Chloroplast degeneration and its inhibition by kinetin in detached leaves of *Cichorium intybus* L.

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Abstract

In the chicory (*Cichorium intybus* L. var. *sativum* cv. Polanowicka) leaves two types of chloroplasts are present differing by their degree of osmiophilicity of the thylakoid inside. This type of differentiation of chloroplasts has so far been found only in several plant species.

The process of chloroplast degeneration in darkness is described. In osmiophilic chloroplasts at certain stage of degeneration minutely layered giant grana were found. Kinetin markedly inhibited the process of chloroplast degeneration, and after prolonged treatment even stimulated the stacking process of grana thylakoids.

INTRODUCTION

Detached leaves of some plants when kept in darkness gradually turn yellow (M o t h e s 1960, S h a w and M a n o c h a 1965). This process is an external symptom of ageing to which correspond definite changes in the fine structure of mesophyll cells (B u t l e r and S i m o n 1968). The protoplast organelles most sensitive to lack of light and at the same time most resistant are chloroplasts (D o d g e 1970, D e V e c c h i 1971). Ultrastructural changes in the chloroplasts of senescing leaves have been described for a number of species. These changes may differ slightly in their sequence and in the "senescence model" (S h a w and M a n o c h a 1965, R o u x and M c H a l e 1968, D o d g e 1970, L j u b e š i ć 1968, D e n n i s et al. 1967, M i t t e l h e u s e r and V a n S t e v e n i n c k 1971).

As shown by preliminary studies of the chicory leaves — most of their chloroplasts exhibited a rarely observed structure characterized by an osmiophilic inside of the thylakoids such as has been described

for *Phleum* chloroplasts by Ledbetter and Porter (1970) in their atlas with the annotation "anomalous image". On account of the different chloroplast structure it seemed interesting to perform more precise fine structure investigations, both of their degeneration and its inhibition by kinetin, which is a known agent inhibiting the process of leaf yellowing (Richmond and Lang 1957, Mothes 1960, Shaw and Manocha 1965, Sveshnikova et al. 1971, Młodzianowski and Ponitka 1973).

MATERIAL AND METHODS

The leaves of *Cichorium intybus* L. var. *sativum* cv. Polanowicka were used in the experiments. *Cichorium* is a biennial plant, and the leaves were taken from the flower stalks, thus from plants in the second year of vegetation. The material was collected directly from a field culture in late autumn. The petioles of cut off leaves were placed together with about 1/3 of the leaf blade length into a kinetin solution of 20 mg/l concentration. The same material placed in water served as control. The leaves were kept in a semidark place (light intensity 2 lux) at 24°C for 12 days.

For investigation of the structure, small section of leaf blades were taken from the outset material as well as after 6 and 12 days of the

Fig. 1. Detached leaves of *Cichorium intybus* immersed to 1/3 of blade in water (left) and in kinetin solution (right).

The part of the leaf immersed in kinetin was dark green (in figure dark zone). The dark colour at the edge of the control leaf is its browning and drying up part

Fig. 2. Detached leaves of *Cichorium intybus* after 12 days of the experiment.

The leaf on the left (control) has preserved its green colour in the apical part and the immersed part has degenerated. The leaf on the right (kinetin) died back in its apical part and remained green in the kinetin solution

Fig. 3. Chloroplast of the outset material with a spindle-shaped profile. Grana (G) visible, regularly arranged stroma thylakoids (arrow) and plastoglobuli (Pl.) $\times 18\,500$

Fig. 4. Fragment of mesophyll cell with chloroplasts arranged along walls in outset material. CW — cell wall. $\times 10\,000$

Fig. 5. Chloroplast fragment from outset material with thylakoids showing an electron-transparent inside.

M — mitochondrion, Px — peroxisome, R — cytoplasmic ribosomes $\times 23\,000$

Fig. 6. Chloroplast fragment from outset material with electron-transparent thylakoid inside (arrows).

Pl — plastoglobuli, R — cytoplasmic ribosomes, r — chloroplastic ribosomes, Tp — tonoplast, S — starch. $\times 35\,000$

Fig. 7. Chloroplast fragment from outset material with electron-opaque (osmiophilic) thylakoid inside (arrow).

Notations as in fig. 6. $\times 32\,000$

Fig. 8. Disappearance of grana (G) and formation of long thylakoids (Th) in chloroplasts, with electron-transparent inside in yellow zone of leaf from control material (after 6 days of the experiment).

Pl. — plastoglobuli. $\times 20\,000$

Fig. 9. Chloroplast with electron-transparent inside of thylakoids, from control material. There are exclusively long thylakoids (Th) and plastoglobuli (Pl) in the yellow leaf (after 12 days of the experiment). $\times 18\,000$

Fig. 10. Cell fragment with nucleus visible (N) and chloroplasts in the cytoplasm layer along the wall in the yellow zone of a leaf from the control material after 6 days of the experiment.

CW — cell wall. $\times 12\,000$

Fig. 11. Chloroplast with thylakoids showing osmiophilic inside and osmiophilic stroma.

Giant grana (GG) and plastoglobuli (Pl) not differing in osmiophilicity from stroma. Control material after 6 days of experiment. $\times 28\,000$

Fig. 12. Chloroplast with densely packed thylakoids.

N — nucleus, M — mitochondrion, r — plastid ribosomes Control material as in Fig. 1. $\times 50\,000$

experiment. The leaf samples were fixed in 5 per cent glutaraldehyde in 0.1 M phosphate buffer pH 7 for 12 h at 4°C. After washing out the fixative the material was treated with 2 per cent OsO₄ in the same buffer for 2 h at room temperature. The fixed material was dehydrated in an ethanol and propylene oxide series and embedded in Epon 812. Sections were cut on a Tesla and LKB ultramicrotome. The sections were counterstained with uranyl acetate and lead citrate after Reynolds (1963). For observation and electronograms a JEM 7A electron microscope was used.

RESULTS

Changes in leaf colour

Leaves treated with kinetin preserved longer their green colour as compared with the controls. The deep green regions lay most frequently in zones of various width along the veins and in the part of the leaf blade immersed in the kinetin solution. The leaf on the right in Fig. 1 has a well delineated green zone (the dark part on the photo) corresponding to the level of kinetin solution in which the leaf was immersed. The control leaves usually turned yellow from the blade edges. Some of them preserved the green colour in the apical part for as long as 12 days, whereas the part immersed in water degenerated. In the kinetin solution, on the contrary, the apical part of the leaf died back (it was dry and brown) and the lower part soaked in the growth regulator solution remained green (Fig. 2).

Fine structure of the outset material

In the outset material, beside chloroplasts with thylakoids with a light inside (Figs 5 and 6), chloroplasts could be seen in which the thylakoid content was osmiophilic (dark) (Fig. 7, cf. also Figs 19 and 20). Most cells contained only chloroplasts in which the inside of the thylakoids was osmiophilic. The chloroplast profiles were spindle-shaped independently of the degree of osmiophilia of the thylakoids (Figs 3 and 4). The grana were well developed, however, their height did not as a rule exceed one half of the plastid diameter. The stroma thylakoids showed regular features (Figs 3 and 5), or they were slightly swollen (Fig. 6). In the matrix, beside single starch grains (Figs 6 and 7) plastoglobuli were present with a diameters of about 150 nm (Figs 6 and 7). Plastid and cytoplasmic ribosomes were also visible (Figs 5 and 6). Chloroplasts occurred in a narrow cytoplasm layer along the cell wall (Fig. 4), frequently in close contact with the mitochondria and peroxysomes (Fig. 5).

Chloroplast degeneration

Various stages of chloroplast degeneration were observed in the yellowing and yellow zone of leaves in the control material and that treated with kinetin, at the points from which probably metabolites had been transferred to the centres activated by kinetin.

In the chloroplasts of the control material in which the thylakoid inside was osmiophilic, a gradual reduction of grana was observed (Fig. 8) until there remained elongated single thylakoids exclusively, running parallel to the long axis of the plastids (Fig. 9). The chloroplast profiles were more oval and smaller. Starch grains were not observed. Plastoglobuli were not more numerous than in green leaves, their diameters, however, were larger, although their maximal size was only 350 nm.

In the initial phase of degeneration, in the chloroplasts with an osmiophilic inside of the thylakoids, there could be seen, against the background of osmiophilic stroma, thylakoids with a still higher osmiophilicity. Some of them formed densely packed, minutely layered stacks others ran singly along the entire plastid (Figs 10 — 12). In this stage of degeneration plastoglobuli exhibited the same degree of osmiophilicity as the stroma (Figs 11 and 13). In some mesophyll cells well preserved nuclei, mitochondria and ribosomes could still be seen (Figs 10 and 12). In the yellow zone of leaves treated with kinetin (Fig. 1), degenerating forms of plastids similar to those noted in the control material were seen (Fig. 13). Beside the plastids, tonoplast, plasmalemma and a very thin cytoplasm layer with mitochondria and some few ribosomes were visible.

In the final phase of degeneration the number of thylakoids decreased, while plastoglobuli became more numerous (Figs 14 and 15) and more osmiophilic than in the preceding phase, whereas the osmiophilicity of

Fig. 13. Chloroplasts arranged along wall cell in very thin cytoplasm layer from yellow zone of leaf in kinetin-treated material (see Fig. 2).

M — mitochondrion, Pl — plastoglobuli, P — plasmalemma, Tp — tonoplast, CW — cell wall.
× 15 000

Fig. 14. Fragment of chloroplast with well visible osmiophilic thylakoid inside. More advanced stage of degeneration than in fig. 13. Control material after 12 days of experiment. × 50 000

Fig. 15. Only free thylakoids (Th) and plastoglobuli (Pl) in most plastids of yellow leaves. Control material after 12 days of experiment. × 20 000

Fig. 16. Last stage of plastid degeneration — bursting of envelope (E) and falling out of plastoglobuli (Pl) and thylakoid remains (Th) into cell lumen in control material after 12 days of experiment. CW — cell wall. × 28 000

Fig. 17. Chloroplast from kinetin-treated material after 6 days of experiment. Electron transparent thylakoid inside of very high grana is visible. $\times 20\,000$

Fig. 18. Chloroplast with dark thylakoid inside. Kinetin-treated material after 12 days of experiment.

M — mitochondrion, Pl — plastoglobuli, G — grana, S — starch, Th — stroma thylakoids, R — cytoplasmic ribosomes. $\times 25\,000$

Fig. 19. Well preserved thylakoid structures with electron-transparent inside. Kinetin-treated material after 12 days of experiment. Wide partitions — the site of thylakoid junction are visible. $\times 66\,000$

Fig. 20. High granum with osmiophilic thylakoid content in leaf treated for 12 days with kinetin. $\times 44\,000$

the stroma diminished (Fig. 15). Finally the envelopes burst and the plastid content in the form of plastoglobuli and remains of thylakoids fell into the cell lumen (Fig. 16).

Effect of kinetin

Under kinetin treatment, in both above described types of chloroplasts a well preserved internal structure was observed, resembling the outset material. On some sections, as late as 12 days from the beginning of the experiment, starch grains could be seen (Fig. 18). As compared with the outset material, the grana were even larger, their height occupied frequently the whole chloroplast diameter (Fig. 17 and 18). The grana and stroma thylakoids exhibited a regular and normal structure (Figs 18 and 19). Particularly noteworthy are the wide partitions — the sites of junction of the thylakoids in the grana (Fig. 19), in which according to Weier and Benson (1966) most of the chlorophyll is localized. In chloroplasts of the kinetin-treated material plastoglobuli were much less numerous, and on many sections they could not be detected at all (Fig. 17). In the plastid matrix ribosomes could be distinguished (Figs 19 and 20). The cytoplasm and mitochondria were well preserved after 6 as well as 12 days of the experiment (Figs 17 and 18).

DISCUSSION

The type of plastids with osmiophilic content in the thylakoids, described in this paper is rare, hence Ledbetter and Porter (1970) report it as abnormal, and Arnolt and Smith (1960) refer to this type of plastids in sunflower as to the effect of viral infection. A similar chloroplast structure is, however, described in *Ocimum basilicum* in control plants which during the experiments were treated with viruses and 3-amino-1,2,4-triazole (Favali and Conti 1970). Ljubešić (1970) observed the osmiophilicity of the stroma of some mesophyll plastids of *Rubus fruticosus*, whereas only young plastids exhibited an osmiophilic inside of the thylakoids in the same material. In the opinion of this author the darkening (osmiophilicity) of certain plastid areas is connected with the method of fixation (glutaraldehyde, OsO_4). This does not, however, rule out chemical differences in the plastids which under the influence of the same fixative give different pictures. It is possible that in the investigated chicory cultivar the different forms of plastids were due to mutation. Similar differentiation of plastids and their changes were, namely, observed in chlorophyll mutants of *Weigela florida* (Po-

szwińska — in press). In the chicory cultivar here studied no differences occurred in the colour of the leaves as they did in *Weigela*. The process of degeneration of chloroplasts with a light inside of their thylakoids occurred similarly as in Brussel sprouts (Dennis *et al.* 1967) and kohlrabi (Młodzianowski and Kwintkiewicz 1973). Chloroplasts with an osmophilic inside of the thylakoids degenerated similarly, however, an additional stage of densely packed and elongated giant grana seems to occur here. Grana of this type were observed in the chloroplasts of many plants treated with antibiotics (Döbel 1963, Margulies 1966).

The phenomenon of intensive greening of the leaf part in contact with the kinetin solution observed in the present study is known from earlier observations on tobacco leaves (Möthes 1960). It is considered that these sites represent attraction or "accumulation" centres drawing metabolites from the remaining part of the leaf (Möthes 1960).

Kinetin had an inhibitory effect on plastid degeneration in chicory leaves, independently of the type of their structure, and this agrees with the observations of other authors (Shaw and Manocha 1965, Dennis *et al.* 1970, and other papers mentioned in the introduction).

On the basis of the observations here described it would seem that the chloroplast structure in leaves deprived of light and treated with kinetin was even more perfect than that in the outset material, since the grana were higher, the stroma thylakoids had a more regular course, and the smaller number of plastoglobuli presumably indicated the utilization of the material contained in them for building the plastid thylakoids. This seems to point an enhanced synthesis of membraneous material as compared with the outset material. A similar phenomenon has been observed in chloroplasts of regreening tobacco leaves owing to the remove of stem above senescing leaves (Ljubešić 1967) and chloroplasts of senescing kohlrabi leaves treated with kinetin (Młodzianowski and Kwintkiewicz 1973).

Since the preservation of grana or their preservation and enlargement in the chloroplasts of chicory and kohlrabi leaves degenerating in darkness is the most spectacular effect of cytokinins, it may be concluded in the light of the latest biochemical and structural studies that kinetin is involved in the synthesis of some particles joining the thylakoids in stacks (Goodenough and Staehelin 1971), and with the photosystems which are probably localized in them (Park and Sane 1971, Kirk 1971, Arntzen *et al.* 1972). This would, however, require confirmation in direct investigations.

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*Degeneracja chloroplastów i jej hamowanie kinetyną w odciętych liściach
Cichorium intybus L.*

Streszczenie

U badanej odmiany hodowlanej cykorii (*Cichorium intybus* L.var.*sativum* c.v.Polanowicka) występowały dwa rodzaje chloroplastów różniących się stopniem osmoofilności wnętrza tylakoidów. Sekwencja zmian ultrastrukturalnych tych plastydów w czasie ich degeneracji przy niskiej intensywności światła przypomina w ogólnym zarysie zmiany występujące w czasie starzenia odciętych liści kapusty brukselki (Dennis i inni 1967) i kalarepy (Młodzianowski i Kwintkiewicz 1973). Jedynie w chloroplastach o osmoofilnej zawartości tylakoidów tworzyły się w pewnej fazie degeneracji gęsto uwarstwione grana olbrzymie.

Kinetyna wyraźnie hamowała proces degeneracji chloroplastów liści zebranych późną jesienią. Wydaje się nawet, że chloroplasty po 12 dniach doświadczenia miały wyższe grana i lepiej zachowane tylakoidy stromy niż chloroplasty liści wyjściowych. Rybosomy chloroplastowe w materiale kinetynowym były lepiej zachowane niż w kontrolnym, jednak porównanie wielkości ich populacji z materiałem wyjściowym na podstawie skrawków nie jest pewne.