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Association of Plant p40 Protein with Ribosomes Is Enhanced When Polyribosomes Form during Periods of Active Tissue Growth[1]

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p40s are acidic proteins of eukaryotic cells occurring either free in the cytoplasm or in association with ribosomes, the latter occurring in both monosomes and polysomes. p40s may play a role in the regulation of protein synthesis, although the exact mechanism is not known. Leaves of all 10 plant species examined here, including both monocots and dicots, contained proteins detected on immunoblots with *Arabidopsis thaliana* p40 antiserum. The number and apparent size of the protein bands were variable even among closely related species. Abundance of p40 relative to ribosomal content during soybean (*Glycine max* L.) seed germination and during seed and leaf development was examined. p40 abundance correlated with periods of active tissue growth and high polysome content. The plant growth regulator indole acetic acid caused an increase in polysome formation in etiolated pea (*Pisum sativum* L.) plants and a concomitant recruitment of p40 into polysomes. Subcellular localization at the microscopy level indicated that the pattern of p40 staining is very similar to that for RNA, except that p40 is excluded from the nucleus. These data suggest that p40 is an accessory protein of the ribosome that might play a role in plant growth and development.

A recently recognized class of acidic proteins that associate with ribosomes is called p40. These proteins are conserved among eukaryotes as distantly related as mammals (Makrides et al., 1988; Yow et al., 1988; McCaffery et al., 1990; Grosso et al., 1991; Tohgo et al., 1994), yeast (Davis et al., 1992; Ellis et al., 1994), *Drosophila* (Melnick et al., 1993), sea urchin (Rosenthal and Wordeman, 1995), *Hydra* (Keppel and Schaller, 1991), and plants (Axelos et al., 1993; García-Hernández et al., 1994). Although in most species the size of the protein predicted from nucleotide data is about 33 kD, the proteins tend to migrate as if their size was about 40 kD on SDS-PAGE, hence the name p40.

Initial studies identified p40 as the 67-kD laminin receptor found in vertebrate basement membranes (Wewer et al., 1986; Yow et al., 1988), although subsequent investigations have called into question the relationship between p40 and the receptor (Grosso et al., 1991; Yang et al., 1992). Lower organisms and plants are not known to contain laminin, and the majority of p40 from several species has been clearly shown to be in the cytoplasm, either in association with ribosomes (McCaffery et al., 1990; Auth and Brawerman, 1992; Davis et al., 1992; García-Hernández et al., 1994; Togho et al., 1994; Rosenthal and Wordeman, 1995) or the cytoskeleton (Keppel and Schaller, 1991). Although the data reported from several laboratories suggest that p40 is involved in protein synthesis, the exact function of p40 remains unresolved. It has been hypothesized that p40 acts during translation initiation (Auth and Brawerman, 1992), perhaps in association with translation initiation factor eIF-4A (Yang et al., 1992). Alternatively, it could be involved in tRNA binding (Davis et al., 1992) or in regulating the activity of translation elongation factors (García-Hernández et al., 1994).

In animals, p40 proteins and/or mRNAs have been identified in most tissues examined (Yenofsky et al., 1982; Rao et al., 1989; Rabacchi et al., 1990; Grosso et al., 1991; Yang et al., 1992; Melnick et al., 1993; Rosenthal and Wordeman, 1995). The protein is especially abundant in cells with high metabolic activity, such as embryonic tissues, and the mRNA was expressed preferentially in tumor cells (Yenofsky et al., 1982; Yow et al., 1988; Satoh et al., 1992). p40s have been implicated in cell tumorogenesis, neurite formation (Yang et al., 1992), embryonic retina development (Rabacchi et al., 1990), and oocyte formation and development (Melnick et al., 1993; Rosenthal and Wordeman, 1995).

Relatively little is known about the role of p40 in plants. Here we have investigated the regulation of p40 abundance during development in a variety of plant tissues. We found that p40 is especially prevalent in tissues undergoing active cell division and cell expansion. We also studied ribosome dynamics in several developing systems and determined that in all cases there was a clear relationship between

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polyribosome formation and the amount of p40. More importantly, the plant growth regulator IAA induced a recruitment of p40 into polysomes during periods previously shown to correspond to high levels of protein synthesis. The results suggest that p40 might play a role in polysome formation and/or translational activity during growth and development.

MATERIALS AND METHODS

Plant Material

Species used in this study were oat (*Avena sativa* L. var Ogie), wheat (*Triticum aestivum* L. var Arapahoe), corn (*Zea mays* L. N211), alfalfa (*Medicago sativa* L. var Jubilee), red clover (*Trifolium pratense* L. var Redman), soybean (*Glycine max* L. Merr. var Hobbit), common bean (*Phaseolus vulgaris* L. var Contender), pea (*Pisum sativum* L. var Alaska), *Arabidopsis thaliana* L. (Heynh) ecotype Columbia, and cucumber (*Cucumus sativus* L. var Earlypik 14 Hybrid). Tissues used are noted for each figure.

Tissue for the comparison of p40 among different plant species was obtained from seedlings 1 to 2 weeks after planting in potting soil. Seedlings were grown in a controlled-environment chamber under a 12-h photoperiod at 23°C. Young tissue was obtained from the interior basal 1 cm of monocot leaves or from the youngest expanding leaf of dicot seedlings. Fifty milligrams fresh weight of tissue were ground immediately at room temperature with a mortar and pestle in 400 μL of 2× SDS-PAGE sample loading buffer (0.8 M Tris-HCl, pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol). After centrifugation for 10 min in a microfuge, 10 μL of each sample were electrophoresed for 4 h at 18 mA on a 12.5% polyacrylamide gel (9 cm long × 12 cm wide × 0.75 mm thick) before blotting.

For other experiments, soybean seeds were surface sterilized with 10% sodium hypochlorite and germinated at 23°C under a 12-h photoperiod on germination paper soaked in water. For the germination studies, embryos and cotyledons were harvested at various times after imbibition and immediately immersed in liquid nitrogen. For the leaf and seed developmental studies, plants were grown in pots in the greenhouse at 25°C with a 16-h photoperiod. Maize seedlings were grown in the dark or under a 12-h photoperiod at 23 to 25°C for 10 d. The second leaf was harvested, cut transversely into three parts (basal, middle, and apical), frozen, and stored at −80°C until used.

For the experiment with IAA, pea seedlings were grown in the dark for 8 d. Plants with their third internode 2 to 5 cm long were aged by removing the plumules and applying petroleum jelly to the stems (Schuster and Davies, 1983). Pea stem tissue aged for 2 d was used for the auxin studies. Petroleum jelly was removed from the stem apices and replaced with the same containing IAA (1%, w/w). Apical 1-cm segments were harvested immediately (control) and at various times thereafter.

Subcellular Fractionation of Embryo Axes and Cotyledons

Equal weights of tissue were homogenized in 10 volumes of buffer U (200 mM Tris-HCl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$) (Abe and Davies, 1991). The homogenates, referred to as the total extracts, were then filtered through nylon cloth, and the filtrates were centrifuged for 10 min at 27,000*g*. Aliquots of 4.5 mL of the supernatants were then layered over a 0.5-mL pad of 60% Suc in buffer B (50 mM Tris-HCl, 20 mM KOAc, 10 mM MgOAc) (Larkins and Davies, 1975) and centrifuged for 2 h at 250,000*g* to yield a high-speed pellet, referred to as the "polysome pellet" and the clarified "supernatant." The polysome pellets were resuspended in 200 μL of buffer U, and 40 μL were layered over a linear 15 to 60% (w/v) Suc gradient in buffer B, centrifuged for 1 h at 250,000*g*, and monitored at 254 nm on an Isco (Lincoln, NE) UA-5 gradient monitor. Total protein content of each fraction was measured by the bicinchoninic acid assay (Pierce). An equal volume of each sample was fractionated by 12.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted with Arabidopsis p40 antisera, as described below. Western blots were scanned densitometrically and quantified with NIH-Image (National Institutes of Health, Bethesda, MD) software.

Isolation and Analysis of Polysomes

Tissues were homogenized in 5 to 10 volumes of buffer U and centrifuged for 2 min at 15,000*g*, and 300 μL of supernatant were layered directly over a linear 15 to 60% (w/v) Suc gradient in buffer B, centrifuged for 1 h at 250,000*g*, and monitored at 254 nm on an Isco UA-5 gradient monitor. In some cases, gradients were manually separated into 10 fractions (20 drops each). The amount of p40 in each fraction was determined as described below.

Areas under the polysome profile curve corresponding to monosomes and ribosomal subunits ($M + S$), polysomes (P), and total ribosomal material ($M + S + P$) were determined. The percentage of material in active polysomes was then calculated as $P/T \times 100$.

Immunodetection of p40

Tissues were homogenized in buffer U and the proteins were separated on 12.5% polyacrylamide gels and electrotransferred to nitrocellulose. Rabbit antisera to Arabidopsis p40 have been described previously, and blots were treated as described (García-Hernández et al., 1994) using a 1:6000 dilution of the secondary antibody (goat anti-rabbit IgM coupled to alkaline phosphatase, from Sigma) and developed via a color reaction by incubation in AP buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5) containing 0.33 g/L *p*-nitroblue tetrazolium chloride, and 0.17 g/L 5-bromo-chloro-indolyl phosphate (Sigma). Protein from Suc gradients was precipitated overnight in 0.3 M NaCl and 75% acetone at −20°C, and the resulting pellet was resuspended in SDS-PAGE loading buffer. Immunoblots were scanned densitometrically and quantified with NIH-Image software. In some cases, the amount of p40 was determined by comparison to a known amount of an Arabidopsis p40 fusion protein expressed in *Escherichia coli* that was included in each gel as an internal standard.

Cellular Localization

A. thaliana plants were grown for 6 to 8 d on Petri plates as described by Smith et al. (1994). Arabidopsis seedlings

were fixed by pouring fixative (4% [w/v] paraformaldehyde, 1 mM $CaCl_2$, and 50 mM Pipes) directly on the seedlings on the plates. Seedlings were fixed for 2 h at room temperature, rinsed in the above solution without the fixative (3 × 10 min), dehydrated in an ethanol series, infiltrated with butyl-methyl-methacrylate containing 10 mM DTT, and polymerized under long-wavelength UV light at 4°C for 4 h, essentially as described by Baskin et al. (1992). Semithin sections (0.5 μm) were cut on an ultramicrotome (Leica Ultracut-S) and collected on silanized slides (Gottlieb and Glaser, 1975).

Sections were extracted in acetone for 10 min and rehydrated in PBS containing 0.05% (v/v) Tween 20 and then incubated for 10 min in PBS containing 1% Tween 20. Some sections were stained in thiazole orange (0.1 mg mL^{-1} in distilled water; Molecular Probes, Eugene, OR) for 15 min, rinsed briefly, and mounted in a commercial antifade (Vectashield, Vector Laboratories, Burlingame, CA). Other sections were incubated in antibody against p40 (1:300 dilution) for 2 h at 37°C and rinsed in PBS-0.05% Tween (3 × 10 min), and then in secondary antibody (1:200 dilution, goat anti-rabbit immunoglobulin, conjugated to cy-3; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at 37°C. After rinsing in PBS-Tween (3 × 10 min), sections were mounted in Vectashield. Sections on control slides were incubated in PBS-Tween in place of the primary antibody. A Zeiss Axioplan was used for microscopy. Fluorescence from thiazole orange was examined with a standard cube for fluorescein and from cy-3 with a standard cube for rhodamine. For Nomarski images, a 1.4-numerical aperture condenser was oiled to the slide.

RESULTS

Electrophoretic Comparison of p40 in Different Plant Species and Tissues

Immunoblot analysis of protein extracts on a fresh weight basis (Fig. 1) indicated the presence of p40 proteins in all 10 plant species examined. The apparent size of the plant p40 homologs varied from about 39 kD in Arabidopsis to about 45 kD in common bean. The number of bands from each species also varied, some (e.g. corn and wheat) having several bands that spanned a wide size range, and

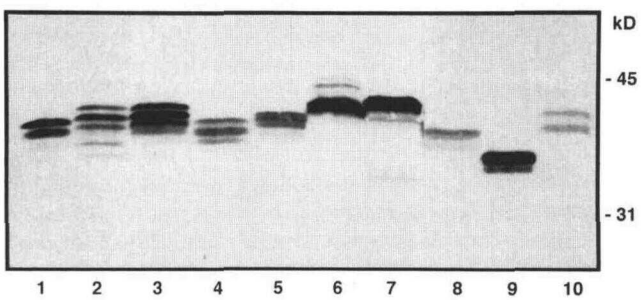

Figure 1. Presence of p40 in various plant species. Western blot analysis of total leaf protein extracts from oat, wheat, corn, alfalfa, red clover, soybean, common bean, pea, Arabidopsis, and cucumber for lanes 1 to 10, respectively.

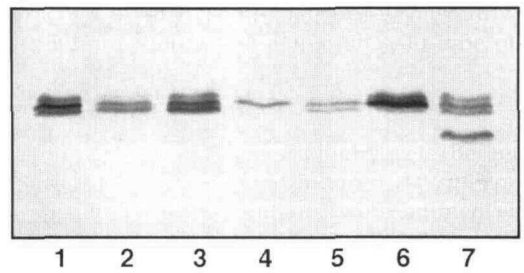

Figure 2. Presence of p40 in soybean tissues. Western blot analysis of total protein extracts (30 μg) from different organs of 8-d-old soybean seedlings. Lane 1, Whole 1-cm epicotyls; lane 2, apical 1-cm hypocotyl; lane 3, apical meristem; lane 4, cotyledon; lane 6, root tip; and lane 7, expanding primary leaf. Protein from mature flowers is shown in lane 5.

others (e.g. soybean and common bean) in which essentially all of the protein detected was in two bands of nearly identical mobility.

We investigated the presence of p40 in different organs in 8-d-old soybean seedlings and in flowers. The immunoblot in Figure 2 shows that p40 was present in all organs of the soybean seedling that were examined, but its abundance relative to total protein was higher in actively growing tissues (young epicotyls, lane 1; apical meristems, lane 3; root tips, lane 6; and expanding primary leaves, lane 7), whereas it was less prevalent in more quiescent organs (hypocotyls, lane 2; cotyledons, lane 4; and mature flowers, lane 5). There was also variation in the number of protein bands and the intensity of each band on the immunoblot among the different organs. In cotyledons, only one band of about 43 kD was resolved; in roots, two bands of about 43 and 44 kD were evident; and in all other organs, three bands were present (about 42, 43, and 44 kD). The additional band corresponding to a protein of only about 35 kD was consistently observed in expanding leaves and may be a degradation product. Whether the different bands in these tissues correspond to different isoforms of the same polypeptide, or to closely related gene products cross-reacting with the antiserum, is not known.

Abundance of p40 in Soybean Flowers and Developing Seeds

We carried out a series of studies on different tissues at various stages of development. These were designed with a double aim: first, to determine the abundance of p40; and second, to investigate the polyribosome content (Davies and Abe, 1995). Isolated ribosomes were fractionated on Suc gradients, and the recorded polysome profiles were used to determine the percent of ribosomes present in polysomes (denoted as $P/T \times 100$).

During the early stages of seed development (1- to 6-mm seeds), characterized by active cell division, the level of p40 both on a per unit fresh weight and a per unit protein basis was relatively high and then declined as seed development progressed (Fig. 3A). During the period of active cell expansion (6–8 mm), the relative concentration of p40 remained almost constant, whereas the amount of p40 per

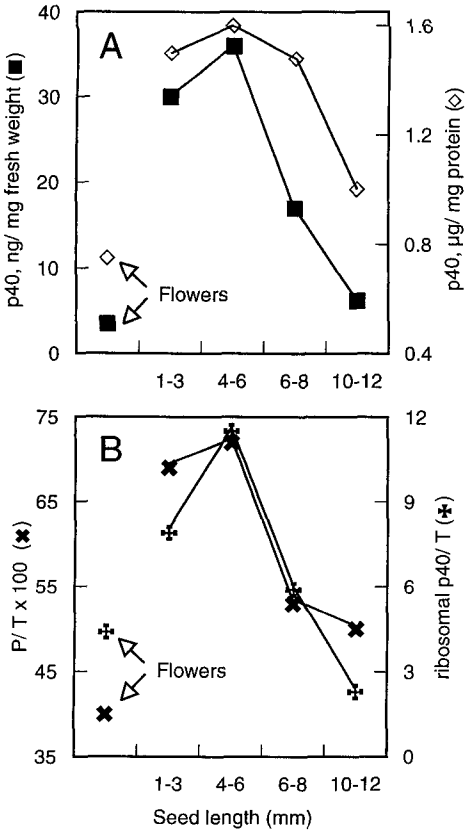

Figure 3. Abundance of p40 and polysome formation in soybean flowers and developing seeds. A, Abundance of p40 in whole extracts on a fresh weight and total protein basis. Values were obtained by comparison to an internal standard (see "Materials and Methods"). B, Abundance of ribosome-associated p40 standardized to the total amount of ribosomal material (p40/T). P/T × 100 is the ratio of the A_{254} for polysomal material to the total absorbance derived from Suc gradients. The experiment was performed twice with similar results.

unit fresh weight decreased about 2-fold. At the midmaturation stage (10–12 mm), during which cell growth declined, the relative concentration of p40 declined about 1.5-fold and the amount of p40 per unit fresh weight declined an additional 3-fold. For comparison, the values of p40 in flowers at the time of fertilization are shown.

When the proportion of ribosomes in polysomes was examined (Fig. 3B), a similar pattern was seen. Polysomes accounted for a maximum of 72% of total ribosomal material when seeds were 4 to 6 mm in size, after which the level declined sharply. This shift in polysomes was accompanied by a parallel loss of p40 per total ribosomal material (Fig. 3B). These results demonstrate that p40 is more abundant during periods of active cell division and cell expansion, periods in which the level of polysomes (and presumably protein synthesis) is highest. The amount of p40 per ribosome also correlates with the proportion of polyribosomes in tissues.

p40 Content during Germination of Soybean Seeds

We previously observed that in Arabidopsis, p40 existed in two forms in the cytoplasm: easily pelletable (ribosomal) and nonpelletable (soluble or nonribosomal) (García-Hernández et al., 1994). The proportion of each form was somewhat variable, depending on the growth rate and metabolic activity of the tissue. Therefore, we examined the relative amount of the two forms of p40 in both the cotyledon and embryo axis during imbibition of dry seed.

The amount of soluble p40 per unit cotyledon (Fig. 4A) increased about 2-fold in the first 36 h of imbibition and then declined gradually through d 8. In contrast, the level of ribosome-associated p40 per unit cotyledon also increased about 2-fold during the first 2 d, but then continued increasing throughout the 8-d experiment. This suggests the occurrence of a net shift of p40 from the soluble to the ribosome-associated pool. Soluble p40 was more abundant than ribosome-associated p40 throughout the experiment. The pattern of increase for the amount of ribosome-associated p40 paralleled the growth (fresh weight) of cotyledons.

The results were similar for the embryo hypocotyl-radicle axis. The level of soluble p40 per unit axis (Fig. 4B) increased about 3.5-fold during the first 2 d of imbibition, whereas the ribosome-associated p40 (Fig. 4B) increased about 13-fold over the same period. Again, soluble p40 decreased after this time, whereas p40 associated with ribosomes increased slightly. As expected, axis fresh

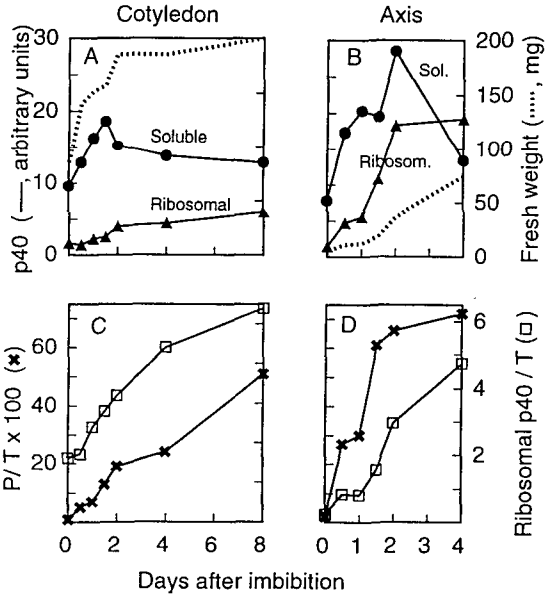

Figure 4. Abundance of p40 and polysome formation in germinating soybean seeds. Soybean seeds were allowed to imbibe in water for up to 8 d. Embryo axes were dissected out when dry (0 time) or every 12 h after imbibition began. Supernatant and polysome fractions from the embryo axes and the cotyledons were obtained, and the amount of p40 in each fraction was determined as described in "Materials and Methods." A, Soluble and ribosome-associated p40 per unit cotyledon and fresh weight of cotyledons. B, Same as in A for embryo axes. C, Proportion of polysomes (P/T × 100) and amount of ribosome-associated p40 per total ribosomal material (p40/T) in cotyledons at different times after imbibition. D, Same as in C for embryo axes. The experiment was performed three times with similar results.

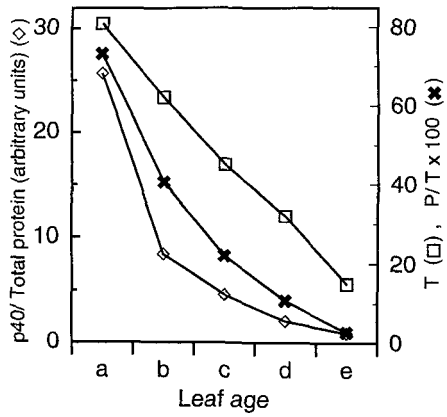

Figure 5. Abundance of p40 and ribosome content in soybean leaves of different ages. Soybean plants were grown in the greenhouse under a 16-h photoperiod for 6 to 8 weeks. Leaves were harvested from the top, nonexpanded leaves (a), to the bottom, fully expanded leaves (e). T, Total ribosomal material; P/T × 100, proportion of ribosomal material in polysomes. The experiment was performed twice with similar results.

weight increased during the experiment due to cell enlargement, followed by cell division.

Dry cotyledons contained abundant amounts of total ribosomes (not shown), but less than 2% were in polysomes (Fig. 4C). Polysome formation began soon after imbibition and increased to about 50% of the total ribosomal material during the first 8 d of imbibition. The ratio of ribosome-associated p40 to p40/*T* also increased about 2.5-fold from dry to 8-d-imbibed cotyledons, closely paralleling polysome formation.

Similar results were found for the embryonic axes of germinating seeds. The dry axes contained abundant ribosomes (not shown), but as in dry cotyledons, the proportion of polysomes was very low (2%) (Fig. 4D). After imbibition, the polysome content of embryonic axes increased markedly, comprising about 70% of the total ribosomal material after 4 d. Over the same period, the ratio of ribosomal p40 to total ribosomal material increased about 20-fold. These results demonstrate that the number of p40 molecules associated with ribosomes increased as polyribosomes formed, possibly by recruiting p40 from the soluble pool.

Abundance of p40 during Leaf Development

To further investigate the role of p40 during plant development, we examined its presence during both soybean and corn leaf development. In soybean, the amount of p40 per unit total protein extracted (Fig. 5) was highest in the youngest, nonexpanded leaves and declined sharply in older, fully expanded leaves. When the ribosomes of these tissues were analyzed, there was a similar decrease in the total ribosomal material and in polysomes ($P/T \times 100$) with age.

Previous observations suggested that light influenced the level of p40 in plant tissues. To address this question, corn plants were used because they develop in the dark through the four- to five-leaf stage. Maize plants were grown in complete darkness or under a 12-h photoperiod for 10 d, at which time both groups of plants had developed three leaves. To study the spatial distribution of p40, the second leaf of each set of plants was harvested and cut into three regions: basal (youngest region), middle, and apical (oldest region), and each region was analyzed as in Figure 5. The results presented in Figure 6 show that the level of p40 relative to total protein varied in the different regions of the corn leaf and was affected by light. The amount of p40 decreased markedly from the youngest, basal region of the leaf to the oldest, apical region in both dark- (6-fold) (Fig. 6A) and light-grown plants (30-fold) (Fig. 6B). Polysome content ($P/T \times 100$) decreased in the same manner with age in both sets of plants, whereas the amount of total ribosomal material (*T*) decreased somewhat slower. Light-grown plants had a lower content of p40 and ribosomal material than did plants grown in the dark in each respective region of the leaf, although the proportion of polysomes was about the same.

Effect of IAA on p40 Content and Polysome Formation

The data presented so far show that p40 changes in abundance during plant growth and development, processes known to be governed by plant hormones, especially auxins. Thus, the possibility that auxin directly affects the abundance or distribution of p40 was examined. We used aged pea epicotyls, a system in which IAA has been shown to cause polysome formation and increase protein synthe-

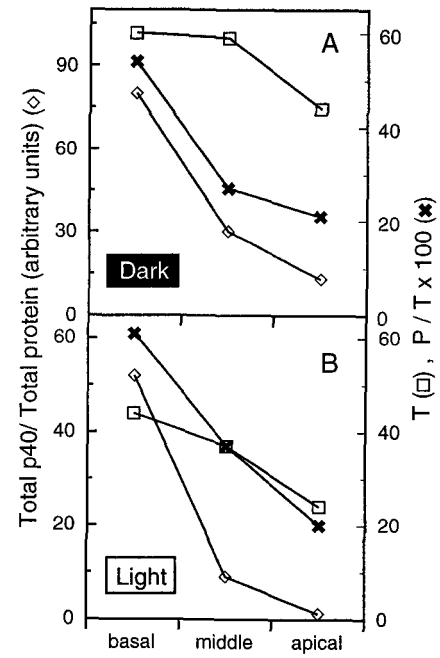

Figure 6. Spatial distribution of p40 and polysome content in light- and dark-grown corn leaves. Maize seedlings were grown in the dark (A) or under a 12-h photoperiod (B) for 10 d. The second leaf of each set of plants was cut into three regions: basal (youngest), middle, and apical (oldest). The experiment was performed twice with similar results.

sis both a few hours after treatment and before measurable changes in growth (Davies and Larkins, 1972).

Intact etiolated pea epicotyls that had been aged for 2 d were left untreated (control) or were treated with IAA and harvested after 3, 6, 9, or 12 h. Ribosomes from an equal weight of fresh tissue were then fractionated on Suc gradients, and 10 fractions from each gradient were collected and analyzed for the presence of p40. The proportion of total p40 located in the regions corresponding to the subunits and monosomes (Fig. 7A, fractions 2–4) decreased progressively from 54% prior to treatment to 10% after 9 h of auxin treatment. Although polysomes increased about 50% during this time (Fig. 7B), the amount of p40 present in polysomes (Fig. 7, A, fractions 5–10, and B) doubled, increasing from 46 to 90% of the total p40. Total p40 decreased only slightly during the experiment. Thus, IAA caused an increase in the number of p40 molecules per ribosome in the polysomal fraction. By 12 h of auxin treatment this trend had reversed and p40 increased slightly in monosomes and subunits and decreased slightly in polysomes.

The accumulation of p40 in polysomes after IAA treatment could be due to (a) an increase in the total amount of p40, (b) a shift from the soluble pool to the ribosomal pool, (c) a shift from the ribosomal subunits and monosomes to polysomes, or (d) a combination of these. IAA treatment did not significantly affect the total amount of p40 in these experiments. Recruitment entirely from the soluble p40 pool to ribosomes seems unlikely, since the amount of soluble p40 in these tissues was very low, judged by its absence from the top of the gradients (Fig. 7A). Hence, a shift of p40 from monosomes to polysomes seems likely.

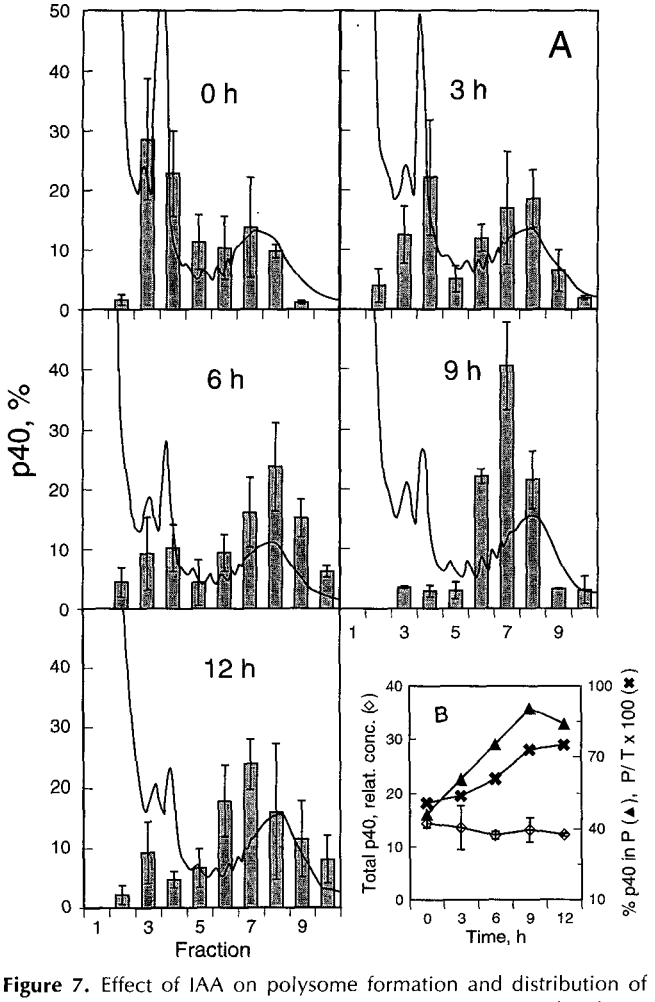

Figure 7. Effect of IAA on polysome formation and distribution of p40. Aged pea epicotyls were left untreated or were treated with 1% IAA in petroleum jelly for 3, 6, 9, or 12 h. A, Distribution of p40 on polysome gradients. Bars indicate the amount of p40 in each fraction expressed as percentage of the total ribosomal-associated p40. Error bars indicate SD of two independent experiments. The continuous line (polysome profile) represents A_{254}. B, Effect of IAA on the total amount of p40 recovered from Suc gradients (Total p40), proportion of p40 in polysomes (%p40 in P), and percent ribosomal material in polysomes (P/T × 100).

Subcellular Localization of p40

We used Arabidopsis roots to localize p40 at the light microscope level because of their strong reactivity with our antibody, which was made against bacterial-expressed Arabidopsis p40 (García-Hernández et al., 1994). When stained with anti-p40, bright fluorescence was observed in all tissues of the root. Figure 8A shows typical results for the apical region of the root, where cells are actively dividing. For this panel, 100 ASA film (Kodak) was exposed for 9 s, indicating the high level of staining. Controls, in which the primary antibody was omitted, had barely visible levels of autofluorescence (not shown). The staining appeared to be cytosolic, with no staining in nuclei, cell wall, or vacuoles. A similar cytosolic pattern of staining was also observed in elongating and differentiating regions of the root (not shown). To compare the anti-p40 staining pattern with the localization of ribosomes, we used the RNA stain thiazole orange. The pattern of staining seen with thiazole orange (Stankovic et al., 1993) (Fig. 8B) was similar to that of anti-p40, but thiazole orange also stained the nucleolus.

The cytosolic nature of the anti-p40 staining is clearly shown in a higher-magnification view of a group of cells at the basal end of the zone of cell division (Fig. 8C). Furthermore, neither the tonoplast nor the plasma membrane was appreciably labeled, insofar as areas where cytosol is excluded between appressed membranes showed no staining (Fig. 8C, thin arrows). The bright fluorescence in the cells is frequently interrupted by large and small spaces. The cytosol appears similarly interrupted with thiazole orange staining (Fig. 8B, inset). Comparison of paired anti-p40 and Nomarski images (Fig. 8, C and D) suggests that some of these spaces are less dense than their surroundings (e.g. thick arrow), whereas others are denser (e.g. double arrowhead). The less-dense spaces are probably vacuoles, whereas the heavier ones are presumably other types of organelles such as plastids, mitochondria, or Golgi. Both types of spaces exclude p40 staining, as expected for a cytosolic marker.

DISCUSSION

p40 appears to be ubiquitous in plants, and the Arabidopsis p40 antiserum produced a strong signal in each of the diverse plant species tested. This is consistent with

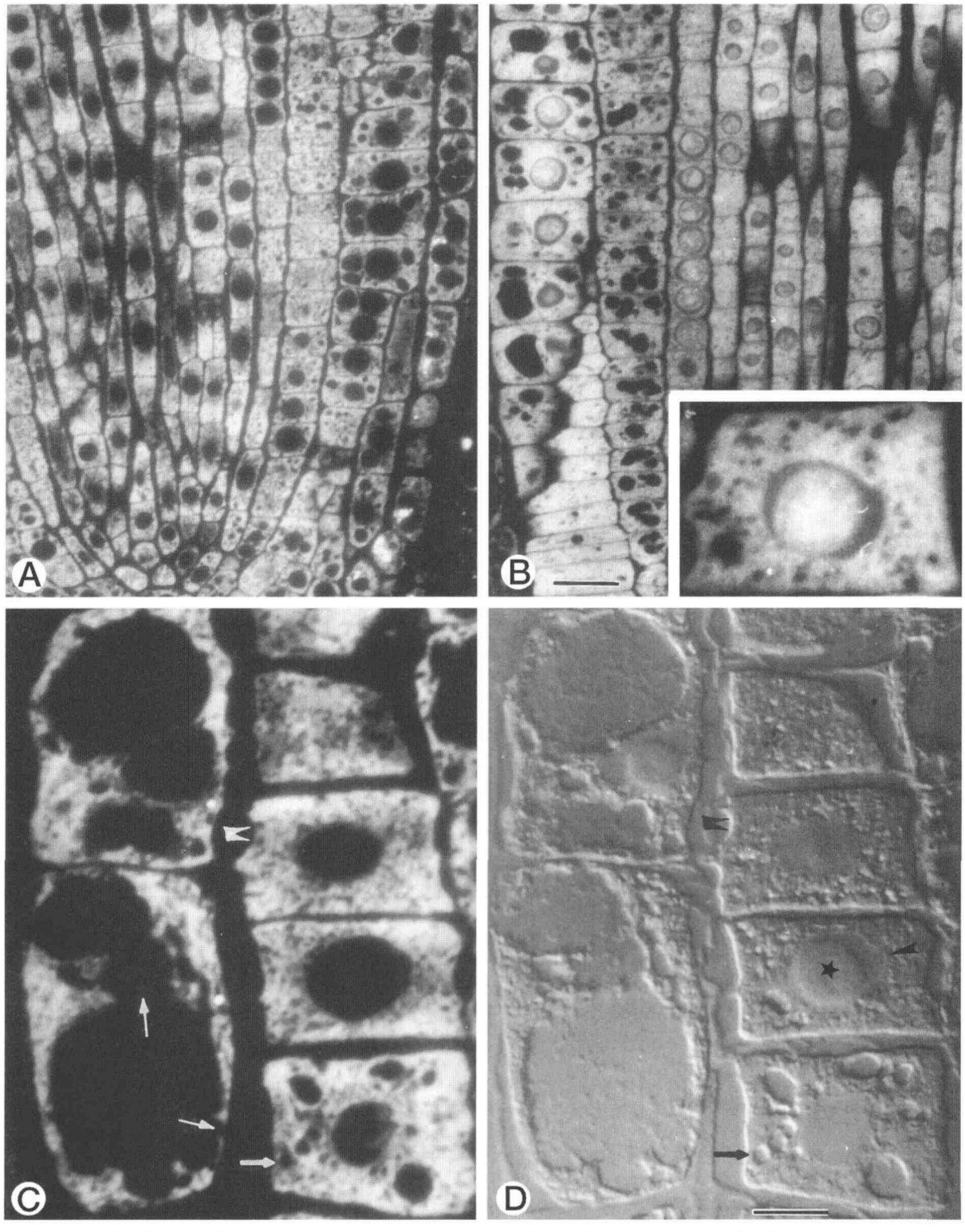

Figure 8. Subcellular localization of p40. Photomicrographs of semithin methacrylate sections of Arabidopsis roots, showing distribution of anti-p40 reactive material and of RNA. A, Section stained with anti-p40 and detected with secondary IgG conjugated to cy-3. B, Section from the same root stained with thiazole orange to localize RNA. Inset shows a higher-magnification view of a single cortical cell. C and D, Paired fluorescence and Nomarski images of a section stained with anti-p40. Thin white arrows (C) point to places where cytosol is largely excluded between two membranes by oppression and no staining is detected. Examples are shown of gaps in the anti-p40 staining that correspond to dense inclusions (double arrowheads in C and D) and an empty or light inclusion (thick arrows in C and D) in the Nomarski image. A, 1175×; bar = 10 μm. B, 1175×; bar = 4.4 μm; inset, 2670×. C and D, 2340×, bar = 5 μm. Star in D indicates cell nucleus.

previous studies that identified p40 in several eukaryotic organisms as distant phylogenetically as plants (Axelos et al., 1993; García-Hernández et al., 1994), mammals (Makrides et al., 1988; McCaffery et al., 1990; Grosso et al., 1991), *Hydra* (Keppel and Schaller, 1991), *Drosophila* (Melnick et al., 1993), yeast (Davis et al., 1992), and sea urchin (Rosenthal and Wordeman, 1995). The ubiquity of p40 in eukaryotes and the similarity among the proteins for which sequences have been deduced suggest that p40 plays a fundamental role in cell metabolism.

The electrophoretic variability of p40 even among closely related plants was somewhat surprising considering the high level of sequence conservation across the plant and animal kingdoms. The composition of p40 among legumes was as varied as between legumes and more distantly related plant species. Animal p40s also vary in size, with mobilities on SDS-PAGE ranging from 33 kD in *Hydra* (Keppel and Schaller, 1991), 43 kD in mouse (Auth and Brawerman, 1992) and yeast (Davis et al., 1992), to 46 kD in chick (Rabacchi et al., 1990). The functional significance of this variation is not known. No significance can be ascribed to possible quantitative variation among plant species examined here because it is not known if the Arabidopsis p40 antiserum reacts equally with all p40 proteins.

Our previous work (García-Hernández et al., 1994) indicated that only a single band was detected with our p40 antiserum for both Arabidopsis and corn, compared to the multiple bands detected here. This difference was partially due to the use of a longer SDS polyacrylamide gel in this study, giving greater resolution. However, tissue-specific variation may also be a factor. Leaves were used in this study, whereas suspension-cultured cells and endosperm were used previously for Arabidopsis and corn, respectively. It is likely that at least some of the heterogeneity of p40 within a species is the result of multiple genes, since Arabidopsis has at least two transcribed genes (García-Hernández et al., 1994), the second cDNA having recently been cloned and sequenced (P. Staswick, unpublished data).

Our results indicate that in plants, as in animals, p40 is more abundant in young or actively growing tissues. This was true for soybean developing seeds, germinating cotyledons and embryonic axes, and young seedlings. Developmental studies on older plants also confirmed that p40 was more prevalent in younger soybean leaves and in the youngest basal portion of corn leaves. Heterogeneity in size and number of p40 protein bands was also evident among soybean tissues, but the significance of this result is not known.

In general, there was a correlation among higher cellular p40 level, increased fraction of ribosomes in polysomes, and an increase in the proportion of total p40 that was associated with ribosomes (as opposed to soluble p40). An increase in polysomes often, although not necessarily, corresponds with increased protein synthesis. For example, protein synthesis increases dramatically in developing soybean seeds at midmaturation stage (Goldberg and Barker, 1989; Bewley and Black, 1994), and this was the time period when the level of polysomes and the association of p40 with ribosomes was maximal in our study (Fig. 3B). Although the correlative nature of these results does not allow us to conclude that p40 plays a direct role in protein synthesis, the results are consistent with that possibility.

In soybean cotyledons, p40 was found at lower levels than in the embryo axes after 2 d of imbibition, and the soluble-p40 was always higher than the ribosome-associated p40. In contrast, in embryo axes, which had a higher proportion of polysomes, the total amount of p40 was higher than in cotyledons, and the proportion of ribosome-associated p40 was also higher. In fact, in tissue with the highest proportion of polysomes, the relative amount of ribosome-associated p40 surpassed that of the soluble p40. Since the total amount of p40 per unit embryo decreased during this period (not shown), p40 must have been recruited from the soluble form to the ribosome-associated form. In addition, the fact that the amount of p40 per ribosome increased (Fig. 4, C and D) corroborates our previous hypothesis that p40 is not a core ribosomal protein, which would be expected to maintain a constant stoichiometry with ribosomes.

The analysis of ribosomal material (T and P/T) in soybean leaves of different ages showed a correlation between levels of p40 and total ribosomal material and polysome content, since all decreased with age. The results obtained with corn leaves (Fig. 6) pointed to a closer correlation between p40 and amount of polysomes (P/T), rather than to the content of total ribosomes (T). That is, active ribosomes in polysomes contain more molecules of p40 than do inactive ribosomes (monosomes). This is consistent with previous suggestions that p40 may play a role in translation (García-Hernández et al., 1994).

The fact that the level of p40 in etiolated tissue was higher than in green tissue, even though P/T values were lower for etiolated than for light-grown tissue, could be explained if p40 were present primarily or exclusively on cytoplasmic ribosomes. Chloroplast ribosomes would thus "dilute" the pool of cytoplasmic ribosomes that contain p40. In fact, we could not detect the presence of p40 either in mitochondria or in chloroplast extracts (not shown).

Further evidence that p40 plays a role in plant growth and development came from an investigation of auxin-mediated growth response. IAA caused a dramatic net shift in p40 from monosomes and ribosomal subunits to polysomes (Fig. 7, A, bars, and B) during polysome formation (Fig. 7, A, line, and B). Moreover, the maximum accumulation of p40 in polysomes occurred during the period that preceded growth, for which previous studies have shown that maximum amino acid incorporation and RNA translatability occur (Davies and Larkins, 1972)

The mechanism(s) of auxin action on growth and development is (are) still unclear. However, auxin-mediated growth induction requires the synthesis of RNA and protein (reviewed by Guilfoyle, 1986). Exogenous application of auxin has also been reported to alter the pattern of protein synthesis (Zurfluh and Guilfoyle, 1980; Schuster and Davies, 1983; Meyer et al., 1984; Gantt and Key, 1985; Pérez et al., 1987; Edelmann and Schopfer, 1989; Edelmann and Kutchera, 1993), the translatability of mRNAs (Davies and Larkins, 1972; Zurfluh and Guilfoyle, 1982; Gantt and

Key, 1983), the accumulation of rRNA (Melanson, 1978), and the phosphorylation of certain ribosomal proteins (Pérez et al., 1987, 1990). Auxin also caused an increase in the coordinated expression of ribosomal proteins (Gantt and Key, 1985; Gao et al., 1994). However, to our knowledge, this is the first report of a ribosome-associated protein whose recruitment into polysomes is elicited by auxin. These results suggest that p40 may be an important factor in the long-term growth responses evoked by auxin.

The subcellular immunolocalization of p40 in Arabidopsis roots (Fig. 8) confirmed the exclusively cytosolic distribution of p40. Moreover, the staining of p40 was similar to that of RNA (ribosomes), with the only exception being the lack of staining in nuclei. Although all root tissues exhibited staining with this antiserum, it is possible that tissue-specific gene expression occurs. We now know that extensive regions of at least two Arabidopsis p40 genes are identical in sequence (P. Staswick, unpublished data). Thus, the polyclonal antisera used here, which was derived from one gene, would undoubtedly react with both gene products. Antisera specific to unique regions of each protein may reveal additional details about p40 function in Arabidopsis.

We previously suggested (García-Hernández et al., 1994) that p40 was not a core ribosomal protein for two main reasons: first, because of its acidic pI; and second, because it can be found in two pools, soluble and ribosome associated. The work presented here supports the hypothesis that p40 is an accessory component of ribosomes. Ribosomal protein genes are differentially expressed in plants (Marty and Meyer, 1992; Taylor et al., 1992; Gao et al., 1994), and the synthesis of rRNA and ribosomal protein mRNAs seems to be coordinated (Gantt and Key, 1983, 1985). Although no studies exist on plants, in other eukaryotes ribosomal protein accumulation is coordinated with ribosome formation and occurs stoichiometrically with ribosomes (Kaspar et al., 1993). Here we have shown that the amount of p40 per ribosome varies during development. The absence of p40 in the nucleus is also consistent with p40 being an accessory protein involved in ribosome function rather than a core protein that is assembled in the nucleus.

To summarize, we have shown that the amount of p40 varies in plants during development. The level of p40 correlated with the extent of polysome formation and the growth regulator IAA caused recruitment of p40 into polysomes in parallel with polyribosome formation. The cytosolic localization of p40 indicated that p40 associates with ribosomes after their assembly. We suggest that p40 may play an important role during plant growth and development, possibly by regulating the activity of ribosomes.

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