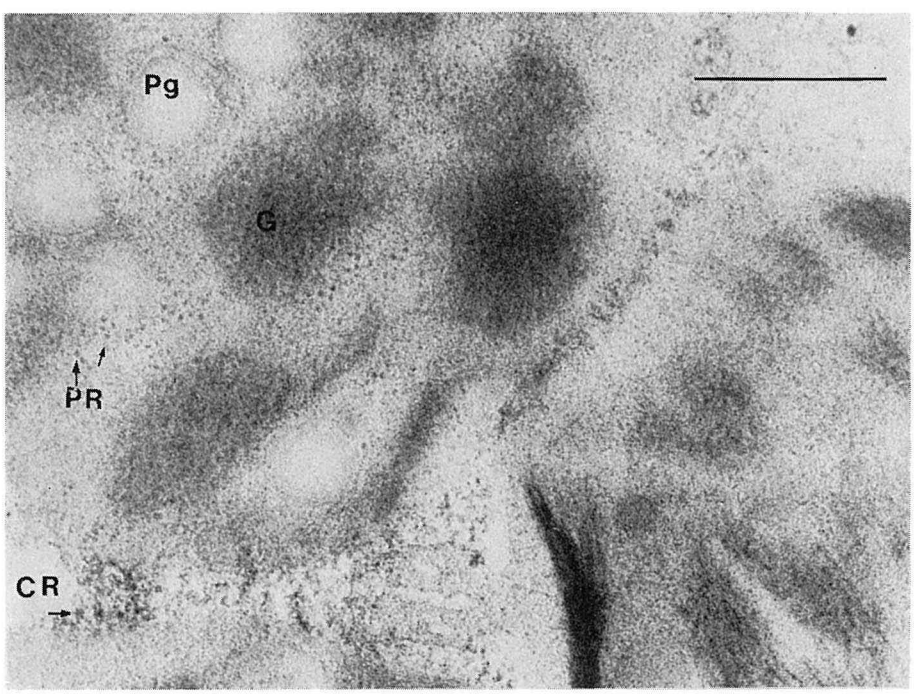

Fig. 5 B

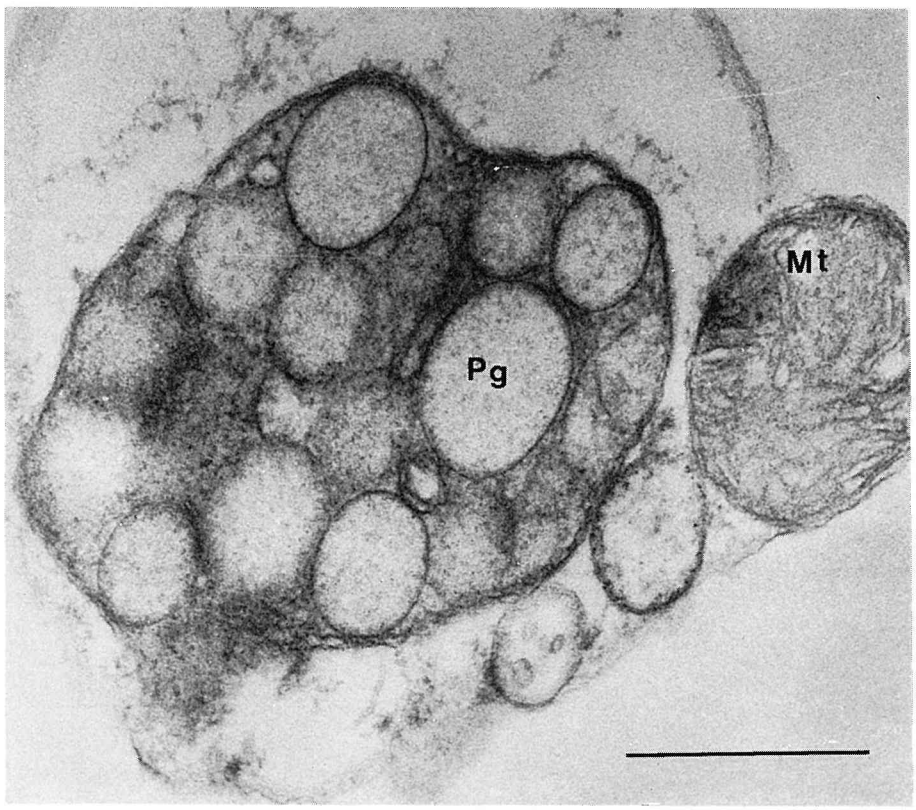

Fig. 6

Ribosomal RNA

The ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA at 28 days was higher in leaves treated with BA throughout than in untreated controls (Table 1). In leaves treated with BA only at early stages (until 10 days), the ratio declined to about the same level as that in untreated controls. When leaves were treated with BA from a late stage (22 days), the ratio attained a value much higher than that in untreated controls but less than that in throughout BA-treated leaves.

Ultrastructure

Although the day at which primary leaves of untreated controls became yellow-green varied from plant to plant, the number of plants whose primary leaves reached this stage showed a maximum at 36 days. Once leaves became yellow-green, they turned completely yellow in about two days. The stages of yellow-green, more

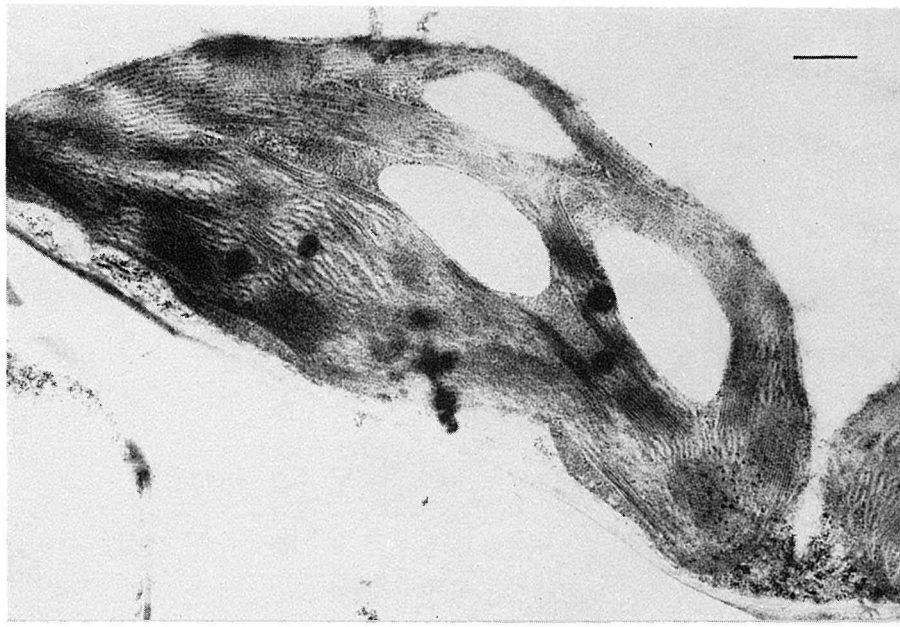

Fig. 7 A

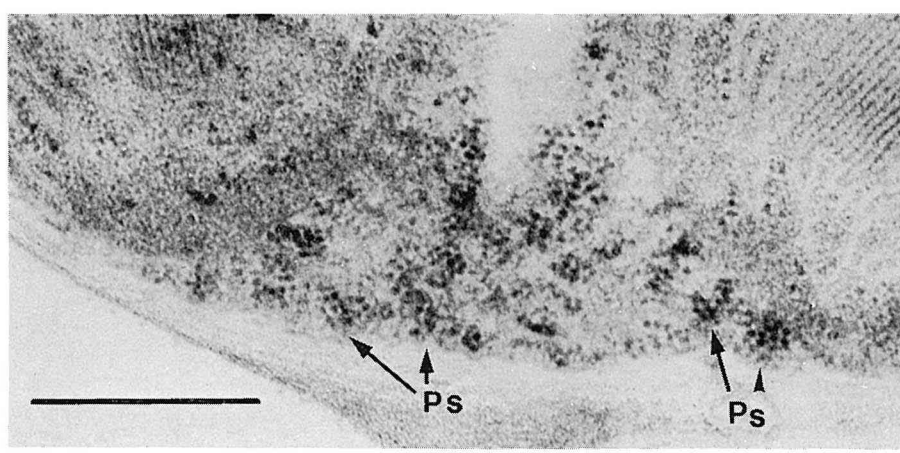

Fig. 7 B

yellowish green, and yellow leaves were referred to as last stage I, II, and III, respectively (Naito et al. in preparation).

Chloroplasts were the first organelles which showed symptoms of senescence. At last stage I, chloroplasts became smaller than those in green leaves. Intergrana

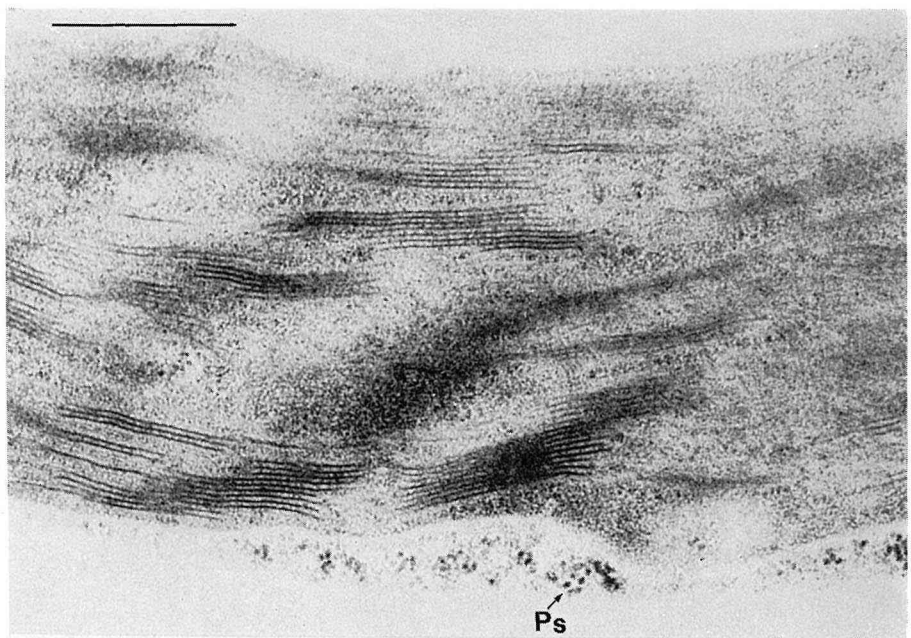

Fig. 8

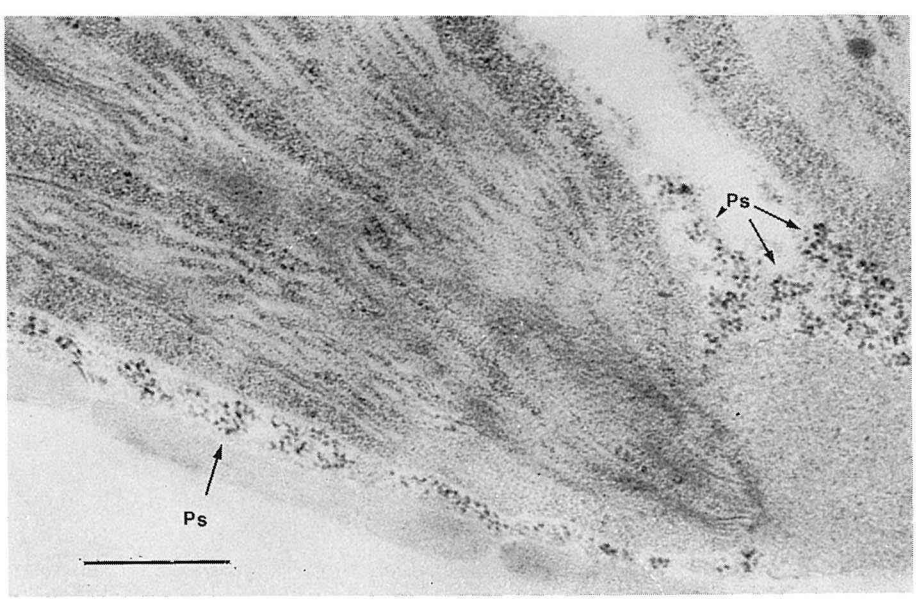

Fig. 9

lamella disintegrated and the orientation of grana became random (Figure 4). No pronounced changes were observed with other organelles at this stage. Chloroplast ribosomes and cytoplasmic ribosomes are still present. At last stage II, chloroplasts became smaller than those at last stage I (Figure 5). Parts of grana disappeared: Grana with smaller number of thylakoids disintegrated earlier. Plastoglobuli grew larger. Both cytoplasmic and chloroplastic ribosomes were still detectable. At last stage III, chloroplasts contained no grana but were filled with large plastoglobuli (Figure 6). Both cytoplasmic and chloroplastic ribosomes were not detected anymore.

Throughout or late BA treatment efficiently prevented the yellowing of leaves and the degeneration of chloroplasts. In these samples, no sign of disintegration of chloroplasts was seen even at 36 days (Figure 7). There were few polysomes in the cytoplasm in cells of leaves just before receiving the late BA treatment, i.e. at 22 days (Figure 8), compared with those in younger leaves (Figure 9). The late treatment markedly increased the number of polysomes in the cytoplasm.

Discussion

There are differences between metabolism of senescing leaf discs and intact leaves reported in the literature (see Kende 1971, Beevers 1976, and Thimann 1978). Hydrolase activities increased with advance of senescence in detached leaves, whereas in attached leaves they decrease with age. In various excised tissues, cytokinins depressed the increases in DNase, RNase, and protease activities, and prevented the decrease of DNA, RNA, and protein contents, thus delaying the loss of chlorophyll,

Abbreviation used in Figures 4–9: PR-chloroplast ribosome, CR-cytoplasmic ribosome, G-grana, Mt-mitochondrion, Pg-plastoglobule, Ps-polysome. Solid lines represents 0.5 μm.

Figure 4. Chloroplast and cytoplasm of yellow green leaf (last stage I). A, Intergrana lamella have disappeared and the orientation of grana is random. B, Ribosomes are present in chloroplast and cytoplasm.

Figure 5. Chloroplasts and cytoplasm of more yellowish green leaf (last stage II). A, Chloroplasts shrunk than at stage I. Parts of grana have disintegrated. B, Ribosomes are still present in chloroplast and cytoplasm.

Figure 6. Chloroplast, mitochondrion, and cytoplasm of yellow leaf (last stage III). Chloroplast and rounded, being filled with large glastoglobuli. All grana have been disintegrated. No ribosomes are seen in chloroplast and cytoplasm. Mitochondrion is still present.

Figure 7. Chloroplast and cytoplasm of 36-day-old leaf which received the late BA treatment. A, Intergrana lamella are present. Grana are normally oriented. B, Numerous polysomes are visible in cytoplasm. Ribosomes are seen in chloroplast.

Figure 8. Chloroplast and cytoplasm of leaf just before receiving the late BA treatment (22 days). Very few polysomes are present in cytoplasm. Grana are normally oriented.

Figure 9. Chloroplasts and cytoplasm of 13-day-old leaf. A large number of polysomes are present in cytoplasm.

the ultimate stage of senescence. In attached leaves, however, BA increased these hydrolase activities or prevented their decrease.

In intact leaves, the first indication of senescence was the decrease in RNA content (Figure 1, see also Naito et al. 1978). RNA content responded more promptly to application or deprival of BA treatment than protein and chlorophyll contents (Naito et al. 1978). Such prompt response to BA application was observed not only with RNA content but also with RNA synthesis and RNase activity. When BA was applied at early stage, it stimulated RNA synthesis and RNase activity and increased RNA content. Late BA treatment also increased RNA content and synthesis activity concomitant with an alleviation of the decrease in RNase activity. On the other hand, when BA treatment was stopped at the early stage RNA content and synthesis decreased, though RNase activity continued increasing for a while.

BA stimulated RNA synthesis and RNase activity by 30% and 100%, respectively. From these figures, BA seemed to stimulate RNA decomposition more than RNA synthesis. However, RNase activity was assayed *in vitro* under optimal conditions, hence its value should be regarded as a potentiality which may not be fully realized under *in vivo* conditions. To the contrary, RNA synthesis was assayed *in vivo*. Actually RNA content was increased by BA. It is, therefore, adequate to conclude that BA stimulated both synthesis and decomposition of RNA but more the former than the latter, resulting in an increase in RNA content. Similar results were also obtained with the relationship among the activities of protein synthesis (Naito et al. in preparation), protease (Naito et al. 1979a), and protein content (Naito et al. 1978) in BA-treated leaves. It also seems to be the case with chlorophyll. BA stimulated the activities of enzymes in chlorophyll synthesis [δ-aminolevulinic acid synthesizing enzyme and δ-aminolevulinic acid dehydratase (Naito et al. 1980)], and chlorophyll breakdown [chlorophyllase (Phillips et al. 1969)], accompanied by an increased accumulation of chlorophyll in intact bean leaves.

Many authors (Kuraishi 1968, Shibaoka and Thimann 1970, Tavares and Kende 1970, Martin and Thimann 1972) claim that cytokinins retard senescence of excised tissues by inhibiting the decomposition of chlorophyll, DNA, RNA, and protein, while some (Osborne 1962, Wollgiehn 1965, 1967) ascribe the cytokinin effect to its action in stimulating the synthesis of those substances. The results with intact leaves suggest that the role of cytokinins in retarding senescence is the maintenance of RNA and protein (Naito et al. in preparation) synthesis rather than the prevention of decomposition of these substances.

Takegami (1975) reported that the amount of chloroplastic ribosomal RNA decreased earlier than that of cytoplasmic ribosomal RNA in senescent tobacco leaf discs in the dark, and that BA retarded this decrease as well as chlorophyll loss. Dayer and Osborne (1971) showed that detached senescent leaves of *Xanthium* did

not re-green in the presence of kinetin, whereas those of *Vicia* and *Nicotiana* did. They attributed the difference in the kinetin effects to the fact that chloroplastic ribosomal RNA is present in senescing leaves of the latter two species but is virtually absent from *Xanthium*. On the other hand, stable chloroplastic ribosomal RNA has been reported as follows.

Studies on the incorporation of radioactive precursors into RNA of leaf discs of wheat, swiss chard, onion (Ingle et al. 1970), and radish (Paranjothy and Wareing 1971) show that chloroplastic ribosomal RNA is synthesized only over a limited period when the leaves are young and that it undergoes a much lower rate of turnover than cytoplasmic ribosomal RNA. Paranjothy and Wareing (1971) reported that fully matured leaves of radish showed some synthesis of cytoplasmic ribosomal RNA but none of chloroplast ribosomal RNA. Kinetin stimulated the former activity in discs from these leaves, but its effect in retarding chlorophyll loss was still present when the synthesis was inhibited by 5-fluorouracil.

In intact leaves, BA markedly increased the ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA in throughout BA-treated leaves, or even in late BA-treated leaves to some extent. When BA treatment was stopped at the early stage the effect of BA in elevating the cytoplasmic-chloroplastic ribosomal RNA ratio diminished. Cytoplasmic-chloroplastic ribosomal RNA ratios of about 2 were reported for mature leaves of the same material (Treharne et al. 1970). In the present experiments, BA treatment may prevent the decrease in the ratio by preventing the decrease in cytoplasmic ribosomal RNA in senescing leaves. Callow and Woolhouse (1973) showed that before the rapid chlorophyll formation in re-greening leaves of decapitated Perilla plants, cytoplasmic ribosomal RNA started to increase earlier than chloroplastic ribosomal RNA.

As for ribosomal structure, Mittelheuser and Van Steveninck (1971) showed that chloroplast ribosomes disappeared in the dark in detached wheat leaves at 11 days, while cytoplasmic ribosomes did not, and that chloroplast ribosomes were maintained in leaves incubated with kinetin. In contrast, the loss of chloroplast ribosomes did not occur until last stage III in the senescence of intact bean leaves.

I have shown the followings with intact leaves: (1) Rate of RNA synthesis and RNA content decreased with age. BA retarded the decreases or even incresaed these values. (2) The ratio of cytoplasmic ribosomal RNA to chloroplastic ribosomal RNA was maintained at a higher level in BA treated leaves than in untreated controls. (3) BA raised the amount of cytoplasmic polysomes which decreased at late stages in untreated leaves. Choe and Thimann (1975) have reported that the presence of cytoplasm accelerated senescence of chloroplasts. Considering this fact, the results of the present study are in favor of the idea that cytokinins may affect chloroplast metabolism through their action on the nucleus and cytoplasm.

References

Atkin, R. K., Srivastava, B. I. S. (1969) Physiol. Plant. **22**, 742.
Beevers, L. (1976) In: Beevers, L. (ed.): Nitrogen Metabolism in Plants. Pp. 276. Edward Arnold, London.
Callow, M. E., Woolhouse, H. W. (1973) J. Exp. Bot. **24**, 285.
Choe, H. T., Thimann, K. V. (1975) Plant Physiol. **55**, 828.
Dove, L. D. (1973) Phytochemistry **12**, 2561.
Dayer, T. A., Osborne, D. J. (1971) J. Exp. Bot. **22**, 552.
Fletcher, R. A. (1969) Planta **89**, 1.
Ingle, J., Possingham, J. V., Wells, R., Leaver, C. S., Loening, U. E. (1970) Symp. Soc. Exp. Biol. **24**, 303.
Kende, H. (1971) Int. Rev. Cytol. **31**, 301.
Kirby, K. S. (1956) Biochem. J. **64**, 405.
Kuraishi, S. (1968) Physiol. Plant. **21**, 78.
Loening, U. E. (1967) Biochem. J. **102**, 251.
Martin, C., Thimann, K. V. (1972) Plant Physiol. **49**, 64.
Mittelheuser, C. J., Van Steveninck, R. F. M. (1971) Protoplasma **73**, 239.
Muramatsu, M., Fujisawa, T. (1968) Biochem. Biophy. Acta **157**, 476.
Naito, K., Ebato, T., Endo, Y., Shimizu, S. (1980) Z. Pflanzenphysiol. **96**, 95.
————, Iida, A., Suzuki, H., Tsuji, H. (1979a) Physiol. Plant. **46**, 50.
————, Tsuji, H., Hatakeyama, I., (1978) Physiol. Plant. **43**, 367.
————, ————, ————, Ueda, K. (1979b) J. Exp. Bot. **30**, 1145.
Osborne, D. J. (1962) Plant Physiol. **37**, 595.
Paranjothy, K., Wareing, P. F. (1971) Planta **99**, 112.
Phillips, D. R., Horton, R. F., Fletcher, R. A. (1969) **22**, 1050.
Pitt, D., Coombes, C. (1969) J. Gen. Microbiol. **56**, 321.
Reynolds, E. S. (1963) J. Cell Biol. **17**, 208.
Srivastava, B. I. S., Ware, G. (1965) Plant Physiol. **40**, 62.
Schneider, W. C. (1946) J. Biol. Chem. **164**, 747.
Shibaoka, H., Thimann, K. V. (1970) Plant Physiol. **46**, 212.
Smillie, R. M., Krotkov, G. (1961) Can. J. Bot. **39**, 891.
Solmosy, F., Fedorcsak, I., Gulyas, A., Farkas, G. L., Ehrenberg, L. (1968) Eur. J. Biochem. **5**, 520.
Spencer, P. W., Titus, J. S. (1972) Plant Physiol. **49**, 746.
Takegami, T. (1975) Plant & Cell Physiol. **16**, 417.
Tavares, J., Kende, H. (1970) Phytochemistry **9**, 1763.
Thimann, K. V. (1978) Bot. Mag. Tokyo Special Issue **1**, 19.
Treharne, K. J., Steddart, J. L., Pughe, J., Paranjothy, K., Wareing, P. F. (1970) Nature **228**, 129.
Wollgiehn, R. (1965) Flora **156**, 291.
———— (1967) Symp. Soc. Exp. Biol. **21**, 231.