Light and growth substances effects on polypeptide pattern of senescent leaves of barley (*Hordeum vulgare* L.)

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Abstract. Chloroplast polypeptides of barley have been analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate after leaf segment treatments with different light and hormones factors affecting senescence. In some incubations, the inhibitors cycloheximide and chloramphenicol were also included to investigate the role of protein synthesis on polypeptide changes. As a control, the polypeptides of the supernatant fraction of the preparation of chloroplasts were also analyzed. Senescence induced in pure water, or stimulated by abscisic acid or ethylene is accompanied by the increase of a 62 KD supernatant polypeptide. Methyl jasmonate produces an increase of 66 KD supernatant polypeptide. Insignificant changes are produced in the chloroplast polypeptide pattern during senescence. Several other minor polypeptide changes are also detected in chloroplasts and supernatant in response to different treatments. Methyl jasmonate induces supernatant and chloroplast polypeptide changes which suggest that it stimulates leaf senescence through a mechanism differing from abscisic acid and ethylene.

Key words: Abscisic acid; Ethylene; Kinetin; Methyl jasmonate; Senescence; UV-light.

Introduction

During senescence most leaf nitrogen is recovered in other plant structures (Thimann, 1980; Stoddart and Thomas, 1982; Peoples and Dalling, 1988). Chloroplast proteins are the main source of exportable nitrogen (Peoples and Dalling, 1988; Thayer *et al.*, 1987). However senescence requires the synthesis of some protein in both cytoplasm (Stoddart and Thomas, 1982) and chloroplasts (Yu and Kao, 1981; Cuello *et al.*, 1984; Martin *et al.*, 1986).

Visible light, through phytochrome (Cuello *et al.*, 1987), and near UV light (Cuello *et al.*, 1989) retard and methyl-jasmonate (Me-JA) accelerates senescence

(Weidhase *et al.*, 1987; Mueller-Uri *et al.*, 1988).

Here, we have studied the effect of different light and hormone factors on the polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) pattern of the polypeptides of chloroplast isolated from senescent barley leaf segments. As a control, we have also analyzed the polypeptides of the supernatant from the preparation of chloroplasts.

Materials and Methods

Barley (*Hordeum vulgare* L. cv. Hassan) was grown for 14 days under an 18-h photoperiod on vermiculite with Cron medium as described by Cuello *et al.* (1987). Four g of 2 cm segments (discarding base and tip) from primary leaves were incubated during 72 h at 26°C in Petri dish with 100 ml of water or different effector solutions.

Light treatments of incubated leaf segments were applied as described previously (Cuello *et al.*, 1987;

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Cuello *et al.*, 1990).

Chloroplast fraction for SDS-PAGE was prepared by homogenizing four g of leaf segments in a Sorvall Omnimixer (10 s at intensity 9) with 25 ml of a 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 strained through two layers of muslin and centrifuged for five min at 200 xg. The pellet was discarded and the supernatant was centrifuged for 10 min at 2500 xg. The chloroplast pellet was washed twice with five ml buffer E and resuspended in one ml of 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM KCl, pH 7.3 plus 0.1 ml mercaptoethanol, 0.25 ml glycerol and 0.075 g SDS. The suspension was heated during 10 min at 70-80°C and stored at -20°C. As a control of chloroplast protein leaked during homogenization and centrifugation, proteins of the supernatant of the first chloroplast precipitation at 2500 xg were precipitated with 15 ml of 25% (w/v) trichloroacetic acid (TCA) (30 min) and centrifuged during 10 min at 10000 xg. The pellet was washed twice with 10 ml ethanol, resuspended in three ml of 60 mM Na_2CO_3, 60 mM mercaptoethanol, 2% (w/v) SDS, 12% (w/v) sucrose and heated as described by Piccioni *et al.* (1982). The suspension was centrifuged for 10 min at 10000 xg and stored at -20°C.

Slab SDS-PAGE of chloroplast and supernatant fractions was carried out with 100 μg protein samples in a LKB vertical unit with 10-20% linear acrylamide (2.5% bis-acrylamide) gel gradient (O'Farrel, 1975) at 18°C and 20 mA constant current. Polypeptides from samples and molecular weight markers were detected by Coomassie staining.

Protein was determined by the method of Lowry *et al.* (1951) after precipitation with 10% (w/v) TCA. Chlorophyll was measured by the method of Arnon (1949).

Results

Usually (Calle *et al.*, 1986) chloroplasts prepared as described in Materials and Methods are sufficiently free of mitochondrial (<5%) and cytoplasmic (<1%) protein. However supernatant fraction includes significant chloroplast contamination which is higher (Calle *et al.*, 1986) when measured by phosphoglycolate phosphatase marker than by chlorophyll marker. Hence, probably, a significant part of stroma fraction is lost during the preparation of chloroplasts (Calle *et al.*,

1986).

When chlorophyll recovered in chloroplast fraction is compared with chlorophyll content in starting leaves, either freshly detached or incubated, we obtain a 23.9 $\pm$ 0.26 % (mean $\pm$ SE of 23 independent extractions) recovery of chlorophyll with the method described here. Around 3.9 $\pm$ 0.05 % of the original leaf chlorophyll is recovered in supernatant fraction but, as we will show, the contamination of supernatant fraction with chloroplast stroma protein is much higher.

Figures 1 and 2 show SDS-PAGE patterns of polypeptides of, respectively, chloroplast and supernatant fractions isolated from leaf segments freshly prepared (lane 3) or after 72 h incubation in the dark in pure water or in the presence of different growth regulators and/or protein synthesis inhibitors. The last ones were applied at concentrations, 10 μM cycloheximide, 310 μM chloramphenicol, which have been described (Peterson and Huffaker, 1975) to inhibit strongly protein synthesis in, respectively, cytoplasm and chloroplasts in barley under similar conditions as those used here.

In respect to freshly detached leaves, leaf segments incubated for 72 h in the dark only produces minor changes in the relative amount of the different chloroplast polypeptides, despite the decrease in whole protein (Fig. 1, lanes 3 and 4). On the other hand, 62 KD polypeptide increases in supernatant fraction after 72 h incubation in the dark (Fig. 2, lanes 3 and 4). This increase is partially inhibited by kinetin (Fig. 2, lane 5) and stimulated by ethylene (Fig. 2, lane 7). The major supernatant polypeptides of 54 and 15 KD, very probably contaminated by chloroplast stroma rubisco, decrease after 72 h incubation in water. The decrease of rubisco polypeptides is more pronounced after Me -JA treatment (lane 8, Figs. 1 and 2). Me-JA treatment produces an increase of 66 KD supernatant polypeptide (Fig. 2, lane 8) (but not of 62 KD polypeptide, which is strongly induced by water, abscisic acid (ABA) or ethylene treatment).

After 72 h of cycloheximide treatment almost all supernatant polypeptides higher than 55 KD disappear (Fig. 2, lanes 1 and 4). Chloramphenicol treatment has almost no effect on the polypeptides of the supernatant fraction (Fig. 2, lanes 2 and 4). Me-JA is the growth regulator showing the highest effect on polypeptide patterns, particularly of the chloroplast fraction (Fig. 1, lanes 4 and 8). Me-JA decreases many chloroplast
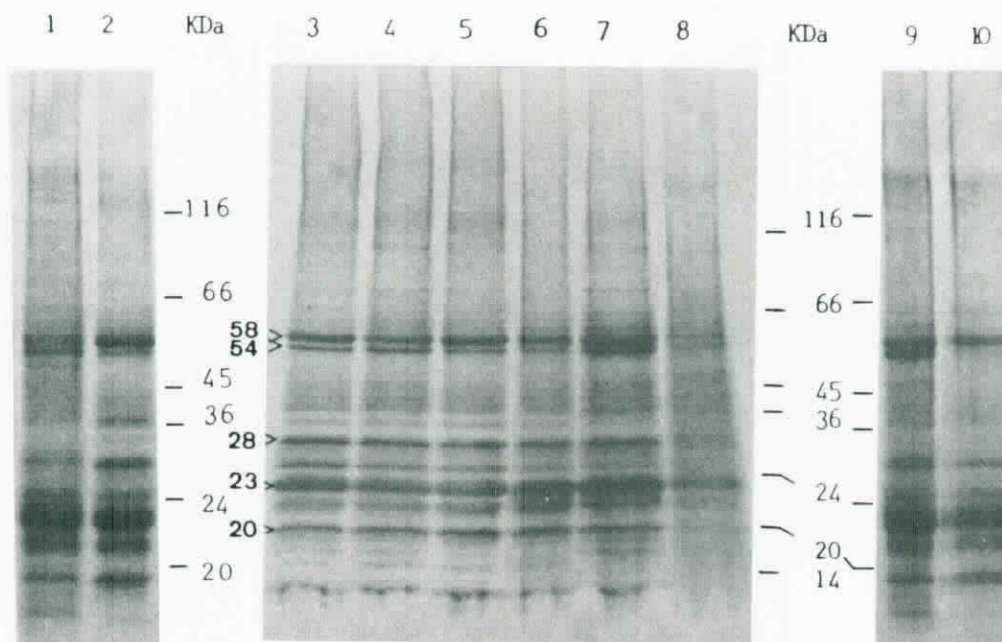
Fig. 1. SDS–PAGE of polypeptides of chloroplast fraction isolated from freshly detached leaves (3) and leaf segments incubated for 72 h in the dark in: 10 μM cycloheximide (1), 310 μM chloramphenicol (2), pure water (4), 14 μM kinetin (5), 34 μM ABA (6), 69 μM ethylene (applied as ethrel) (7), 45 μM Me–JA (8), 10 μM cycloheximide plus 45 μM Me–JA (9) or 310 μM chloramphenicol plus 45 μM Me–JA (10). Marks (>) indicate the polypeptides cited in text.
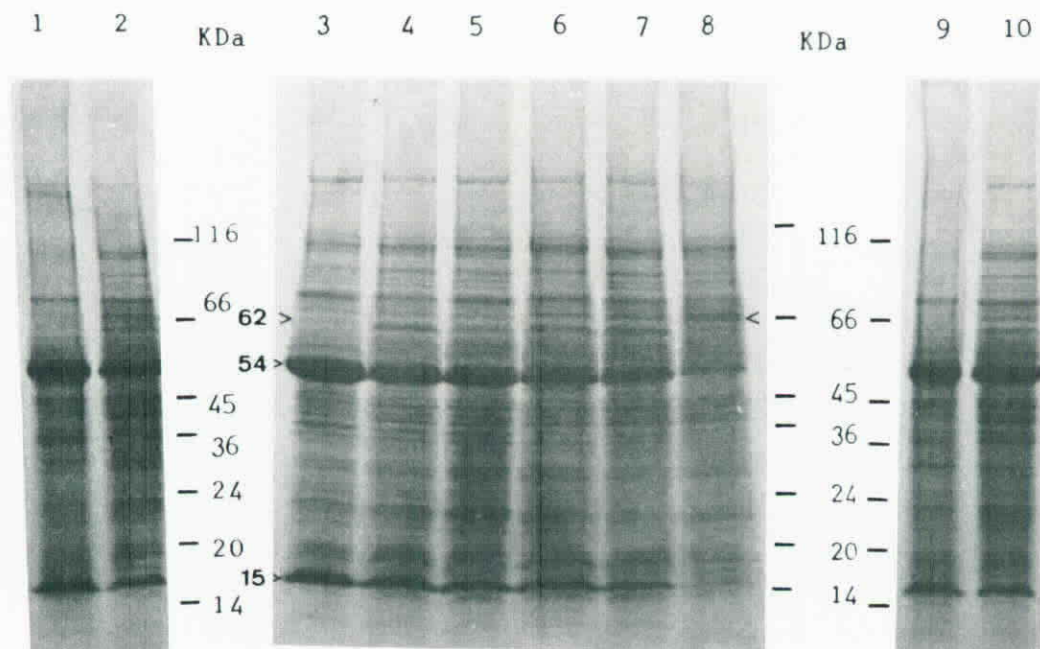

Fig. 2. SDS–PAGE of polypeptides of supernatant fraction isolated from freshly detached leaves (3) and leaf segments incubated for 72 h in the dark in: 10 μM cycloheximide (1), 310 μM chloramphenicol (2), pure water (4), 14 μM kinetin (5), 34 μM ABA (6), 69 μM ethylene (applied as ethrel) (7), 45 μM Me–JA (8), 10 μM cycloheximide plus 45 μM Me–JA (9) or 310 μM chloramphenicol plus 45 μM Me–JA (10). Marks (>) indicate the polypeptides cited in the text.
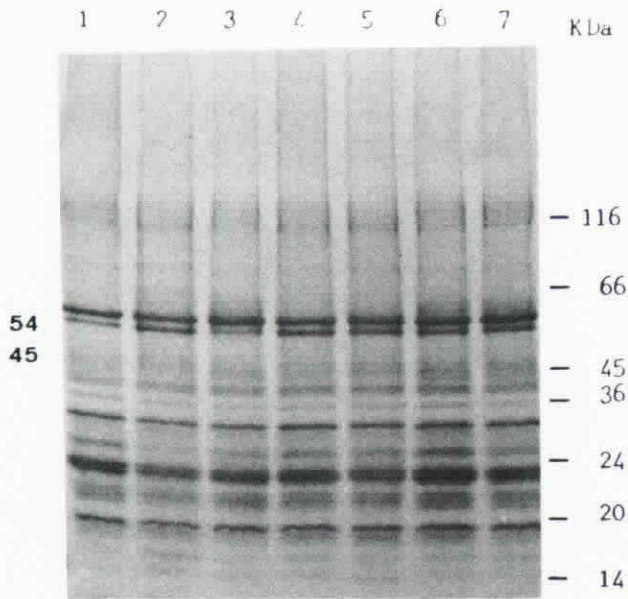
Fig. 3. SDS PAGE of polypeptides of chloroplast fraction iso-
lated from freshly detached leaves (1) and leaf segments
incubated for 72 h in pure water in: continuous dark (2),
dark interruption with brief red (4) and red followed far
-red (5) light, and photoperiodic UV (3), continuous red
(6) and continuous white (7) light. Marks (>) indicate
the polypeptides cited in the text.

Fig. 4. SDS PAGE of polypeptides of supernatant fraction iso-
lated from freshly detached leaves (1) and leaf segments
incubated for 72 h in pure water in: continuous dark (2),
dark interruption with brief red (4) and red followed far
-red (5) light, and photoperiodic UV (3), continuous red
(6) and continuous white (7) light. Marks (>) indicate
the polypeptides cited in the text.

polypeptides (20, 23, 28, 54, 58,... KD) and increases sev-
eral other. In the case of supernatant polypeptides, Me
-JA strongly reduces the levels of the 54 and 15 KD
polypeptides (lanes 4 and 8, Fig. 2). Simultaneous appli-
cation of cycloheximide inhibits most of the effects of
Me-JA on polypeptide changes (lanes 8 and 9, Figs. 1
and 2).

Light treatments affect senescence of leaf seg-
ments, however, they only produce minor changes in
chloroplast polypeptide pattern. Photoperiodic UV
treatment induces a disminution in chloroplast 54 and
45 KD polypeptides (Fig. 3, lanes 2 and 3). On the other
hand, concerning the leaf segments incubated in contin-
uous dark, only continuous white light decreases sev-
eral high molecular weight polypeptides of 62, 86, 90
and 92 KD in supernatant fraction (Fig. 4, lanes 2 and
7). Surprisingly, brief red light, which like continuous
white light retards leaf senescence, does not induce any
changes of polypeptide patterns in the supernatant and
chloroplast fractions. Moreover, continuous dark, and
brief red or red followed far-red light produce the
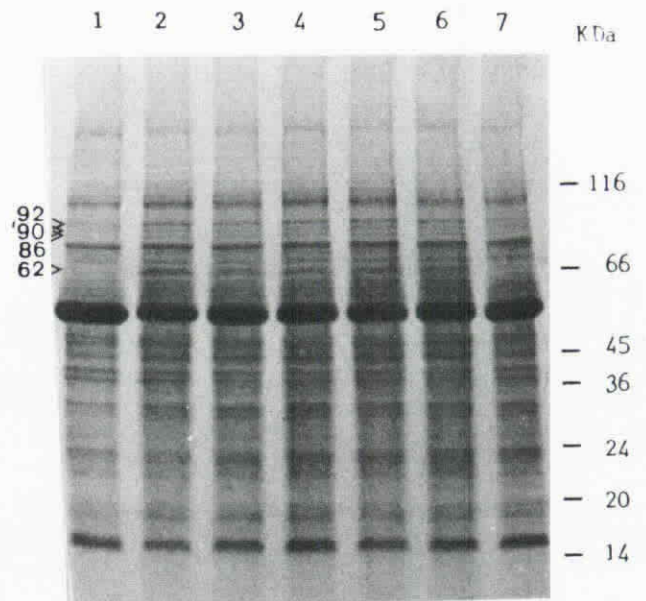same effect (Figs. 3 and 4, lanes 2, 4 and 5).

Discussion

The loss of chloroplast stroma protein during
chloroplast isolation is a common problem which, prob-
ably, is enhanced in senescent leaves (Stoddart and
Thomas, 1982). In our work, more rubisco was obser-
ved in supernatant fraction than in chloroplast frac-
tion. However, separate analysis of supernatant and
chloroplast fractions together protein synthesis in-
hibitor assays, provides sufficient indication on subcel-
lular location and synthesis of some polypeptides
whose levels change during senescence or in response
to different light or hormone treatments affecting
senescence.

Thus the probable extrachloroplast 62 KD polype-
ptide increases during senescence. Its increase is inhib-
ited by continuous white light, kinetin (both senescence
retarding factors) and cycloheximide but not by chlor-
amphenicol. However, ethylene, but not Me-JA (both
senescence accelerator regulators), stimulates the pro-
duction of this polypeptide. One possibility is that it
corresponds to one of the endoproteinases (Thayer *et*

al., 1987) which increases during senescence, but, obviously, this would not account for the degradations induced by Me-JA. Though Bricker and Newman (1980) have described the increase of a 62.6 KD polypeptide during senescence of soybean cotyledons, however, it occurs in thylakoids.

Brief red light does not show any effect on the 62 KD polypeptide when it is induced in the dark; but, given that phytochrome (in the far-red form) does retard senescence more in young than in old leaves (Cuello *et al.*, 1989; Cuello *et al.*, 1990), this fact suggests that phytochrome may be more important in the stimulation of the biogenetic processes than in the retardation of the senescence degradative processes (Cuello *et al.*, 1989). Continuous red light does not affect the polypeptide patterns, however, in our experiments its irradiance was smaller than that of continuous white light (3.2 as opposed to 13.5 W m^{-2}).

Kawasaki and Takeuchi (1989) have described the increase of 38 KD thylakoid polypeptide induced by senescence in spinach leaves. However, we found, clearly, a similar chloroplast polypeptide (of 39 KD) only during senescence of barley leaves attached to plants (unpublished results).

The strong effect of Me-JA on chloroplast and supernatant polypeptides seems to be dependent on protein synthesis in both cytoplasm and chloroplasts, as it is inhibited by both cycloheximide and chloramphenicol. Particularly, the 66 KD polypeptide induced by Me-JA seems to be synthesized in cytoplasm as described by Weidhase *et al.* (1987) and Mueller-Uri *et al.* (1988) who analyzed leaf crude extracts from barley grown for 7 days in continuous light. Our results indicate that the 66 KD polypeptide is extrachloroplastic.

On the other hand, the different effects of ABA and Me-JA on chloroplast and supernatant polypeptide patterns suggest different action mechanisms for both senescence accelerators. Cuello *et al.* (1990) have described that the senescence stimulation of Me-JA is stronger in young than in old leaves whereas ABA promotes senescence more in old than in young leaves. One possibility is that Me-JA acts as stress instead of hormonal factor (Parthier, 1990).

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光照和生長素對大麥(*Hordeum vulgare* L.) 老化葉片內多肽類型的影響

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利用不同光照和影響老化的激素處理大麥葉部，然後以膠體電泳分析其葉綠體之多肽類型，並在一些實驗中，以環己醯胺和氯黴素等抑制劑，探討在多肽變化中蛋白質合成的角色。同時，分析製備葉綠體時其上層液之多肽以作爲對照。純水誘導老化或以離層酸或乙烯刺激老化時，其上層液內 62 KD之多肽隨之增加，黃素馨酸甲酯(Methyl jasmonate)則可造成上層液內 66 KD之多肽增加。在老化期間，葉綠體多肽類型的改變並不顯著。根據不同的處理，葉綠體和上層液內可偵測到其他多肽的一些變化。由於，黃素馨酸甲酯可誘導上層液和葉綠體內多肽類型之改變，故推測這種物質引起葉片老化之機制不同於離層酸及乙烯之作用機制。