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Synthesis of chloroplast proteins by barley leaf segments: effects of senescence induction and kinetin treatment

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SUMMARY

Synthesis of chloroplast proteins associated with induced leaf senescence in barley (*Hordeum vulgare* L., var. Hassan) was studied. Leaf segments were incubated for 18–27 h in the dark, floating on distilled water (senescence induction) or on kinetin (senescence retardation). Radioactivity was readily incorporated into plastid protein (followed after different incubation times of leaf segments with [^{14}C] labelled amino acids) and increased during the first 3 h of incubation. Radioactive plastid polypeptides were analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and fluorography. Several polypeptides (48, 38, 28, 23 and 20 kDa), were synthesized very actively when leaf segments were incubated in the dark, but synthesis was low or absent in greening leaves or when senescence was retarded by kinetin. The results confirm that synthesis of some senescence-specific chloroplast polypeptides is impaired by kinetin treatment.

Key-words: chloroplasts, *Hordeum vulgare*, kinetin, protein synthesis, senescence.

INTRODUCTION

During the first stages of leaf senescence, protein synthesis remains active in both the cytoplasmic and chloroplastic cell compartments. The synthesis of specific proteins encoded in the chloroplast and in the nuclear DNA is required for senescence (Yu & Kao 1981; Cuello *et al.* 1984; Thomas 1987). The synthesis of proteins by isolated chloroplasts increases early and transitorily during natural (Martín *et al.* 1986) and detachment-accelerated senescence (García *et al.* 1983). This fact has allowed us to identify several polypeptides synthesized specifically during leaf senescence by isolated chloroplasts (Guéra *et al.* 1989; Martín & Sabater 1989; Vera *et al.* 1990).

The question arises whether the proteins synthesized by isolated chloroplasts are the same as the proteins synthesized '*in vivo*' during leaf senescence. In a preliminary

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Abbreviations: chl = chlorophyll; K_{18} = leaf segments incubated 18 h in the dark in the presence of 50 μM kinetin; K_{27} = leaf segments incubated 27 h in the dark in the presence of 50 μM kinetin; Se_{18} = leaf segments incubated 18 h in the dark floating on distilled water; Se_{27} = leaf segments incubated 27 h in dark floating on distilled water; YL = greening leaves.

approach, we have established the assay conditions for radioactive protein labelling in senescent leaf segments of barley. The polypeptides labelled when senescence is induced by leaf detachment and incubation in the dark were analysed by SDS-PAGE and compared with those labelled when senescence is retarded by leaf treatment with kinetin.

MATERIALS AND METHODS

Culture conditions and leaf treatments

Barley seeds were sown on vermiculite, regularly watered with Cron medium (Bond 1951) and grown at 23°C for 14 days, under an 18 h photoperiod of low intensity of illumination, *c.* 15 W m^{-2} (12·7 W m^{-2} PAR), to prevent the accumulation of starch (Martín & Sabater 1989). Etiolated leaves were obtained from barley seeds sown on wet vermiculite during 6 days in darkness at 23°C. Greening leaves were prepared from etiolated leaves by incubation of detached leaves on water during 18 h under the same light conditions at 23°C.

For senescence induction treatment, 2 or 5 g of 20 mm segments from the central portion (discarding 20 mm base and 20 mm tip), of the oldest leaf of 14-day-old plants (about 120 mm in length, containing *c.* 1·42 mg chlorophyll g^{-1}) were extensively washed with sterile water and then incubated for 18 or 27 h at 23°C in the dark floating on 20 ml of sterile distilled water (Se_{18} and Se_{27} leaves, respectively) or 14 μM kinetin (K_{18} and K_{27} leaves) in sterile 100 ml conical flasks closed with cotton.

Labelling of leaf segments with radioactive amino acids

Unless otherwise stated, 3 h before completion of the 18 or 27 h total incubation time of leaf segments, 14–70 kBq of [^{14}C] amino acids mixture (about 5 GBq $nmol^{-1}$, Amersham) in 3 ml of distilled water were added. The incubation was terminated by extensive washing with distilled water at 0–5°C. Some experiments were carried out in the presence of chloramphenicol (which inhibits protein synthesis on 70S chloroplastic ribosomes) or cycloheximide (which inhibits protein synthesis on 80S cytoplasmic ribosomes). When employed, the protein synthesis inhibitors were added to the radioactive amino acids mixture. Values of radioactivity incorporated into proteins were derived from the average of 3–4 independent experiments which did not differ significantly ($\leqslant 10\%$).

Isolation of chloroplasts

Appropriate amounts of incubated leaf segments were homogenized for 10 s at 0–5°C in a Sorvall Omni-mixer with 5–8 volume of a freshly prepared buffer (E) containing 0·35 M sucrose, 25 mM Na-HEPES, 2 mM Na_2-EDTA and 2 mM Na-isoascorbate (pH 7·6). The homogenate was pressed through four layers of muslin and centrifuged for 5 min at 200 ***g***. The supernatant fluids were centrifuged for 10 min at 2000 ***g***. The pellet was resuspended in buffer E (one fifth of the volume formerly used) and pelleted as above. Routine analysis for bacterial contamination showed that under these conditions less than 1% of total particles present in chloroplast preparations are visualized after Gram staining. The chloroplast preparation was free of mitochondria ($< 5\%$) and cytoplasm ($< 1\%$) (Calle *et al.* 1986). At least 70% of the chloroplasts were intact as measured by the ferricyanide reduction assay or by ultracentrifugation in hypertonic sucrose gradient (Guéra *et al.* 1989). After the first centrifugation at 2000 ***g*** the supernatant fluids were also collected.

Proteins of chloroplast and supernatant fractions were precipitated with 6% (w/v) trichloroacetic acid. The radioactive precipitates were treated for radioactive counting by liquid scintillation or for SDS-PAGE as described previously (Martín & Sabater 1989).

SDS-PAGE and fluorography

Slab SDS-PAGE was performed with 10–20% (w/v) linear acrylamide gradients (2·5%, w/w, bisacrylamide) with the buffer system of O'Farrell (1975) as described previously (Martín & Sabater 1989). Radioactive gels were impregnated with Amplify (Amersham) dried and fluorographed with MAFE RP-X-1 film, exposed for 30–90 days at −80°C. Molecular weight markers were detected by Coomassie brilliant blue staining.

Other determinations

Chlorophyll was determined according to Arnon (1949). Proteins were determined according to Lowry *et al.* (1951) after precipitation with trichloroacetic acid.

RESULTS AND DISCUSSION

Incorporation of amino acids into proteins by leaf segments

Barley leaf segments incorporated externally supplied radioactive amino acids into proteins of chloroplast and supernatant fractions. We measured the incorporation of radioactivity into chloroplast protein and supernatant fraction protein after addition of radiolabelled amino acids. For senescence and kinetin treated leaves, the leaf segments were collected 6 hours after the termination of the dark phase of photoperiod on the 14th day after sowing. Thereafter, leaf segments were incubated for 18 or 27 h in the dark under conditions explained in Materials and Methods. At different times (1–6 h before termination of the total incubation time), 14 kBq of radiolabelled amino acids were added to the leaves. Typical results shown in Figure 1 indicated that chloroplasts incorporated more radioactivity than supernatant fractions on a protein basis (Bq mg protein^{-1}). The incorporation of radioactivity was dependent on the physiological state of the leaves. Greening and 27 h incubated leaf segments of 14-day-old plants incorporated more radioactivity into chloroplast and supernatant fraction protein than 14-day-old leaf segments incubated for 18 h, with or without kinetin. Radiolabelled amino acids were readily incorporated into trichloroacetic acid precipitable material during the first 3 h of incubation. For longer incubation time with radioactive amino acids, the incorporation of radioactivity increased slowly or diminished, probably because of the degradation of radioactive protein. Thus, the observed increase of radioactivity incorporated into chloroplast proteins of kinetin treated or greening leaves after 3 h of incubation in the presence of radiolabelled amino acids could be due to a lower level of protein breakdown in the kinetin-treated or greening leaves than in the senescent chloroplasts. However, the possibility that these differences were caused by differences in the uptake of the labelled amino acids into the leaf tissue for leaves treated under different physiological conditions cannot be ignored.

Proteins synthesized by leaf segments incubated for 18 h in the dark were probably required for subsequent leaf senescence (Cuello *et al.* 1984). Thus, most of the following experiments were carried out with 14-day-old barley leaf segments incubated during 18 h with or without kinetin in the dark (K_{18} and Se_{18} leaves, respectively). To minimize the

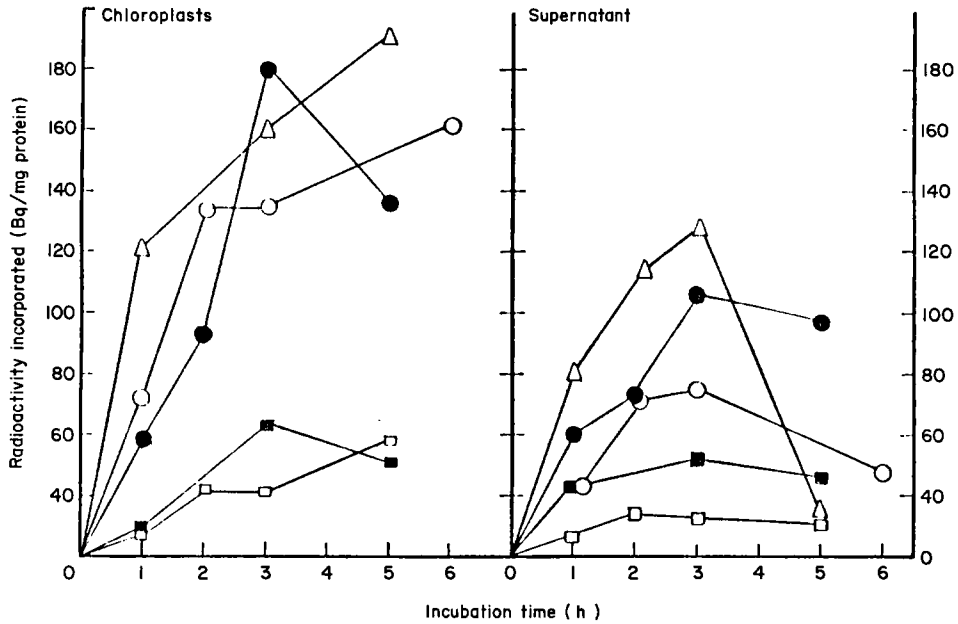

Fig. 1. Effect of the incubation time on the incorporation of [^{14}C] amino acids in chloroplast and supernatant proteins by leaf segments. Two g of greening (△), 18 h incubated senescent (■) or kinetin-treated (□) and 27 h incubated senescent (●) or kinetin-treated (○) leaf segments were supplied with 15 kBq of [^{14}C] amino acids at the indicated times before completion of the 18 or 27 h incubation. Radioactivity was measured in trichloroacetic insoluble materials of chloroplast and supernatant fractions.

Table 1. Effects of chloramphenicol and cycloheximide on the incorporation of radioactive amino acids into proteins by barley leaf segments. Chloroplasts and supernatant fractions were prepared after incubation of 2 g of greening or senescent (Se_{18}) leaf segments during 3 h with 15 kBq of [^{14}C] amino acids and, when indicated, chloramphenicol or cycloheximide. The percentage of radioactivity incorporated with inhibitors are indicated in parentheses

		Radioactivity incorporated (Bq × mg^{-1} protein)		
Leaves	Fraction	Control	+6 mol m^{-3} chloramphenicol	+0·6 mol m^{-3} cycloheximide
Greening	Chloroplast	178	39 (22)	171 (96)
	Supernatant	130	60 (43)	64 (49)
Senescent	Chloroplast	88	28 (32)	52 (59)
	Supernatant	55	47 (85)	25 (46)

effect of protein breakdown, only the last 3 h of total incubation time in the dark were carried out in the presence of radioactive amino acids.

Effects of chloramphenicol and cycloheximide on protein synthesis

The incorporation of radioactivity was impaired by inhibitors of protein synthesis, both in greening and in senescent (Se_{18}) leaves (Table 1). As many chloroplast proteins are

synthesized by 80S cytoplasmic ribosomes and as, until now, it was not possible to obtain cytoplasmic preparations free of broken chloroplast material (mainly stromal products), the lower but significant inhibition by chloramphenicol and cycloheximide of radioactivity incorporation in supernatant and chloroplast fractions respectively, are not surprising. It is well known (Cashmore 1976; Ellis 1982), that incorporation of radioactive amino acids into proteins of leaf tissue is strongly inhibited when leaves are treated with chloramphenicol. On the other hand, inhibition of radioactivity incorporation into supernatant fraction could also be explained by the presence, not only of stromal contamination, but also of mitochondria in this fraction. Moreover, chloramphenicol may indirectly inhibit protein synthesis on 80S ribosomes by affecting several ATP-yielding processes, and the synthesis of proteins in chloroplasts is affected by the synthesis of chloroplast proteins in cytoplasm (Ellis 1982). In any case, our results showed clearly that both, protein synthesis in chloroplasts and incorporation of cytoplasmically synthesized proteins into chloroplast remained very active during the first hours of senescence induction.

Synthesis of specific chloroplast polypeptides

The chloroplast proteins synthesized by leaves after different treatments were analysed by SDS-PAGE and fluorography. For these experiments total chloroplast material recovered from leaves was loaded on the electrophoresis gel. Differences in the recovery of chloroplast material from one preparation to another explain the differences of label present in each lane. Despite this, it is clearly shown in Figure 2 that when leaves were incubated for 3 h in the presence of 25 kBq of radioactive amino acids, Se_{27} leaves synthesized three major polypeptides of Mw 48, 38 and 30 kDa. Nevertheless, K_{27} leaves and greening leaves (YL) synthesized two main polypeptides of Mw 43 and 36 kDa, that clearly differed in their molecular weight from those synthesized by Se_{27} leaves. On the other hand, a polypeptide of about 30 kDa is synthesized by greening leaves but is not synthesized by K_{27} leaves.

To study the polypeptides synthesized by Se_{18} and K_{18} leaves, they were incubated in the presence of higher levels of radioactive amino acids (70 kBq). Three polypeptides of 48, 38 and 30 kDa were some of the main products synthesized by Se_{18} leaves (Fig. 2, Se_{18}, 0), but were not among the major polypeptides synthesized by K_{18} leaves (as described above for Se_{27} leaves). Several polypeptides synthesized by K_{18} leaves, as those of 43 and 66 kDa, were also synthesized by Se_{18} leaves. On the other hand, the 36 kDa protein synthesized actively by kinetin treated leaves was barely synthesized by the senescent ones. In addition some other polypeptides synthesized very actively by Se_{18} leaves (28, 20 kDa) were not detected in K_{18} leaves.

Therefore, longer incubation time increased the synthesis, but did not affect the general pattern of the chloroplast polypeptides detected in senescent leaves. Nevertheless, when the leaves were incubated in the presence of kinetin, the pattern of synthesized chloroplast polypeptides showed always significant differences when compared to that of senescent leaves.

After 3 h incubation with radioactive amino acids, Se_{18} leaf segments were washed and incubated 2 additional hours in the absence of amino acids to determine the stability of chloroplast polypeptides synthesized by leaf segments incubated in water. As is shown in Figure 2 (Se_{18}, 2), most of the polypeptides synthesized by senescence induced leaves were still present two hours after their synthesis. High and medium molecular weight polypeptides (including the 48 and 30 kDa polypeptides) were rather stable under the conditions

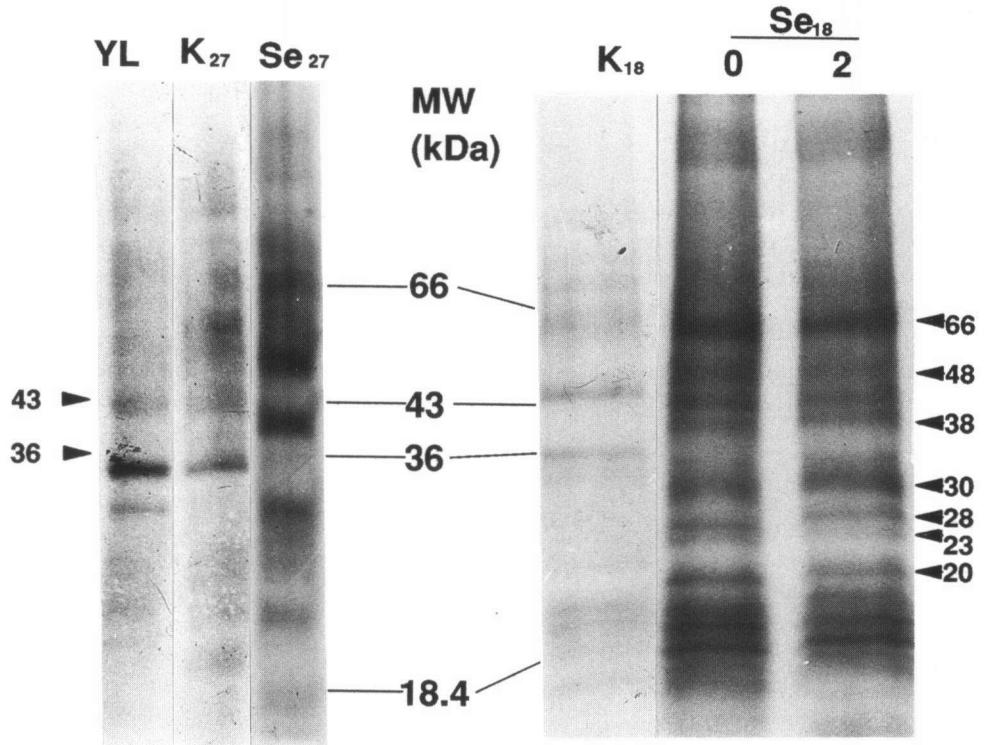

Fig. 2. SDS-PAGE and fluorography of labelled chloroplast polypeptides. Chloroplasts were isolated from 5 g leaf segments incubated under the conditions described in Materials and Methods. Total chloroplast isolated trichloroacetic acid precipitable material loaded per lane: YL, 840 Bq; K_{27}, 840 Bq; Se_{27}, 1500 Bq; K_{18}, *c.* 1200 Bq; Se_{18} (0,2), *c.* 2000 Bq. Marks (>) correspond to the main polypeptides cited in the text.

of this experiment. Apparently, low molecular weight polypeptides (28, 23 and 20 kDa) were subjected to a faster breakdown. The 48 kDa and the 30 kDa polypeptides were not accumulated after the two additional hours of incubation. These results suggest that these proteins might be '*de novo*' synthesized proteins rather than degradation products from higher molecular weight proteins.

Our results demonstrate that during the first stages of induced senescence, when the breakdown of chlorophylls and proteins is still not significant (Martín *et al.* 1986; Schellenberg *et al.* 1990), the synthesis of chloroplast proteins is very active and even is increasing when the treatment is extended from 18 to 27 hours (Fig. 1). Probably, several polypeptides synthesized '*de novo*' are responsible for hydrolytic activities which increase during senescence (Stoddart & Thomas 1982); however, it is difficult to know the function of each polypeptide identified in Figure 2. Results presented in this work are in accordance with previous results of our laboratory that showed for 'in-vitro' protein synthesis experiments with isolated chloroplasts (Guéra *et al.* 1989, Martín & Sabater 1989) that thylakoidal polypeptides of 66, 48, 43 and about 30 kDa, are the main products synthesized by senescent chloroplasts. Chloroplast polypeptides synthesized actively by senescent but not by kinetin-treated or greening leaf segments (48, 38, 28, 23 and 20 kDa) could play a specific role in senescence.

The results of protein synthesis with leaf segments described here confirm previous ones with isolated chloroplasts (García *et al.* 1983; Martín *et al.* 1989; Ohya & Suzuki 1990),

indicating that the retardation of senescence by kinetin is associated to changes in the synthesis of chloroplast proteins.

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