The role of cytokinins in the development and metabolism of barley leaves

I. The effect of cytokinins on leaf senescence

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Abstract

The loss of chlorophyll during dark-induced ageing of barley leaves was prevented to a similar extent by kinetin and the natural cytokinin, 6- Δ^2 -isopentenylaminopurine (2iP). A concentration of 400 μ M appeared to be optimal in both cases. In the presence of 2iP the structure of mesophyll cells in dark-incubated leaves was maintained unchanged. A distinct correlation has been found between the retardation by 2iP of the chlorophyll loss, the rate of 32 P-incorporation into nucleic acids and the maintaining of the RNase activity at a low level. The importance of the RNA synthesis and the rate of its degradation in the cytokinin action in barley leaf senescence will be subjected to future experiments.

INTRODUCTION

Detached leaves of various plant species in which cytokinins inhibit or even reverse ageing processes are model objects frequently used in studies on biological activity of these substances. A specially convenient material for experiments are leaves of grasses which can be readily grown in laboratory conditions from germinating caryopses. Among them, barley leaves were the object of several studies and relatively numerous facts are known concerning their response to the cytokinin treatment (Srivastava and Ware 1965, Kulaeva et al. 1967, Atkin and Srivastava 1968, 1969, 1970). In spite of that, some basic informations, regarding e.g. the dose-response and the structural aspects of the leaf ageing and cytokinin treatment are still missing. Therefore, before starting a series of experiments on the effect of cytokinins on the metabolism and develop-

ment of barley leaves, it seemed necessary to carry out a detailed preliminary investigation on the effects of a broad range of concentrations of these substances and of various times of treatment on changes in the chlorophyll level, leaf ultrastructure, incorporation of precursors into nucleic acids and ribonuclease activity.

MATERIAL AND METHODS

Seedlings of barley (*Hordeum vulgare* L., cv. Damazy), after 2 days of germination in darkness, were grown for 8 days in continuous white fluorescent light of ca. 500 lux. First leaves of about 10 cm length were used for experiments. Detached leaves were placed with their petioles in water (control medium) or in cytokinin solution and kept in darkness for 1-5 days.

Two cytokinins, kinetin and 6- Δ^2 -isopentenylaminopurine (2iP) in concentrations of 0.1 — 1 600 μ M were tested for their effect on leaf senescence.

Chlorophyll estimation.

Chlorophyll was extracted by homogenizing 0.15 g of leaf samples in cold 80 per cent acetone. The extracts were clarified by centrifugation (3 min., 800 $\times$ g), and their absorption was determined at 645 and 663 nm. Concentrations of chlorophylls a and b were calculated according to the method described by Kirk (1968).

Incorporation of (32 P) orthophosphate into nucleic acids.

Five hours before the end of dark incubation, the immersed parts of petioles were cut off and the leaves placed in fresh incubation media to which (32 P) orthophosphate (20 μ Ci/ml) was added. The nucleic acids were prepared from 3 g of leaves by the phenol-detergent method according to Parish and Kirby (1966). Radioactivity of samples containing 100 μ g of nucleic acids was measured in toluene scintillator in USB-2 scintillation counter.

Estimation of RNase activity.

A modified method of Tuve and Anfinsen (1960) was used for ribonuclease determination. Enzyme was extracted with 0.1 M tris-HCl buffer, pH 7.5, containing 0.01 M NaCl (2 ml of buffer per g of leaf tissue). The reaction mixture containing 0.5 ml of the enzyme extract, 0.5 ml of a 1 per cent solution of yeast RNA in 0.1 M acetate buffer, pH 5.0, and

2 ml of 0.1 M acetate buffer, pH 5.0, was incubated at 37° C for 30 min. The reaction was stopped by adding 2 ml of MacFadyen's reagent. The mixture was kept for 30 min. at 5° C and centrifuged at $10\,000 \times g$ during 10 min. The supernatant was diluted in a ratio of 1 : 6 and the extinction was measured at 260 nm.

Electron microscopy.

The leaves were fixed in 5 per cent glutaraldehyde solution in 0.1 M cacodylate buffer, pH 7.0, at 4° C for 24 h and postfixed in 2 per cent OsO_4 in the same buffer at 4° C for 2 h. After washing in the buffer, the material was dehydrated by ethanol, acetone and propylene oxide, embedded in Epon 812 and cut with the LKB "Ultratom III" ultramicrotome. Sections for light microscopy were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate (Reynold 1963) and examined with the JEM 7A electron microscope.

RESULTS AND DISCUSION

Cross section of a leaf being the initial material for experiments is demonstrated in Fig. 1. It shows features characteristic for C_3 -grass leaves, with more or less uniform arrangement and structure of mesophyll cells. The chloroplasts were situated along the cell walls (Figs. 1 and 2), also the nuclei were localized in the thin layer of cytoplasm adjacent to the cell walls.

Considerable amounts of condensed chromatin, in which granular components could be distinguished, were present in the nuclei (Fig. 3). The chloroplasts were regularly fusiform, with only sporadically appearing peristromium projections (Fig. 4). They were uniform in structure, with

Figs. 1—2. Freshly harvested light grown leaves (initial material for experiments). 1. Fragment of a cross sectioned leaf. Epoxy section stained with toluidine blue. $200 \times$

2. Mesophyll cells with chloroplasts along the cell walls. Ultrathin epoxy section.
8 000 $\times$

Figs. 3—4. Freshly harvested light grown leaves (initial material for experiments).
Mesophyll cells

3. Cell nucleus with a conspicuous condensed chromatin. 22 000 $\times$

4. Chloroplast with a normally developed thylakoid system and with a peristromium projection 14000 $\times$

the system of thylakoids well developed. The grana usually consisted of 8 compartments (only occasionally they formed high stacks of over 25 compartments). On some sections, nucleoid-like areas of lower electron density and with DNA fibrils were visible within the stroma. The plastoglobules were few and small.

The level of chlorophyll in the detached leaves kept in the darkness decreased distinctly after few days (Fig. 5). For further analyses four

Fig. 5. Changes in the chlorophyll a and b levels during dark incubation of detached leaves in water. Chlorophyll extracted with 80% acetone and determined by the method of Kirk (1968).

days of dark incubation were selected, when ageing processes were considerably advanced. Changes in the mesophyll cell ultrastructure mainly concerned plastids. The number of plastids per cell distinctly decreased (cfr. Figs. 2 and 6), whereas the size of plastoglobules increased (Fig. 10).

The grana were almost completely replaced by a system of uniform long lamellae, running along the plastids (Fig. 10). They were slightly swollen and their content was electron dense. The process of chloroplast degeneration occurred according to the "type B" of chloroplast ageing (Brussel sprouts, kohlrabi) described by Młodzianowski (1974).

Kinetin and 2iP in 80-800 μM concs. inhibited the fall in the levels of the chlorophylls a and b (Figs 8 and 9). Optimal concentrations in both cases were 400 μM . The natural cytokinin 2iP was approximately as effective in retarding the chlorophyll loss as was kinetin. This result is different from that obtained by Varga and Bruinsma (1973) who found that senescence was delayed in oat leaves by kinetin and benzyladenine, but not by the natural cytokinins, zeatin and 2iP. One can assume that there are differences between the ageing of the oat and barley leaves with regard to the rate of breakdown of the natural cytokinins inside the leaf cells. At 1600 μM conc., 2iP intensified the disintegration of chlorophylls, whereas kinetin still kept the chlorophylls on the control level. Since at the most favourable concentrations, i.e. 400 μM , no differences were observed between the effects of kinetin and 2iP, for further experiments the natural cytokinin was used.

In leaves treated with the cytokinin, the mesophyll cells showed a well preserved structure (Fig. 7) resembling the initial material. Only the plastoglobules in the chloroplasts of the 2iP treated material showed a tendency to concentrate within some regions of the stroma (Fig. 11). Compared with the initial material, the grana were similar or even larger (the compartment number per granum was up to 23). This is in agreement with many studies on leaf ageing in which especially the grana have been reported to be stabilized in the presence of a cytokinin, as compared with their tendency to disintegrate in the control material (e.g. Dennis et al. 1967, Młodzianowski and Kwintkiewicz 1973, Młodzianowski and Ponitka 1973). The cytokinins were also found to retard the loss of chloroplast ribosomes (Mittelheuser and Van Steveninck 1971, Młodzianowski and Kwintkiewicz 1973). It is believed that cytokinin may be a factor responsible for the synthesis or inhibition of degradation of protein particles which according to Goodenough and Staehelin (1971) are in turn responsible for the linking of the thylakoid membranes into grana.

The effect of the cytokinin on the incorporation of precursor into nucleic acids and on the activity of RNase was examined using three 2iP concentrations, 100, 200 and 400 μM . The results are presented in Fig. 12. The highest incorporation of the ^{32}P -orthophosphate in the total nucleic

Figs. 6—7. Detached leaves after 4 days of dark incubation

6. Water control. Mesophyll cells with a decreased chloroplast number. 5 000 $\times$

7. 2iP, 400 uM. Mesophyll cells rich in chloroplasts. 4 000 $\times$

Fig. 8. The effect of kinetin on the chlorophyll a and b levels in detached leaves after 4 days of dark incubation. Chlorophyll extracted with 80% acetone and determined by the method of Kirk (1968)

Fig. 9. The effect of 2iP on the chlorophyll a and b levels in detached leaves after 4 days of dark incubation. Chlorophyll extracted with 80% acetone and determined by the method of Kirk (1968)

acids was obtained with the concentration of 400 μM : it was three times as high as in the water control. On the contrary, the activity of the RNase was lowest in leaves incubated in this 2iP concentration. The results thus showed a distinct correlation between the retardation by 2iP of the chlorophyll loss during the dark-induced ageing of barley leaves, the rate of precursor incorporation into the nucleic acids and maintaining the RNase activity at a low level. Some authors connect the maintaining by cytokinins of a high level of RNA and the delaying of leaf ageing with

Figs. 10—11. Detached leaves after 4 days of dark incubation mesophyll cells
10. Water control. Chloroplast with a uniform system of elongated, electron dense
thylakoids and developing large plastoglobules. A — artefact. 34 000 $\times$

11. 2iP, 400 uM. Chloroplast with a normal differentiation of the thylakoid system,
with large, well developed grana and with the plastoglobules showing a tendency to
concentrate in one region of the stroma. 16 000 $\times$

their stimulation of RNA synthesis (e.g. Kulaeva 1967, Fletcher 1969, Mothes 1972, Zwar 1973), whereas other think that this effect results only from prevention of the RNA degradation by keeping the

Fig. 12. The effect of 2iP on the incorporation of ³²P-orthophosphate into nucleic acids and on the RNase activity in detached leaves after 4 days of dark incubation. The labelled orthophosphate was added 5 h before the end of dark incubation. Nucleic acids were prepared by the phenol-detergent method. RNase was estimated by a modified Tuve and Anfinsen methods (1960)

RNase activity relatively low (cfr. Paranjothy and Wareing 1971). Further experiments are in progress to explain this question in the case of senescing barley leaves.

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Rola cytokinin w rozwoju i metabolizmie liści jęczmienia. I. Wpływ cytokinin na starzenie się liści

Streszczenie

Kinetyna i cytokinina naturalna, 6- Δ^2 -izopentenylaminopuryna (2iP), w podobnym stopniu zapobiegały utracie chlorofilu przez liście jęczmienia podczas ich starzenia się w ciemności. Stężeniem optymalnym obu substancji było 400 μM . Struktura komórek mezofilu pozostawała w ciemności niezmieniona w liściach poddanych działaniu 2iP. Stwierdzono korelację między hamowaniem przez 2iP rozkładu chlorofilu, włączaniem ^{32}P do kwasów nukleinowych oraz utrzymywaniem aktywności RNazy na niskim poziomie. Udział syntezy i szybkości rozpadu RNA w działaniu cytokinin na starzenie się liści jęczmienia będzie przedmiotem dalszych badań.