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Chloroplast Polypeptides Synthesized by Leaf Segments and Isolated Chloroplasts During Senescence in Barley

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Starting from senescent barley (*Hordeum vulgare* L. cv Hassan) leaf segments receiving light and hormone treatments affecting senescence, the plastid polypeptides synthesized by isolated chloroplasts and by leaf segments were analyzed by radiolabelling followed SDS-PAGE and fluorography. Among 20 to 30 polypeptides detected, a few were specifically synthesized (by chloroplasts and/or leaf segments) after each senescence treatment. Apparently, the polypeptides labelled in assays with isolated chloroplasts are truly synthesized *in vivo*, because most of them were also labelled in assays with leaf segments. The comparison of polypeptide profiles, for every senescence treatment, after labelling with isolated chloroplasts or leaf segments, suggests that most plastid polypeptides synthesized during senescence are coded in plastid DNA.

Key words: Barley, Chloroplasts, Hormones, Protein synthesis, Senescence.

The levels of specific poly(A)$^+$-RNAs are different in senescent and non-senescent cotyledons (6). Similarly, the polypeptides synthesized by isolated chloroplasts vary when senescent leaves or cotyledons are treated with light or hormone effectors of senescence (4, 11, 13) and are very different in chloroplasts from greening and senescent leaves (17). These results and the retardation of leaf senescence by inhibitors of protein synthesis (15, 16) suggest that the development of senescence syndrome requires the expression of specific genes controlled at transcriptional (6) and translational level (7). However, proteins synthesized by isolated chloroplasts or by wheat germ system with leaf mRNAs are not necessarily synthesized *in*

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vivo, when specific control mechanisms of protein synthesis and degradation do operate.

To know if the polypeptides labelled after protein synthesis by isolated chloroplasts are synthesized *in vivo*, they have been compared with those synthesized by leaf segments during senescence. Fourteen-day-old barley leaf segments were incubated during 20 h under ten different light and hormone treatments affecting senescence. The plastid polypeptides labelled with [^{14}C]-amino acids after protein synthesis by isolated chloroplasts and leaf segments were analyzed by SDS-PAGE* and fluorography.

Materials and Methods

The growth of barley (*Hordeum vulgare* L. cv Hassan), at 23 °C under an 18 h photoperiod of white light, ca. 15 W m^{-2} (12.7 W m^{-2} PAR), has been described previously (2).

For leaf treatments, 2 g of 20 mm segments, discarding base and tip, of the oldest leaf of 14-day-old plants (ca. 1.42 mg chlorophyll g^{-1}) were incubated for 20 h at 25 °C in the dark with 25 ml pure water or 14 µM kinetin, 35 µM ABA, 70 µM ethylene (as ethrel) or 45 µM methyljasmonate. Sometimes dark was interrupted at 9 h of incubation by 10 min red light (3.2 W m^{-2} peaking at 650 nm, 38 nm half-band width) or 10 min red light followed by 20 min far-red light (13.5 W m^{-2} of a wide band from 700 to 800 nm, which peaks near 800 nm). In other treatments, leaf segments were incubated for 20 h in continuous blue (0.4 W m^{-2} peaking at 450 nm, 12 nm half-band width) or 6 h in near-UV (0.9 W m^{-2} peaking at 355 nm, 35 nm half-band width) followed by 14 h in the dark, as described in detail elsewhere (2, 3).

Leaf radioactive labelling was carried out during the last 3 of the 20 h incubation of leaf segments by adding 110 kBq of [^{14}C]-amino acid mixture (1.85 GBq (mg atom)$^{-1}$, Amersham) in 1.5 ml water. The incubation was ended by washing with distilled water at 0-5 °C. Results (unpublished) showed that radioactivity incorporation in proteins was linear during the 3 h incubation.

For isolation of chloroplasts, incubated leaf segments (2 g) were homogenized for 10 s at 0-5 °C in a Sorvall Omnimixer with 12 ml 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 strained through 4 layers of muslin and centrifuged for 5 min at 200 × g discarding the pellet. The supernatant was centrifuged for 10 min at 2.000 × g. The new pellet with chloroplasts was washed with 6 ml of buffer E. The chloroplast preparation was bacterial contamination free (< 1 % particles after Gram staining) and of mitochondria (< 5 % protein) and cytoplasm (< 1 % protein). At least 70 % of the chloroplasts were intact as measured by the ferricyanide reduction assay or by ultracentrifugation in hypertonic sucrose gradient (7).

When isolated from leaf segments incubated in the absence of radioactive amino acids, chloroplasts were immediately incubated for light-driven protein synthesis as described previously (8) in 300 µl incubation mixture containing 200 kBq of [^{14}C]-amino acid mixture (1.85 GBq (mg atom)$^{-1}$, Amersham) and around 400 µg chlorophyll, at 30 °C during 40 min. In these assays, dark incubation (> 90 %) and 2 mM chloramphenicol (> 90 %) but not 0.1 mM cycloheximide (< 10 %) inhibit protein synthesis (8).

Chloroplast radioactive proteins (synthesized by isolated chloroplasts or labelled *in situ*) were precipitated with 8 %

* *Abbreviations*: ABA, abscisic acid; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

(w/v) final trichloroacetic acid. The radioactive pellets were resuspended for SDS-PAGE in 75 µl 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.* (12).

Slab SDS-PAGE was performed with 10-20 % (w/v) linear acrylamide gradient (2.5 %, w/w, bisacrylamide) with the buffer system of O'FARRELL (10) as described previously (7). Radioactive gels were impregnated with Amplify (Amersham) dried and fluorographed with MAFE-X-1 film exposed for 30 days at −80 °C. Molecular weight markers were detected by Coomassie staining.

Chlorophyll was determined according to ARNON (1).

Results and Discussion

Figures 1-3 show SDS-PAGE and fluorographies of the plastid polypeptides synthesized by isolated chloroplasts (C) and of those labelled *in situ* (S) with [^{14}C]-amino acids during the last three of the 20 h incubation time of each treatment. Circles on C lanes indicate the main plastid polypeptides labelled after synthesis by isolated chloroplasts but not in leaf segments. Bars on S lanes indicate the main plastid polypeptides labelled in assays with leaf segments but not with isolated chloroplasts.

Although differences in relative labelling between C and S lanes (derived from the same leaf treatment) were apparent, and some patterns in S lanes were smeared due to high total protein loading (to compensate for the low protein synthesis activity), most polypeptides labelled after protein synthesis by isolated chloroplasts (particularly in the low molecular weight range) were also labelled when the corresponding leaf segments were incubated with [^{14}C]-amino acids. Few additional polypeptides were labelled in assays with leaf segments (bars), which suggests that

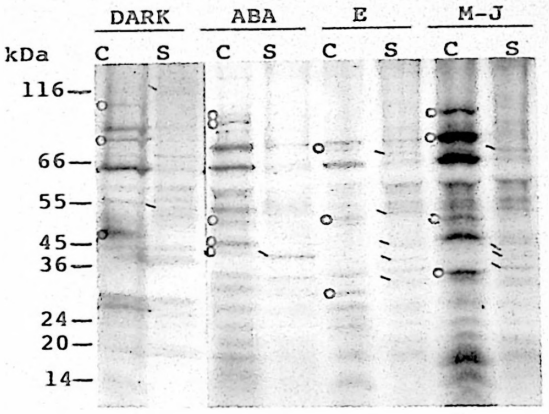

Fig. 1. *Effects of ABA, ethylene and methyljasmonate on the synthesis of chloroplasts polypeptides.*
Leaf segments were incubated 20 h in the dark, dark with ABA (ABA), dark with ethylene (E) and dark with methyljasmonate (M-J). The radioactive polypeptides synthesized by isolated chloroplasts (C) and labelled *in situ* (S) were analyzed by SDS-PAGE and fluorography.

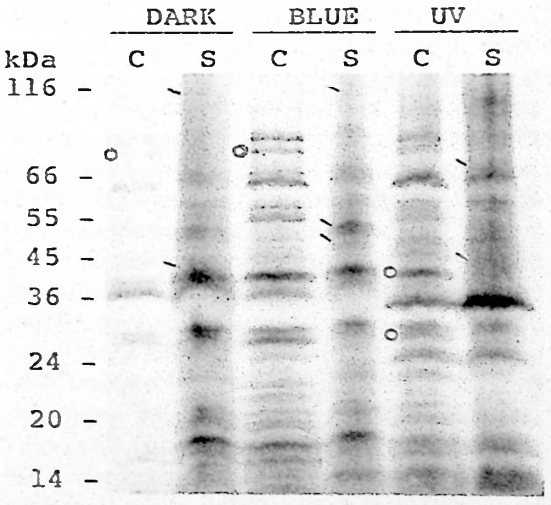

Fig. 2. *Effects of blue and near UV lights on the synthesis of chloroplasts polypeptides.*
Leaf segments were incubated 20 h in the dark, in continuous blue light (BLUE) and in UV light (the first 6 h) (UV). C and S as in fig. 1.

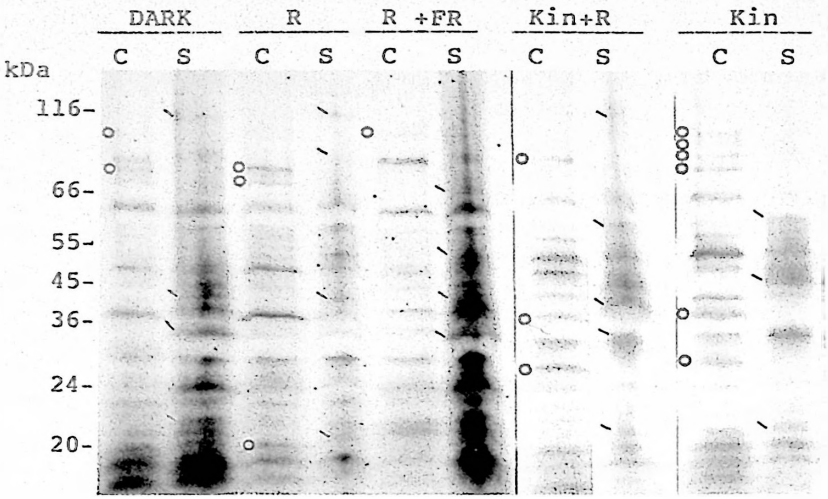

Fig. 3. *Effects of kinetin and phytochrome-related light treatments on the synthesis of chloroplast polypeptides.*
Leaf segments were incubated 20 h in the dark, dark interrupted with red light (R), dark interrupted with red light followed far red light (R + FR), dark with kinetin (Kin) and dark with kinetin and red interruption (Kin + R). C and S as in fig. 1.

most plastid senescence polypeptides are synthesized in chloroplasts and, thus, encoded in plastid DNA.

In agreement with previous results (7, 13), chloroplasts isolated from leaf segments incubated in the dark synthesized a characteristic set of polypeptides (senescence polypeptides) (figs. 1-3). Minor differences with respect to previous results with [^{35}S]-methionine are probably due to the new labelling with [^{14}C]-amino acid mixture. Although the relative labelling of some senescence polypeptides was not always reproducible (e.g. the 48 kDa polypeptide, synthesized by chloroplast isolated from dark incubated leaf segments, shows very low labelling in fig. 2), the polypeptide pattern was satisfactorily reproduced if leaf and chloroplast handling was carefully controlled.

ABA, ethylene and methyljasmonate stimulated the synthesis in leaf segments of a 37 kDa polypeptide (fig. 1). Near-UV, characteristically, stimulates the synthesis of polypeptides of 32 and 24 kDa (fig. 2). Methyljasmonate treatment stimulated the synthesis by leaf segments of a 66 kDa polypeptide and several polypeptides in the range 36-40 kDa, which were not labelled in experiments with isolated chloroplasts. WEIDHASE *et al.* (19) also reported that the treatment of 7-day-old barley leaf segments with methyljasmonate accelerated the symptoms of senescence and stimulated the synthesis of polypeptides having 66, 37 and 23 kDa in leaf segments. Several polypeptides intensely labelled in assays with chloroplasts isolated from leaf segments treated with methyljasmonate were barely detected after labelling of leaf segments (fig. 1). Kinetin treatment of leaf segments (which retards subsequent senescence) changed the pattern of polypeptides synthesized by isolated chloroplasts, as described previously (7), and by leaf segments (fig. 3).

MULLET *et al.* (9) claimed that, in assays of protein synthesis with isolated chloro-

plasts, most labelled low molecular weight polypeptides are artifacts resulting from pausing of ribosomes at discrete sites on plastid mRNA and they are not detected by *in situ* labelling. Clearly, polypeptides labelled in our assays with isolated chloroplasts can hardly be artifacts, probably because they were incubated during 40 min, in the recommendable range (14), while MULLET *et al.* (9) detected low molecular weight intermediates after short incubation times (5-10 min) with [^{35}S]-methionine or under condition of low ATP supply.

The leaf treatment effects on the pattern of polypeptides synthesized indicated that the physiological state of leaf samples must be carefully controlled to obtain reproducible results.

When compared with the senescence accelerated by incubation in the dark of leaf segments, kinetin treatment and red light interruption of the dark (in a phytochrome mediated effect) retard senescence while ABA, ethylene and methyljasmonate accelerate senescence (3) as measured by chlorophyll or protein loss. Continuous blue and near-UV lights retard senescence. Apparently, the effects of these treatments on senescence are mediated by primary effects on plastid protein synthesis.

Polypeptides synthesized by chloroplasts during the first 20 h incubation of leaf segments control subsequent senescence processes (2). To find out the role of senescence polypeptides it may be easier to work with protein synthesis by isolated chloroplasts than with leaf segments. Most available antibodies against plastid polypeptides (synthesized at the juvenile stage) do not react with polypeptides synthesized during senescence (5, 13) (although senescent plastids still contain polypeptides of the juvenile stage). On the other hand, the presence of non-translated mRNAs in senescent chloroplasts (18) makes the identification of plastid genes encoding senescence polypeptides difficult. Hybrid select translation assays (18) suggest that genes for most senescence polypeptides probably map in the single short copy region of plastid DNA.

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Resumen

Partiendo de segmentos de hojas senescentes de cebada (*Hordeum vulgare* L. cv Hassan) que habían recibido tratamientos hormonales y luminosos que afectan a la senescencia, los polipéptidos sintetizados por cloroplastos aislados y por segmentos de hojas se analizan por marcaje radiactivo seguido de SDS-PAGE y fluorografía. Entre 20 y 30 polipéptidos detectados, unos pocos eran sintetizados específicamente (por cloroplastos y/o por segmentos de hojas) después de cada tratamiento que afecta a la senescencia. Aparentemente, los polipéptidos marcados radiactivamente después de ensayos con cloroplastos aislados son sintetizados *in vivo*, ya que la mayor parte de ellos también eran marcados en ensayos con segmentos de hojas. La comparación de los perfiles polipeptídicos para cada tratamiento que afecta a la senescencia, después de marcar cloroplastos aislados o segmentos de hojas, sugiere que la mayor parte de los polipéptidos de cloroplastos sintetizados durante la senescencia están codificados en el DNA de cloroplastos.

Palabras clave: Cebada, Cloroplastos, Hormonas; Senescencia, Síntesis de Proteínas.

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