# Light-regulated protein and mRNA synthesis in root caps of maize

Lewis J. Feldman[1], Birgit Piechulla[2] and Piera S. Sun[1]
[1]*Department of Botany, University of California, Berkeley, CA 94720, USA;* [2]*Institut für Biochemie der Pflanze der Universität Göttingen, Untere Karspüle 2, 3400 Göttingen, Federal Republic of Germany*

## Abstract

Illumination of maize roots initiates changes in mRNA levels and in the activities of proteins within the root cap. Using Northern analysis we showed a 5 – 6-fold increase in the levels of three specific mRNAs and a 14-fold increase in plastid mRNA. This increase is rapid, occurring within 30 minutes of illumination. With prolonged periods of darkness following illumination, messages return to levels observed in dark, control caps. For two species of mRNA illumination results in a reduction in message levels. Light-stimulated increases in the levels of specific mRNAs are proportionally greater than are increases in the activities of corresponding proteins. We suggest that the light-stimulated increase in protein activity in root caps may be preceded by and occur as a consequence of enhanced levels of mRNA. Our work suggests that photomorphogenesis in roots could involve changes in the levels of a wide variety of mRNAs within the root cap.

## Introduction

Light regulates many aspects of growth and development in plants [6, 20]. Most studies of the mechanism of this regulation have been conducted with above-ground portions of the plant, usually with leaves or stems, or occasionally with fruits [1, 10, 16, 19]. Roots, because they typically grow in a subterranean environment, are usually assumed to be indifferent to light. However, within the past 15 years evidence has accumulated showing a role for light in mediating many aspects of root development, including root extension, geosensitivity (i.e., the ability to respond to gravity) and lateral root production [2, 6].

The region of perception of the light is the root cap, a distinctive population of cells located at the tips of roots (Fig. 1). If the cap is excised and then roots are illuminated, light has no effect on root development. The effective wavelength of light indi-cates that this response is phytochrome-mediated [8].

Earlier we showed that light stimulated protein synthesis within the cap and that this synthesis was required for root geosensitivity. If either protein or RNA synthesis was inhibited within the cap, and then this tissue was illuminated, roots no longer showed a sensitivity to light [7, 9]. Thus we concluded that light could modify root development, at least in part, by affecting protein and/or RNA synthesis within the cap. Recent work of Stinemetz *et al.* [17] supports this conclusion. These workers showed that illumination of maize roots caused an increase in the level of the calcium-binding protein calmodulin in the terminal 1 – 2 mm of the root. Since calcium is believed to have a central role in regulating root geotropism, this light-stimulated increase in calmodulin may be important in understanding how light promotes geotropism in roots. With the exception of this work by Stinemetz *et al.* [17] and the work of
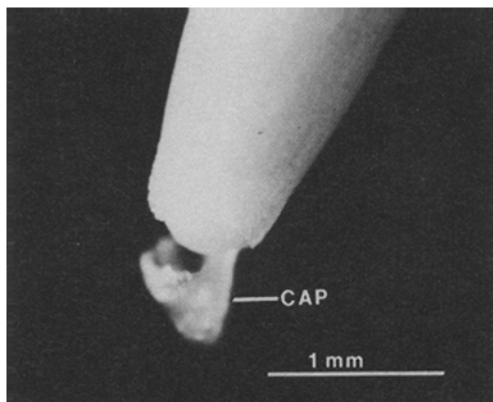
*Fig. 1.* View of root cap tissue following excision from the root proper. Note that the cap can be obtained relatively free from adjacent root tissue.

Poovaiah and Veluthambi [14] showing light-induced protein phosphorylation in roots, there are relatively few reports of the effects of light on the activities of specific root proteins and no reports of light effects on specific mRNAs.

The objectives of this work were twofold: first, to determine whether the effects of light are limited to a few proteins, or to many; and second, to establish whether light-induced changes in protein activities are paralleled by changes at the mRNA level, as our earlier work suggested. For this work we chose to examine the effects of light on the levels of a number of specific mRNAs, as well as the effects of light on the activities of corresponding proteins. Because so little is known of the physiology of the root cap, the selection of mRNA probes was somewhat arbitrary. Our aim was to use probes for mRNAs coding for a variety of structurally and physiologically dissimilar proteins.

In this paper we report that light rapidly modifies the levels of specific mRNAs within the root cap. However, this modification in mRNA is not paralleled by changes of similar magnitude in protein activities.

## Materials and methods

### Materials

Total RNA was obtained from root caps from 2-day-old primary seedling roots of maize (*Zea mays* cv. Merit, Asgrow Seed Company, Tracy, CA). Caryposes (seed) were germinated in the dark at 23 °C on paper towels moistened with distilled water. Forty-two to forty-eight hours after imbibing, seedlings were irradiated with white light from a 40 W incandescent lamp (1.65 W m$^{-2}$) [7], for 10 min, returned to the dark for varying intervals of time (5 – 120 min), and then the root caps were collected using a dissecting microscope and dim (4 × 10$^{-14}$ W m$^{-2}$) green (515 – 575 nm) light as the only illumination (Fig. 1). Immediately after excision from the root, the cap was frozen by placing in contact with a glass slide resting on a block of dry ice. As shown earlier, cap tissue can be obtained relatively free of contamination from any other root tissue [7]. For this work 300 – 500 mg of cap tissue was collected for each time point.

### RNA preparation

Total RNA was isolated as follows. Approximately 0.5 g of tissue was homogenized with a mortar and pestle for 10 min in liquid nitrogen. The tissue was then transferred to a Kontes Duall tissue grinder and homogenized (with 25 strokes) in 5 volumes of buffer containing the following: 50 mM Tris (pH 8), 15 mM EGTA, 0.2 M NaCl, 0.5 mM dithiothreitol, 2% w/v sodium dodecyl sulfate (SDS), 0.1% proteinase K (EM Labs). The homogenate was incubated for 1 h at 40 °C and then enough 2 M KCl was added so that the final concentration of KCl was 0.2 M. The homogenate was centrifuged at 10000 *g*, at 4 °C for 30 min and the supernatant then filtered through 1 layer of GF/A Whatman filter paper (Fisher). To the filtrate enough 10 M LiCl$_2$ was added to bring the final LiCl$_2$ concentration to 2 M. This mixture was incubated at 4 °C overnight and then centrifuged at 10000 *g* at 4 °C for 30 min. The supernatant was discarded and the pellet washed and mixed with 2 M LiCl$_2$, and then centrifuged as before. The supernatant was discarded, the pellet dissolved in 3 ml of 0.2 M NH$_4$OAc pH 5.5 and then centrifuged for 10 min at 10000 *g*. The supernatant was removed, combined slowly with 2.5 volumes of absolute ethanol and then left overnight at −20 °C.

Following the overnight precipitation the mixture was centrifuged for 30 min at 10000 *g* and then the pellet washed with 70% ethanol and recentrifuged. The washing and centrifugation were repeated a second time and then the pellet was dried under vacuum until there were no traces of ethanol. The RNA was then dissolved in 100−200 $\mu$l of water.

## Preparation of hybridization probes

Specific gene probes were used for the hybridization (Table 1). Sources of probes and preparation of plasmid DNA are described in [13]. The cloned inserts coding for the genes of interest were isolated by preparative digestion of the plasmid DNA with appropriate restriction enzymes (see Table 1). DNA fragments were separated by gel electrophoresis and the fragments isolated by electroelution (100 V in 0.1× TBE buffer) [13]. The re-isolated DNA was purified by phenol:chloroform:isoamyl alcohol (25:25:1) extractions and used for nick translation reactions [12] (using dCTP, sp. act. 410 Ci/mmol; Amersham). Specific activities of probes:

$$
\begin{aligned}
\text{25S and 18S rDNA} &= 1.11 \times 10^7 \text{ cpm/}\mu\text{g}, \\
\text{alcohol dehydrogenase} &= 1.97 \times 10^6 \text{ cpm/}\mu\text{g}, \\
\text{16S rDNA} &= 1.14 \times 10^6 \text{ cpm/}\mu\text{g}, \\
\text{aldolase} &= 1.28 \times 10^7 \text{ cpm/}\mu\text{g}, \\
\alpha\text{-amylase} &= 3 \times 10^6 \text{ cpm/}\mu\text{g}, \\
\text{tubulin b} &= 4 \times 10^6 \text{ cpm/}\mu\text{g}, \\
\text{ATPase (alpha)} &= 1.14 \times 10^6 \text{ cpm/}\mu\text{g}, \\
\text{ATPase (beta)} &= 1.3 \times 10^6 \text{ cpm/}\mu\text{g}.
\end{aligned}
$$

## Analysis of RNA

Electrophoresis of 3 $\mu$g of RNA was performed on formaldehyde-containing agarose gels [12]. The amounts of RNA applied to the gels were standardized by spectrophotometric quantitation, quantitation of the fluorescence intensity of cytoplasmic rRNA in ethidium bromide-stained gels, and the relative levels of hybridization with heterologous cytoplasmic rDNA [13]. The RNA was transferred to nylon filters (Hybond, Amersham) and the filters exposed to UV light for 4−5 minutes. Nylon filters were then prehybridized for 4 h at 65 °C in 2× SSC (0.3 M NaCl; 0.03 M sodium citrate, pH 7.0), 1× Denhardt's solution (0.1 g Ficoll, 0.1 g polyvinylpyrrolidone 40, 0.01 g BSA per 500 ml). Unless noted otherwise hybridization with specific probes was carried out at 65 °C for 12−16 h in 2× SSC, 1× Denhardt's solution, 0.5% SDS. Filters were washed at 65 °C in 1 liter of 2× SSC (3 times, 15 min) and 1 liter 1× SSC (3 times, 15 min) and exposed to X-ray film (−70 °C with intensifying screen). After exposure, the films were scanned densitometrically using a Loebl densitometer. Relative amounts of mRNA were determined by peak-area measurements. All hybridizations were repeated at least three times with different filters. In order to ensure that the hybridization signals were in the linear range of the film, films were exposed for varying periods of time. Only those films not showing signal saturation were used in preparing Table 2 and Fig. 2.

## In vitro translation

A wheat germ extract was prepared and used as the cell-free protein synthesis system [18]. The standard reaction mixture (25 $\mu$l total) for the incorporation of amino acids into protein contained 2.5 mM ATP,

*Table 1.* Probes used for hybridizations.

| Plasmid | Restriction fragment | Source[a] | Gene |
|---|---|---|---|
| pHA2 | whole plasmid | pea | 18S and 25S rDNA |
| pZM1154 | 1.0 kb, *Pst* I | maize | aldolase |
| 1 28L | 1.6 kb, *Pst* I | barley | α-amylase |
| pZML84 | 0.9 kb, *Pst* I | maize | alcohol dehydrogenase |
| pTCB8 | 3.3 kb, *Bam* HI | tobacco | 16S and 23S rDNA |
| pATB4 | 1.0 kb, *Kpn* I-*Bam* HI | *Arabidopsis* | tubulin b |
| pTA22 | 0.7 kb, *Eco* RI-*Bam* HI | maize | ATPase (alpha) |
| pcNp10 | 1.5 kb, *Eco* RI-*Sal* I | tobacco | ATPase (beta) |

[a] See [13] for the exact source of the probes.

0.25 mM GTP, 4 mM magnesium acetate, 5 mM phosphoenolpyruvate, 20 mM HEPES (pH 7.6 with KOH), 90 mM potassium acetate, 1 mM dithiothreitol, 10 $\mu$Ci of $^{35}$S-methionine (sp. act. > 600 Ci/mmol; Amersham), 0.025 mM of the 19 common L-amino acids, excluding methionine, 10 $\mu$l of wheat germ extract and 7 $\mu$g of total RNA.

The reaction mixture was incubated at 25 °C for 1 h and 5 $\mu$l of the mixture was then pipetted onto squares of Whatman 3 MM filter paper, which were then pretreated with a drop of 20% (w/v) trichloroacetic acid (TCA). The filters were washed with 5% (w/v) TCA at 80 °C for 15 min, 5% TCA again at room temperature, and then one wash each in ethyl alcohol:diethyl ether (1:1) and in pure diethyl ether. The radioactivity in precipitated protein was then counted in a toluene-based scintillator using a Beckman LS 6800 scintillation counter.

Products from *in vitro* translations were also analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [9]. An equal number of counts (approximately 100000) were loaded per lane on a 12% polyacrylamide gel. Autoradiographs were prepared from the dried gels (Fig. 4).

*Protein assays*

Approximately 200–400 caps (50–100 mg) were used in assays for $\alpha$-amylase, aldolase or alcohol dehydrogenase (ADH). Tissues were homogenized (with 25 strokes) in a Duall tissue grinder (Kontes) at 4 °C in 5–10 volumes of buffer containing 0.1 M glycylglycine pH 7.5, 0.2 M potassium acetate and 50 mM $\beta$-mercaptoethanol. Aldolase, ADH and $\alpha$-amylase activities were measured spectrophotometrically following the protocols of Vanderheiden and Meinhart [22] (for aldolase), Vallee and Hoch [21] (for ADH), and Jones and Varner [11] (for $\alpha$-amylase). A standard curve was prepared for each enzyme; then a dilution series was made of the root cap extract, in order to obtain values falling along the linear portion of the standard curve. Values for aldolase, ADH and $\alpha$-amylase are expressed in terms of units of activity/mg of total root cap protein. For purposes of comparison, values from dark caps have arbitrarily been assigned a value of one. The reaction mixture for the ADH assay was incubated at 25 °C in a 1 ml cuvette which contained 580 $\mu$l of 32 mM pyrophosphate buffer, 200 $\mu$l of 2 M ethanol, 200 $\mu$l of 25 mM NAD and 20 $\mu$l of the enzyme extract. Absorbance measurements were made at 340 nm using a Perkin-Elmer 552 spectrophotometer. The reaction mixture for aldolase contained 0.5 ml of the above homogenization buffer, 370 $\mu$l of 20 mM fructose-1,6-diphosphate (pH 7.5), 100 $\mu$l of NADH (NADH-Na, 2 mM in 1 mM NaOH), 10 $\mu$l of

*Table 2.* Relative levels of specific mRNAs in root caps from maize. mRNA was obtained from dark-grown root caps or from caps illuminated for 10 minutes and then returned to the dark for varying intervals of time. Values for the dark caps have arbitrarily been set at 1.

| Treatment | Hybridization probe | | | | | | |
|---|---|---|---|---|---|---|---|
| | ADH[a] | Aldolase | Amylase | Tubulin b | Plastid 16S | ATPase | |
| | | | | | | alpha | beta |
| Dark control | 1 ±0.2 | 1 ±0.3 | 1 ±0.4 | 1 ±0.3 | 1 ±0.2 | 1 ±0.3 | 1 ±0.3 |
| 10 minutes light | | | | | | | |
| + 5 minutes dark | 1.1±0.4 | 1.2±0.5 | 1.5±0.4 | 1.2±0.4 | 2 ±0.4 | 1.1±0.2 | 1.2±0.2 |
| 10+ 10 | 2.5±0.5 | 3.6±0.6 | 3.6±0.3 | 2.0±0.7 | 3.1±0.7 | 1.1±0.3 | 1.1±0.2 |
| 10+ 20 | 4.3±0.4 | 5.1±0.6 | 4.8±0.6 | 2.1±0.3 | 7.5±1.4 | 0.2±0.2 | 1 ±0.3 |
| 10+ 30 | 5.1±0.6 | 6.2±0.5 | 4.3±0.4 | 2.2±0.5 | 14 ±2.4 | 0.1±0.2 | 1.2±0.2 |
| 10+120 | 1.9±0.2 | 2.1±0.4 | 1.1±0.3 | 1.0±0.4 | 12.3±1.9 | 0.1±0.2 | 0.6±0.2 |

[a] Alcohol dehydrogenase.
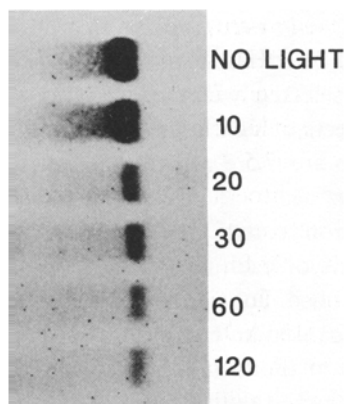
Fig. 2. Autoradiogram (Northern) of total RNA from root caps after 10 minutes of light plus varying periods of darkness (probed with the nick translated gene probe for ATPase-alpha). (The amount of time in darkness is indicated in minutes beside each lane). Three μg of RNA were loaded per lane. Northern blot hybridization conditions are described in the materials and methods.

glycerophosphate dehydrogenase-triosephosphate isomerase (Sigma). The mixture was incubated for 3 min and then 20 μl of the enzyme extract added and absorbance measurements made at 340 nm. To-tal amylase actvity was determined using the starch-iodine method [11]. The reaction mixture contained 0.5 ml starch, 100 μl of the enzyme extract, 0.5 ml of $I_2$, and 2.5 ml of water. The mixture was incubat-ed for 12 min at 30 °C and then the absorbance read at 625 nm. Protein concentrations were measured by the method of Bradford, by using the Bio-Rad pro-tein assay kit [4].

## Results

Northern analysis was performed on total RNA from dark or illuminated root caps of maize. We used a number of hybridization probes and found that except for ATPase (alpha and beta subunits) il-lumination caused an increase in the levels of specif-ic mRNAs (Table 2). Most messages accumulated to a maximum level approximately 30 min after the ter-mination of the 10 min illumination (10+30). For three species of mRNA illumination caused a 5−6-fold increase in relative abundance, but one message, tubulin-b, showed only a 2-fold increase. In contrast, the plastid 16S rRNA increased 14-fold. Two hours after illumination (that is, 10 min of light plus 2 h of darkness = 10+120), the levels of 4 of the light-stimulated messages decreased (Table 2). mRNA for tubulin-b and for α-amylase decreased to levels measured in dark, control caps, while the mRNA for ADH and aldolase also decreased, but to a value about twice that measured in non-illuminated caps. In contrast to this general decline in message levels with increasing periods of darkness, there was only a small decrease in the plastid rRNA, even after 2 h of darkness. For ATPase (alpha) illumination result-ed in a rapid, 10-fold decrease in the relative abun-dance of this message (Fig. 2). The message for the beta subunit of ATPase also decreased as a result of illumination, though the rate of decrease was slower than for the alpha subunit (Table 2). After 2 h of darkness (10+120) the ATPase message levels had not returned to those of the dark, control caps.

Table 3. Relative levels of enzyme-specific (protein) activities in root caps of maize. Activities were measured in dark-grown cap tissue and also in cap tissues illuminated for 10 minutes and then returned to darkness for varying intervals. Values for dark, control caps have arbitrarily been set at 1.

| Treatment | Protein | | |
|---|---|---|---|
| | ADH[a] | Aldolase | α-amylase |
| Dark control | 1 ±0.2 (464)[a] | 1 ±0.3 (69) | 1 ±0.2 (97) |
| 10 minutes light + 30 minutes dark | 1.7±0.4 (768) | 1.4±0.3 (96) | 1.5±0.2 (146) |
| 10+ 60 | 2.1±0.5 (948) | 1.5±0.5 (108) | 1.6±0.4 (155) |
| 10+120 | 1 ±0.4 (452) | 1.6±0.5 (109) | 1.6±0.3 (155) |

[a] Alcohol dehydrogenase.
[b] Values in brackets denote milliunits of activity/mg of total protein.

We also measured the activities of specific enzymes in dark-grown and illuminated root caps (Table 3). Light caused an increase in the activities of the 3 enzymes for which we assayed, with maximum levels reached 30–60 min after illumination. ADH activity was approximately twice that of the dark control, whereas aldolase and $\alpha$-amylase increased 1.5 times as a result of illumination. Increased periods of darkness following illumination resulted in a decrease in ADH activity. After 2 h of darkness (10+120) ADH activity returned to that measured in non-illuminated caps. Aldolase and $\alpha$-amylase, although enhanced only slightly by illumination, did not show significant changes in activity, even after 2 h of darkness.

We also translated in vitro total RNA from dark-grown and illuminated root caps (Fig. 2). The highest label incorporation was obtained using RNA from caps provided 10 min light plus 30 min of dark (10+30) and was approximately 2 × greater than in dark control caps. RNA translated from caps which were illuminated and then provided 2 h darkness (10+120) showed label incorporation nearly identical to that of dark control caps. Analysis of the in vitro translation products by means of SDS-PAGE showed that mRNA from various time points (e.g., 10+30, 10+60) yielded essentially the same pattern of incorporation of $^{35}$S into proteins.

## Discussion

Light regulates development in roots of maize, morning glory, cress and likely in roots of many other species as well [6, 20]. Exactly how the light is transduced into a developmental response is not known, but may involve the redistribution of both calcium and auxin (indole-3-acetic acid) [17]. Earlier we showed that both protein and RNA synthesis must occur in the root cap in order for light to influence root development. If this synthesis was prevented or retarded, light was unable to alter or affect the pattern of root development. We speculated then that illumination may initiate the production of unique proteins and mRNAs associated with various light-regulated developmental responses. However our current data do not support this conclusion.

Analysis of the in vitro translation products (Fig. 4) show essentially identical patterns. No protein(s) appears to be selectively enhanced. Thus we conclude that the effects of light on proteins and mRNAs in the root cap are more general and not limited to a few species.

Illumination results in rapid changes in the mRNA levels of each of the specific mRNAs for which we probed. For five of the species of RNA, illumination caused an enhancement in message levels. For two of the messages however, illumination initiated a marked reduction in message levels. For those messages enhanced by light, maximum levels were attained 20–30 min after illumination (that is, 20–30 min after returning the tissue to the dark). After about 120 min of darkness levels of specific RNAs had declined to dark control, or near-dark control values for all but the 16S light-stimulated rRNA. Even after 2 h of darkness the plastid 16S rRNA remained at a level 12 × greater than found in non-illuminated tissues. The similar kinetics of enhancement shown by all of the light-stimulated RNAs suggest that the induction of these RNAs may involve a common step, whereas the turnover of each species of RNA appears to be independently regulated.

Whereas the majority (five) of the mRNAs increased as a result of illumination, the transcripts for both the alpha and beta subunits of the ATPase decreased. The kinetics for the decrease of the two subunits differs, with the alpha subunit declining more rapidly and to a greater degree than the beta subunit. After 120 min of darkness the alpha subunit had fallen to approximately 1/10 the value seen in dark control caps and the beta subunit had declined to about 60% of the dark control level. These differences in the kinetics between the alpha and the beta subunits may be attributable to the fact that the alpha subunit is mitochondrial-encoded [5] and the beta subunit nuclear-encoded [3]. It is interesting to note that our data on the effects of light on ATPase message levels parallel observations by Serlin et al. [15] who report that red light, via phytochrome, depressed ATPase activity in isolated mitochondria.

In contrast to the often marked effects of light on mRNA levels, the effects of light on protein activity
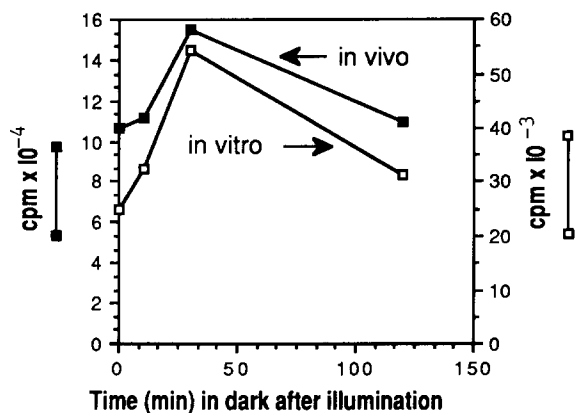
Fig. 3. Radioactivity incorporated into TCA-precipitable protein from both *in vivo* and *in vitro* labeling experiments. *In vitro* labeling was done with total RNA obtained from root caps provided 10 minutes of light and then varying periods of darkness. The *in vivo* labeling represents protein synthesized in intact caps which have been provided 10 min light and then varying periods of darkness. (See [7] for more details). Equal amounts of total RNA were translated for each time point.

are less dramatic. Following illumination, activities of ADH, aldolase and α-amylase within the root cap increased only 1.5−2 times. Whereas changes in mRNA levels were always detectable within 10 min of the light treatment (10+10), the earliest measurable changes in proteins were not seen until about 30 min following illumination (10+30) (Table 3). Because mRNA increases precede the rise in activity for the corresponding proteins these data are consistent with the suggestion that light could affect protein activity in root caps via an alteration in the levels of specific mRNAs. However, because the increases in mRNA are not paralleled by proportionate increases in protein activity, it is also possible that light
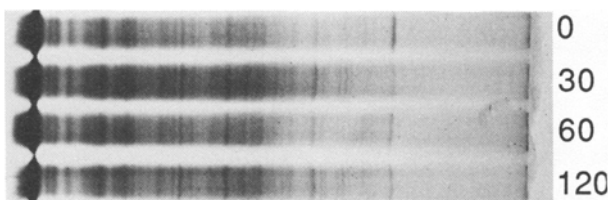


Fig. 4. Autoradiogram of *in vitro* translated proteins run on a 12% polyacrylamide gel. mRNA was obtained from root caps provided 10 min light plus varying periods of darkness. The amount of time (in min) in darkness is indicated beside each lane.

affects protein activity independent of its effects on mRNA.

In a general way the changes in the mRNA population shown by Northern analysis parallel the data from *in vitro* translation (Fig. 3). These data, from translation of total message, show the most counts incorporated into protein derived from message of the 10+30 root caps. We believe that these results with the wheat germ *in vitro* translation system mirror what occurs *in vivo*, in the illuminated root cap. In support of this conclusion we have included in Fig. 3 data showing the kinetics of total protein labeling in intact, illuminated root caps (data from [7]). The parallel in the two curves leads us to conclude that *in vitro* translation data accurately reflect mRNA processing in the intact root cap. Moreover, the similarity in patterns of labeled proteins from the *in vitro* translation (Fig. 4) suggests that light affects many messages and proteins, and not preferentially a few.

In summary, we have shown that illumination of root tissue results in rapid changes of at least seven, and we would suggest probably most pre-existing mRNAs within the root cap. Although we know that mRNA synthesis within the cap is necessary in order for light to affect root development [7, 9], we do not now know whether illumination results in the expression of unique, light-induced messages. Because light triggers simultaneous changes in many messages, it could be argued that light-regulated development in roots is not dependent on a few unique mRNAs or their corresponding proteins, but rather depends on a general increase (or decrease) in the levels of pre-existing mRNAs. Overall our data show that illumination causes a 1.8-fold increase in total translatable mRNA. Because of the rapidity of this response, our results suggest a short transduction chain for light-induced changes in mRNA in the root cap of maize.

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