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Martha T. Hamblin for the degree of Master of Science in Botany and Plant Pathology presented on March 18, 1982.

Title: RIBULOSE BISPHOSPHATE CARBOXYLASE: A MARKER FOR THE GERMINATION PATHWAY IN WHEAT EMBRYOS

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Abscisic acid (ABA) at $10^{-4}$ M prevents the synthesis of the small subunit of ribulose bisphosphate carboxylase (RuBPCase) (E.C.4.1.1.39) in wheat embryos (Triticum aestivum L. var. Yamhill) during the first four days of grain imbibition, as demonstrated by in vivo labelling with $^{35}SO_4^{-2}$. Germinating the embryos in darkness causes a reduced but detectable amount of RuBPCase synthesis, compared to that in embryos grown in the light.

RuBPCase is identified as a 23 kilodalton protein precursor in cell-free translations of wheat leaf RNA as evidenced by its precipitation with antibody to wheat RuBPCase. This precursor is synthesized in cell-free translations using total and poly (A) RNA from shoots of dark- and light-grown embryos, but not in translations primed with RNA from ABA-treated embryos or unimbibed embryos.

Synthesis of the small subunit of RuBPCase appears to be transcriptionally regulated in two separate processes: 1) a low level of synthesis is initiated as part of the germination program, independent of light, and 2) a higher level of synthesis is reached in response to illumination. Incubation of embryos in ABA inhibits both of these processes.

# Ribulose Bisphosphate Carboxylase: A Marker For The Germination Pathway In Wheat Embryos

by

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# RIBULOSE BISPHOSPHATE CARBOXYLASE: A MARKER FOR THE GERMINATION PATHWAY IN WHEAT EMBRYOS

## I. INTRODUCTION

### Rationale

Embryogenesis, germination, and early seedling development involve the precise orchestration of many complex genetically programmed events, corresponding to the appearance of particular proteins. In most higher plants, the progression through embryogenesis to germination is interrupted by a period of desiccation and developmental arrest, which may or may not constitute a true state of dormancy (Walbot, 1978). It is resumed upon exposure of the mature seed to favorable growth conditions which stimulate germination. Once the cell division and tissue formation of early embryogenesis are complete, however, it is possible for young embryos of many species to germinate directly when removed from ovular tissues and cultured in an appropriate nutrient medium. Wheat embryos become capable of precocious germination at 15-20 days post-anthesis (DPA) (King, 1976). Addition of an ovular extract will prevent precocious germination of young cotton embryos (Ihle and Dure, 1972), as will the hormone abscisic acid (ABA). Failure of these embryos to germinate in culture is not the result of a cessation of metabolic activity, however. Embryos exposed to ABA continue to develop, but in ways similar to the normal course of embryogenesis in the ovule, synthesizing storage proteins, lipids, and enzymes, as well as increasing in size and dry and fresh weight (Crouch and Sussex, 1981; Choinski, et al., 1981; Triplett and Quatrano, 1982). King (1976) showed that the ABA content of developing wheat grains starts to

rise at 20-25 days after anthesis, is high from 25-40 days after anthesis, and then drops sharply, coincident with water loss in the grain. Most of the ABA present in the grain is localized in the embryo. The correlation of endogenous ABA levels with the effects of ABA on isolated embryos *in vitro* suggests that ABA is a natural regulator in the intact wheat grain, somehow acting to prevent germination and promote continued embryogenesis leading to developmental arrest.

Given this system in which isolated immature embryos can be cultured *in vitro* and induced to follow one of two possible developmental programs, (i.e. germination or continued embryogenesis) it should be possible to identify the specific gene products which are associated with each of those programs and to study the regulation of those products by ABA. Previous work on this project (Triplett and Quatrano, 1982) has identified wheat germ agglutinin (WGA) as a protein unique to the embryogenic pathway: WGA is synthesized *de novo* beginning at 25-30 DPA (i.e. about midway through grain development *in situ*), and is produced in isolated 18 DPA embryos cultured in the presence of ABA. Eighteen DPA embryos grown without ABA do not accumulate significant amounts of WGA, yet are able to germinate into normal seedlings. WGA, then, seems to be a gene product not essential for germination, which is expressed only in embryos following the pathway to developmental arrest, and has therefore been chosen as a marker protein for that pathway. The overall objective of my research is to look at the synthesis and regulation of the small subunit of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase (E.C.4.1.1.39) (RuBPCase) to determine if it can serve as a useful marker protein for the germination pathway.

## Characteristics of RuBPCase and Its Synthesis

RuBPCase, which catalyzes the first step of the Calvin-Benson $CO_2$-fixation cycle, has been called the most abundant protein on earth, and is certainly the major soluble protein in the green leaf (Kung, 1976). It has been studied extensively and in many plant species from green algae to cereals and dicots; much is known about its structure, genetics, activity, and synthesis. A 16-subunit enzyme, RuBPCase is composed of eight identical large subunits which range in size from 51,000-58,000 daltons, depending on the species, and eight identical small subunits of 12,000-18,000 daltons (Jensen and Bahr, 1977). In wheat, these subunits were determined by SDS polyacrylamide gel electrophoresis to have molecular weight of 53,000 and 13,500 (Yeoh, et al. 1979). The large subunit, which contains the catalytic site of the enzyme, is coded for by chloroplast DNA and synthesized on chloroplast ribosomes. The small subunit is nuclear-encoded and synthesized on cytoplasmic ribosomes, transported across the chloroplast envelope and assembled into the holoenzyme. The function of the small subunit is unknown and is assumed to be regulatory (Kung, 1976). Synthesis of the large and small subunits does not appear to be tightly coupled (Ellis, 1981), although there have been reports which suggest otherwise (e.g. Iwanji, et al. 1975).

Because the small subunit is nuclear-encoded and, therefore, subject to the various levels of control which operate in all eukaryotic cells, it was decided to study the synthesis and regulation of the small subunit only. Questions of assembly and enzyme activation will not be addressed in this thesis. My approach involves characterization of the effects of light and ABA on the pattern of RuBPCase expression

at the level of protein and mRNA, through the use of in vivo labeling and a cell-free translation system. In conjunction with this work, I have partially purified the mRNA for the small subunit of wheat RuBPCase so that it can be used to synthesize a cDNA as a probe to detect low levels of mRNA and to screen a genomic library for the gene sequence. In this way one can determine when the gene is first transcribed in embryos which are committed to germination.

## Discovery that the Small Subunit of RuBPCase is Made as a Precursor

When plant leaf RNA is translated in a cell-free system, none of the major proteins synthesized comigrates with the small subunit of RuBPCase. Roy, et al. (1976) working with wheat, and Dobberstein, et al. (1977), working with Chlamydomonas, found that antibodies to the small subunit precipitated a cell-free product of 20,000 molecular weight (MW), several kilodaltons (kd) larger than the authentic protein, suggesting that the small subunit was synthesized as a larger precursor. Dobberstein, et al. (1977) confirmed this hypothesis by demonstrating that the 20 kd polypeptide was taken up by isolated chloroplasts and processed to its final size. Highfield and Ellis (1978) subsequently showed that this same phenomenon occurs in pea. They found that the processing of the polypeptide can take place in the absence of protein synthesis, which distinguishes it from processing according to the "signal peptide" model. Table I presents data which have been reported on small subunit precursors in various plant species.

Table I. Molecular Weights of the Small Subunit Precursor and Native RuBPCase in Various Species.

| Species | Native | Precursor | Reference |
|---|---|---|---|
| Pea | 14,000 | 20,000 | Highfield and Ellis, 1978 |
| Tobacco | 12,000 | 20,000 | Lett, et al. 1980 |
| *Lemna* | 12,000 | 20,000 | Tobin, 1978 |
| *Chlamydomonas* | 16,500 | 20,000 | Dobberstein, et al. 1977 |
| Cucumber | not reported | 25,000 | Walden and Leaver, 1981 |
| Wheat | 12,000 | 20,000 | Roy, et al. 1976 |
| Spinach | 14,000 | 18,000 | Chua and Schmidt, 1978 |

Regulation of Chloroplast Proteins by Light

Although the primary motive for this research is to study the effects of ABA on embryo development, the role of light in the regulation of a photosynthetic enzyme is also of obvious interest. Low levels of $CO_2$-fixation enzymes are present in etiolated plants, and increase 2-3 fold during the greening process (Kirk and Tilney-Bassett, 1978). Etiolated plants contain no functional chloroplasts and no chlorophyll, but do contain the chlorophyll precursor protochlorophyll, which is very quickly (within one minute) photoreduced to chlorophyll upon illumination. This is followed by loss of the crystalline structure of the prolamellar bodies (1-60 min.) and organization of the thylakoid membranes with their pigment/protein reaction centers. Increase in RuBPCase activity in response to light is much less rapid, having a lag time of 5-6 hours in etiolated barley (Smith, et al. 1974). Synthesis of the subunits as seen by incorporation of $^{14}C$-amino acids, however, increases immediately upon illumination.

The mechanism involved in the light-stimulated synthesis of RuBPCase is not known. A system which is better understood is the light-induction of the chlorophyll a/b binding protein, which is similar to the small subunit of RuBPCase in being cytoplasmically synthesized as a precursor polypeptide and transported into the chloroplast (Apel and Kloppstech, 1978). Unlike the photosynthetic enzymes, the chlorophyll a/b protein is not detectable in etiolated plants. The mRNA is also not detectable in dark-grown leaves of barley (Apel and Kloppstech, 1978), although Cuming and Bennett (1981) report it to be present at low levels in leaves of etiolated

peas. In both systems, however, short pulses of either red or white light are sufficient to induce high levels of mRNA for the chlorophyll a/b protein, suggesting regulation by means of a phytochrome response. The protein itself does not accumulate under these conditions. Presumably this is because chlorophyll synthesis requires continuous illumination with white light, and the protein is not stable unless it is incorporated into the pigment/protein complex. This hypothesis is supported by the fact that mutants lacking chlorophyll b also fail to accumulate the protein even when grown in the light.

The effect of light on synthesis of RuBPCase is not as clear, which is perhaps not surprising since it is not directly involved in the light reactions of photosynthesis. As mentioned above, low levels of enzyme are detectable in etiolated tissue, yet it has been reported that, in pea (Bedbrook, et al. 1980) and duckweed (Tobin, 1978), no translatable mRNA for the small subunit of RuBPCase can be isolated from dark-grown plants. Giles, et al. (1977), on the other hand, found that polysomal mRNA from either dark- or light-grown bean coded for synthesis of a prominent 20 kd polypeptide, presumably the precursor to the small subunit. Light unquestionably stimulates synthesis of the mRNA (Lett, et al. 1980; Walden and Leaver, 1981; Sasaki, et al. 1981; Tobin and Suttie, 1980); the argument concerns the absoluteness of the requirement. The duration of the dark treatment, the stability of the protein, and the stability of the mRNA would all have bearing on the observed relationships between light, protein, and message. Since both the mRNA and the protein appear to be fairly stable, this can make interpretation and comparison of results quite difficult.

Prolonged etiolation is not a normal situation, so it is important

to look at early development under the influence of a normal photoperiod. In such a study of cucumber seedlings (Walden and Leaver, 1978), it was found that chloroplast rRNA and RuBPCase appear in the cotyledons on the third day after imbibition, coincident with the emergence of the cotyledons above ground and the disappearance of enzymes of the glyoxylate cycle, which break down storage compounds. Chlorophyll does not appear until the fourth day. Keeping the plants in the dark does not alter the time of appearance, or level, or RuBPCase, though it does prevent the formation of chlorophyll. After day 4, however, RuBPCase stops accumulating in the dark, and begins to drop at day 6, while light-grown plants continue to accumulate the protein. These studies suggest that very early synthesis of RuBPCase is programmed to occur upon initiation of germination, and will proceed even in the absence of light, but that continued synthesis to attain normal levels does require illumination and involves a different regulation process. Prolonged etiolation may lead to loss of the mRNA transcribed early in germination.

Regulation of RuBPCase by ABA

If it is true that RuBPCase production is part of the germination program of the dry seed, then one can ask whether it is also part of the program of the young embryo before it becomes committed to developmental arrest, whether its synthesis can be controlled by ABA, and, if so, at what level this control takes place. Dry embryos of wheat grains are known to contain stored mRNA, which is recruited into polysomes upon imbibition even in the presence of α-amanitin and cordecypin, inhibitors

of transcription and polyadenylation, respectively (Huang, et al. 1980). Some of these messages may represent residual mRNA for proteins which also function in embryogenesis, while others may be transcribed during embryogenesis specifically to be used at the time of germination. Translation of these latter mRNAs must be suppressed prior to developmental arrest, when the embryo is still actively synthesizing other kinds of proteins. ABA has been implicated in the suppression of these germination mRNAs. For example, germinating cottonseeds will synthesize carboxypeptidase and isocitratase in the presence of inhibitors of RNA synthesis, presumably using stored mRNA transcribed during embryogenesis (Ihle and Dure, 1972; Harris and Dure, 1978). ABA will prevent translation of these mRNAs, by some mechanism which is apparently dependent on RNA synthesis.

There is some controversy as to the role played by stored mRNAs during early germination. Recently it has become apparent that RNA synthesis does take place very early in imbibing embryos, before actual growth resumes (Huang, et al. 1980; Cheung, et al. 1979), and that germination will not take place if this synthesis is inhibited (Jendrisak, 1980). Marked changes in protein patterns are seen between 40 minutes and 6 hours of imbibition, but do not occur in the presence of α-amanitin (Thompson and Lane, 1980), which inhibits mRNA synthesis. Stored prevalent mRNAs do not appear to be involved in the temporal regulation of early development (Brooker, et al. 1978).

ABA may also be involved in regulation of genes which are newly transcribed during germination. The best-known instance of such an effect is the inhibition of the gibberellic acid-induced production of α-amylase in barley aleurone layers treated with ABA (Ho and Varner,

1976). This effect also appears to be dependent on a short-lived RNA.

In this thesis, I will present evidence that the mRNA for the small subunit of RuBPCase is newly transcribed during germination of wheat, and that this transcription can be inhibited when embryos are incubated with ABA.

# II. MATERIALS AND METHODS

## Plant Material

The endosperm portion of mature wheat grains (Triticum aestivum L. var. Yamhill) was removed and the remaining embryo-containing portion was surface-sterilized in 10% Chlorox. Thirty embryos were germinated on filter paper moistened with either five ml of water or five ml of $10^{-4}$ ABA (Sigma) in a nine cm glass petri dish. All glassware and solutions were autoclaved. For dark-grown embryos, the dishes were wrapped in aluminum foil. All dishes were incubated at 27°C in a growth chamber with a 16 hour photoperiod.

## In Vitro Protein Labelling

Twenty-four hours after imbibition, 150 µCi $^{35}SO_4^{-2}$ (43 Ci/mg) was added to each dish of embryos. Four days after imbibition, the embryos or seedlings were harvested, rinsed in water, and frozen at -20°C. Manipulation of dark-grown embryos was performed using a green safe light (CBS Green 545).

Depending on the treatment, 0.14-0.80 grams of plant material were homogenized in five ml 10 mM Tris-HCl pH 9.0, 10 mM glycine, 1% β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride (PMSF). The homogenate was cleared by centrifugation for thirty minutes at 10,000 rpm in an HB-4 Sorvall rotor and dialyzed overnight against one-tenth strength homogenization buffer. All procedures were performed at 4°C. The dialysate was lyophilized and resuspended in 400 µl of water containing 1 mM PMSF.

## Polyacrylamide Gel Electrophoresis and Fluorography

Samples of radioactively labelled proteins were analysed on discontinuous gels containing sodium dodecyl sulfate (SDS) according to the method of Thomas and Kornberg (1975). The running gel was composed of 18% acrylamide, 0.12% bis, in 0.75 M Tris-HCl pH 8.8, 0.1% SDS, while the stacking gel was composed of 5% acrylamide, 0.16% bis, 0.125 M Tris-HCl pH 6.8, 0.1% SDS. The electrode buffer was 0.05 M Tris base, 0.38 M glycine, 0.1% SDS, pH 8.3. Samples were electrophoresed for six hours at a constant voltage of 100-110 V.

Gels were stained with 0.1% Coomassie brilliant blue R250 in water/ethanol/acetic acid (5:5:1) and destained in water/ethanol/acetic acid (73:20:7). For fluorography, the destain solution was removed by soaking in water at least 30 minutes, followed by 30 minutes in 1 M sodium salicylate on a shaker (Chamberlain, 1979). The gels were then dried onto Whatmann 3 MM paper under Saran wrap on a BioRad gel dryer for two hours. Kodak XR-5 x-ray film was exposed to dried gels at -70°C for various lengths of time.

## RNA Extraction

Plant material to be used for RNA extraction (shoots of seedlings grown without ABA, axes of embryos grown with ABA) was harvested and frozen into liquid nitrogen. Dark-grown tissue was collected under green light.

One gram of frozen tissue was ground to a powder in a mortar, with addition of liquid nitrogen during grinding to prevent thawing.

Depending on the water content of the tissue, 2.6-3.5 ml of homogenization buffer (0.2 M Tris-HCl, pH 9.0, 0.4 M NaCl, 25 mM EGTA, 1% SDS) heated to 100°C was added to the frozen powder and mixed for one minute. The remainder of the RNA extraction procedure follows the method of Hall et al. (1978). Proteinase K (1.25 mg per gram tissue) was added to the homogenate, the mortar was covered and incubated in a 37°C water bath for one hour. The homogenate was then made 154 mM KCl and centrifuged for ten minutes at 10,000 rpm in a Sorvall HB-4 rotor. The supernatant was made 2 M LiCl, kept on ice overnight, and centrifuged fifteen minutes at 10,000 rpm. The LiCl precipitate was resuspended in two ml water, made 0.3 M in NaCl and re-precipitated with 3 volumes of 95% ethanol. The RNA was pelleted by centrifugation for 15 minutes at 10,000 rpm, and resuspended in one ml NET (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5) 0.5% SDS. Any insoluble material was removed by centrifugation for 20 minutes at 10,000 rpm. The RNA was stored at -20°C.

Sucrose Density Gradient Centrifugation

Linear 10-40% sucrose gradients in NET 0.2% SDS were formed by layering seven steps of 1.84 ml each in tubes for the SW-40 rotor. The gradients sat at room temperature for five hours prior to the run. RNA samples (1.2 mg each) were ethanol-precipitated in microfuge tubes and dried in a vacuum dessicator. Each sample was dissolved in 50 µl water and 50 µl dimethylsulfoxide, heated to 65°C for five minutes, quick-chilled on ice, and diluted with 500 µl NET 0.2% SDS. Each sample divided among three gradients, 200 µl/tube. The gradients

were centrifuged in a Beckman L5-65 ultracentrifuge for 15 hours at 30,000 rpm at 20°C in an SW-40 rotor.

Gradients were fractionated by pumping from the bottom of the tube through a 50 µl capillary pipette, using a Buchler polystaltic pump set on low, 3.5. Fractions were collected by time, 0.9 minutes per fraction (approximately 0.8 ml) using a Gilson microfractionator. The absorbance at 260 nm was read, fractions were pooled from the three gradients, ethanol precipitated, dissolved in one ml water, and the absorbance at 260 nm was read again. Fractions were then lyophilized and resuspended in water at a concentration of 0.5 µg/µl. These fractions were stored at -70°C.

Oligo (dT) Cellulose Chromatography

Polyadenylated RNA (poly(A)RNA) was prepared by oligo (dT) cellulose chromatography. Three hundred mg of oligo (dT) cellulose (Sigma) was suspended in binding buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% SDS) and poured in a three ml disposable syringe plugged with glass wool. The column was used at room temperature and stored at 4°C in 0.02% sodium azide when not in use.

One mg of RNA in one ml NET 0.5% SDS was heated to 65°C for two minutes, quick-chilled on ice, and applied to the column. When all the sample had entered the column, unbound RNA was washed through with several column volumes of binding buffer, until the $A_{260}$ of the eluate was less than 0.1. Unbound RNA was reapplied to the column, which was again washed with binding buffer until the $A_{260}$ of the eluate was less than 0.05. Bound RNA was eluted with water and collected in

0.6 ml fractions. The peak fractions as determined by $A_{260}$ were pooled (1.2-1.8 ml total), ethanol precipitated, and resuspended in 1 ml water. Absorbance was again determined, the samples were lyophilized, dissolved in water at a concentration of 0.25 µg/µl, and stored at -70°C.

## In Vitro Translation

A rabbit reticulocyte lysate kit from New England Nuclear was used for in vitro translations. Translations were carried out as described in the kit protocol, except that the amount of L-[$^{35}$S]-methionine (New England Nuclear) used was 25 µCi per 25 µl assay (specific activity = 1155-1226 Ci/mmole) and reaction mixtures were not vortexed. Gradient-fractionated total cellular RNA was added to a final concentration of 100 µg/ml in the reaction, while poly (A) RNA was added to a concentration of 30-60 µg/ml.

Incorporation of $^{35}$S-methionine was determined by spotting an aliquot (1-2 µl) of the translation mixture onto Whatmann 3 MM paper, soaking in 10% TCA for thirty minutes, boiling in 10% TCA ten to fifteen minutes, and rinsing with 10% TCA followed by acetone. The dried filters were counted in Omnifluor in a Beckman LS 6800 scintillation counter.

## Isolation of RuBPCase and Preparation of Antibodies

RuBPCase was isolated from mature wheat leaves by the method of Hall and Tolbert (1978). The protein thus obtained was separated into large and small subunits by SDS polyacrylamide gel electrophoresis. The small subunit was eluted from the gel and used to prepare antibodies

in rabbits as described by Cashmore (1976). A 50% ammonium sulfate precipitate, dialyzed against 0.0175 M sodium phosphate, pH 6.3, was used in the immunoprecipitation procedure described below.

Immunoprecipitation

Immunoprecipitation of the small subunit of RuBPCase was carried out using Staphylococcus aureus cells (IgG sorb from the Enzyme Center, Boston, MA) as described by Martial et al. (1977). S. aureus cells were washed and resuspended in NET, 0.05% Triton X-100, 1 mg/ml bovine serum albumin, 2 mM methionine. Translation mixes (5-15 µl) were adjusted to a total volume of 20 µl with NET 0.05% TX-100. Eight µl S. aureus cells were added to each sample, incubated five minutes at room temperature, and centrifuged one minute in a Beckman microfuge. The supernatant was transferred to a fresh tube, 20 µl antibodies were added, and samples were incubated overnight at 4°C or one hour at room temperature. IgG-antigen complexes were recovered by the addition of 300 µl S. aureus cells and 30 minutes incubation on ice. The cells were pelleted, washed three times in the buffer containing BSA and methionine, and resuspended in NET 0.05% TX-100. The samples were transferred to clean tubes and centrifuged again. The pelleted cells were suspended in 40 µl SDS gel sample buffer, heated five minutes at 80°C to dissociate bound protein, and centrifuged again. One tength of the supernatant was spotted on Whatmann 3 MM paper and counted. The remainder was analyzed by SDS gel electrophoresis as described above.

# III. RESULTS AND DISCUSSION

## Expression of RuBPCase *in vivo*

Treatment with $10^{-4}$ M ABA will prevent precocious germination of 20-day-old wheat embryos (Triplett and Quatrano, 1982). The same concentration of ABA, however, will not entirely prevent initiation of germination of embryos from mature grains. As reported by Mansfield (1981), the radicle and coleoptile do elongate somewhat under these conditions, although they are very much reduced in size compared to those of the control plants. The reduced shoots do not become green even in the light when mature grains are incubated in ABA. Mature grains germinated in the dark produce seedlings that are morphologically similar to those grown in light except that they are slightly smaller and are not green.

To compare the expression of genes in mature embryos incubated under these conditions, the embryos or seedlings were labelled *in vivo* with $^{35}SO_4^{-2}$, and proteins were extracted in a Tris-glycine buffer (pH 9.0) which removes most soluble proteins including RuBPCase. Comparison of these proteins is shown in Figure 1. Both subunits of RuBPCase are clearly visible in the extract from light-grown embryos (Lane 1), faintly visible in the extract from dark-grown embryos (Lane 2), and not clearly detectable in the extract from ABA-treated embryos (Lane 4). These results indicate that ABA is preventing RuBPCase synthesis, even the low levels seen in embryos germinated in the dark.

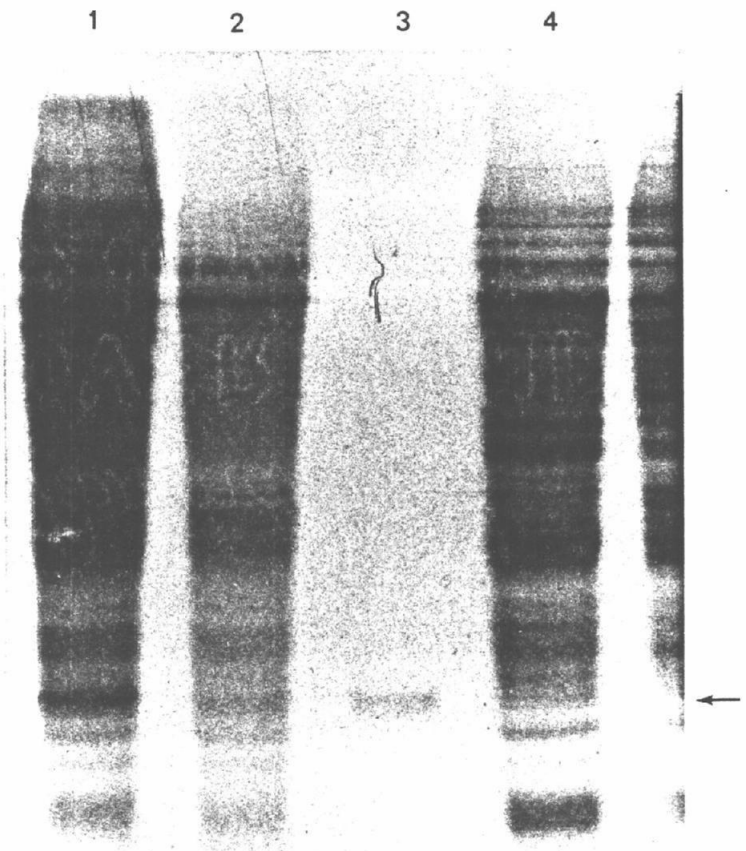



Figure 1. Fluorogram of proteins synthesized in vivo by germinating wheat embryos in different environments.

1. Control embryos, germinated in light, no ABA
2. Embryos germinated in darkness, no ABA
3. Purified small subunit of wheat RuBPCase
4. Embryos germinated in the presence of ABA

## Identification of the Putative Precursor to the Small Subunit of RuBPCase

I wanted to determine the level of mRNA for the small subunit of RuBPCase and to see whether it reflects the pattern of RuBPCase synthesis exhibited in Figure 1. I chose the technique of *in vitro* translation to determine the levels of mRNA for the small subunit and immunoprecipitation to confirm its synthesis as an *in vitro* translation product of that mRNA.

It is clear from the literature that the cell-free product of small subunit mRNA would be a precursor polypeptide several kilodaltons larger than the small subunit, and that it should be a major product of *in vitro* protein synthesis programmed by RNA from green leaves. For identification of this precursor polypeptide in a cell-free translation system, RNA from green leaves of 6-day-old seedlings was extracted and fractionated on linear 10-40% sucrose gradients. RNA fractions from the gradient were translated *in vitro*, and the electrophoretically separated translation products were detected by fluorography (Figure 2). The size of the proteins made clearly correlates with the sedimentation rate of the RNA; the higher the sedimentation rate of the RNA fraction, the larger the proteins synthesized *in vitro*. Lanes 10 and 11 both contain a very prominent protein of approximately 23 kd, which is somewhat larger than the size most commonly reported for the precursor to the small subunit (see Table I in Introduction). Roy et al. (1976) found a 20 kd protein among translation products of wheat leaf polyribosomes which may correspond to the precursor to the small subunit.

To confirm that the 23 kd protein was RuBPCase, translation products were immunoprecipitated using antibody made against wheat RuBPCase. Figure 3 shows that the 23 kd protein was selectively precipitated from the total translation products (Lane 6b). In addition to the 23 kd protein, two smaller proteins of approximately 18 kd and 16 kd were precipitated. These bands appear in the immunoprecipitates of both fractions 9 and 10 (lanes 5b and 6b), and do not correspond to major bands in the total translation products (lanes 5a and 6a). Some non-specific antibody binding does occur, as shown by the precipitate obtained using preimmune serum (Lane 5c), but these two minor precipitate bands are reacting specifically with the antibody. This is not an artifact of the rabbit reticulocyte system, since an identical pattern is seen in the immunprecipitation of proteins synthesized in the wheat germ cell-free system (data not shown). The smaller polypeptide appears to comigrate with authentic small subunit (Lane 4), so perhaps some processing is taking place _in vitro_. A similar pattern of immunoprecipitated proteins has been reported by Cashmore et al. (1978) and Sasaki et al. (1981) in translation of pea leaf RNA.

The 23 kd precursor polypeptide, henceforth referred to as P23, is the only major product formed by translation of RNA fraction 11 (Figure 2, Lane 7). Thus, the sucrose density gradient centrifugation has resulted in an RNA fraction substantially enriched for the mRNA for this particular protein. Further purification, (e.g. agarose gel electrophoresis) should yield an mRNA suitable for use in making a cDNA for the message. Such a probe is an essential tool for doing

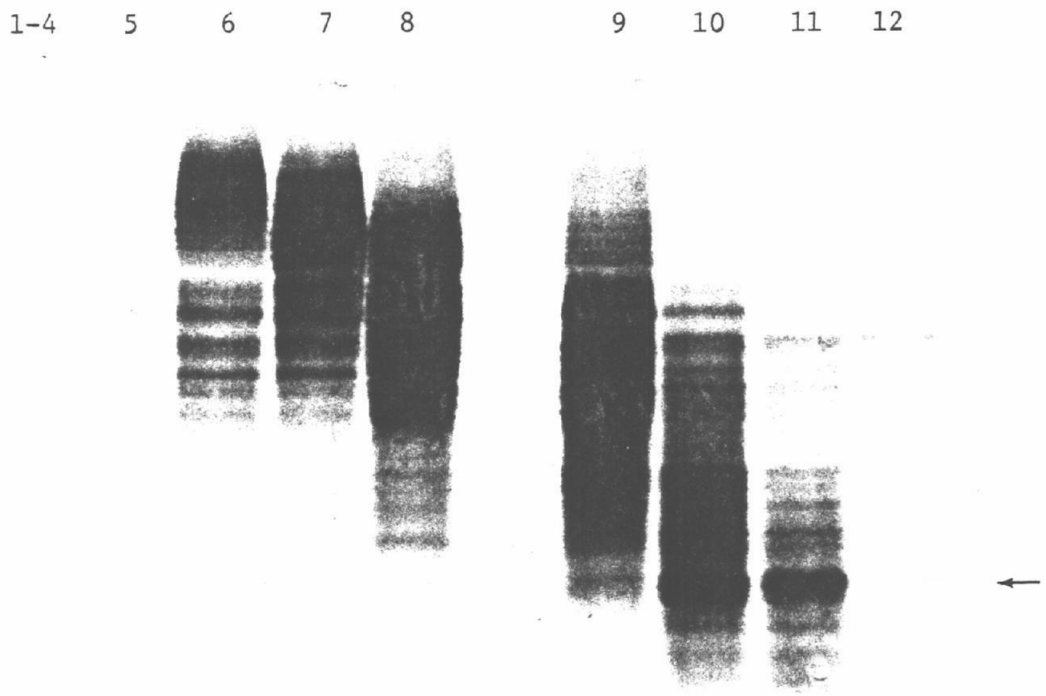



Figure 2. Cell-free translation of total cellular RNA fractionated on sucrose density gradients. Fractions 1-4 contain the most rapidly sedimenting RNA, fraction 12 contains the most slowly sedimenting RNA. Each lane shows the proteins synthesized by an equivalent amount of RNA. The arrow indicates the 23kd putative precursor to the small subunit of RuBPCase.

Figure 3. Immunoprecipitation of cell-free translation products using antibody to the small subunit of RuBPCase.

1a. Total translation products, globin mRNA.

1b. Immunoprecipitate of 1a.

2a. Total translation products, fraction 8 wheat leaf RNA.

2b. Immunoprecipitate of 2a.

3. Molecular weight standards: Bovine serum albumin (66,000); β-Lactoglobulin (18,400); Lysozyme (13,400)

4. Purified small subunit of RuBPCase from wheat.

5a. Total translation products, fraction 9 wheat leaf RNA.

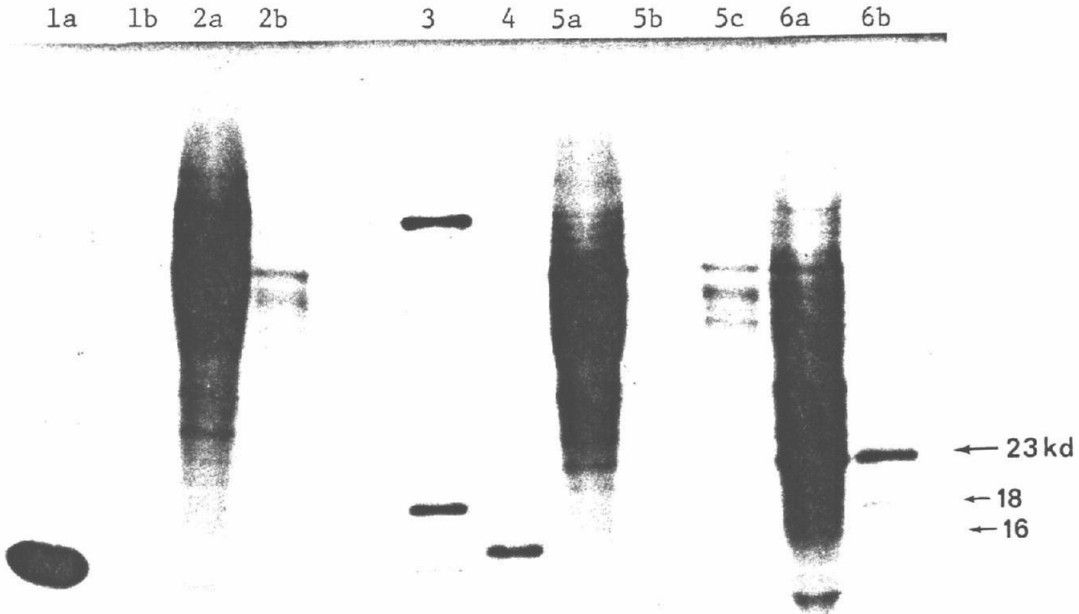
5b. Immunoprecipitate of 5a.

5c. Precipitate of 5a using preimmune serum.

6a. Total translation products, fraction 10 wheat leaf RNA.

6b. Immunoprecipitate of 6a.

Arrows indicate the 23kd, 18kd, and 16kd polypeptides selectively precipitated by anti-RuBPCase.



sensitive assays to determine the level at which gene expression is controlled.

Characteristics of RNA from Dark-Grown and ABA-Treated Tissue

Having identified P23 as the precursor to the small subunit of RuBPCase from green leaves of wheat, I then used the same approach to look for P23 mRNA activity in RNA from my experimental material, light and dark-grown shoots and ABA-treated axes. By comparing mRNA activity with the synthesis of RuBPCase observed in vivo (Figure 1), I could determine whether this mRNA might be regulated at the translational level.

Although great care was taken to treat all tissue and RNA samples in exactly the same way, the RNA characteristics varied considerably depending on the germination conditions of the embryos (Table II). The high yield of RNA per gram fresh weight of unimbibed embryos and ABA-treated axes compared to that of shoots from control and dark-grown seedlings is probably due primarily to the greater density of the tissue. This cannot explain, however, the great increase in yield of ABA-treated axes compared to unimbibed embryos. This is interesting in light of the fact that ABA-treated embryos appear morphologically to develop very little. In addition, their poly (A) RNA content decreases 85% compared to the dry seed. The very high template activity of the poly (A) RNA which is present in ABA treated tissue suggests that the decrease in % poly (A) RNA is not a result of non-specific degradation. The observed differences in mRNA activity reported in Table II were seen

Table II. Comparison of RNA from Unimbibed Embryos, Light-Grown Shoots, Dark-Grown Shoots, and ABA-treated Axes.

| Tissue Source | mg of total RNA per g. fresh wt. | % Poly(A) RNA | mRNA Activity of Poly (A) RNA; cpm $^{35}S$ incorporated/ g RNA |
|---|---|---|---|
| unimbibed embryo | 3.1 | 3.10 | 753,000 |
| 4-day-old shoots, control | 2.4 | 1.35 | 798,000 |
| 4-day-old shoots, dark | 2.2 | 1.35 | 1,461,000 |
| 4-day-old axes, +ABA | 5.2 | 0.45 | 1,809,000 |

when all RNA preparations were translated under identical conditions. The possibility that different mRNA populations might have different optimal conditions for translation was not investigated.

Comparison of the sedimentation profiles of total cellular RNA preparations are shown in Figure 4. The three RNA preparations were run on identical sucrose gradients (tRNA and 5.8S RNA are not seen in these profiles because the LiCl step employed in RNA isolation is selective for precipitation of high molecular weight RNA). RNA from dark-grown shoots and ABA-treated axes both show two distinct rRNA peaks, 18S and 28S. The two profiles are very similar except that dark-grown shoots have more rapidly sedimenting RNA. RNA from shoots of the control plants, on the other hand, shows only one extremely broad peak. This may be due to a greater proportion of hetero-disperse RNA (i.e., processing intermediates) as a result of increased transcriptional activity, since RNA accumulation during leaf development proceeds more rapidly in the light (Grierson and Covey, 1975). In addition, cells from light-grown shoots contain higher levels of chloroplast rRNA, which sediments at 23S and 16S. These organellar RNA could obscure the 28S and 18S cytoplasmic rRNA peaks.

Influence of ABA and Darkness on the Presence of the mRNA for P23 in Total Cellular and Poly (A) RNA

Fractions 1-14 of each of the RNA preparations shown in Figure 4 were translated in the reticulocyte lysate cell-free system. P23 appears to be present in all three sets of translations (Figure 5,

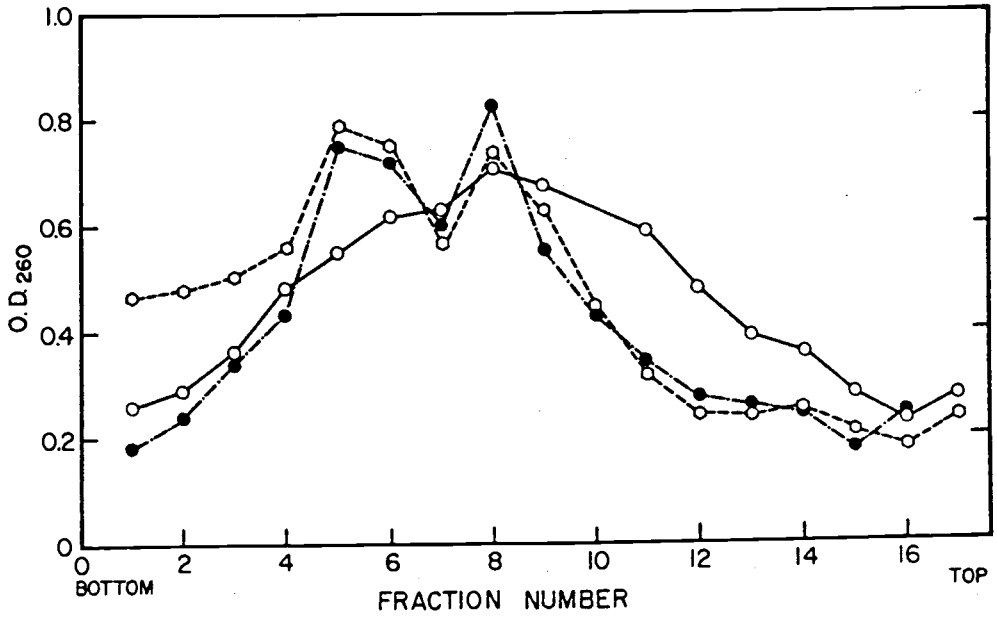



Figure 4. Sedimentation profiles of total cellular RNA from germinating wheat.

o——o RNA from shoots of light-grown embryos.

o- - - -o RNA from shoots of dark-grown embryos.

●—·—·—● RNA from axes of ABA-treated embryos.

Figure 5. Cell-free translations of RNA fractionated on sucrose density gradients. RNA fractions shown in Figure 4 were translated in a cell-free system. Arrows indicate the position of the putative precursor of RuBPCase.

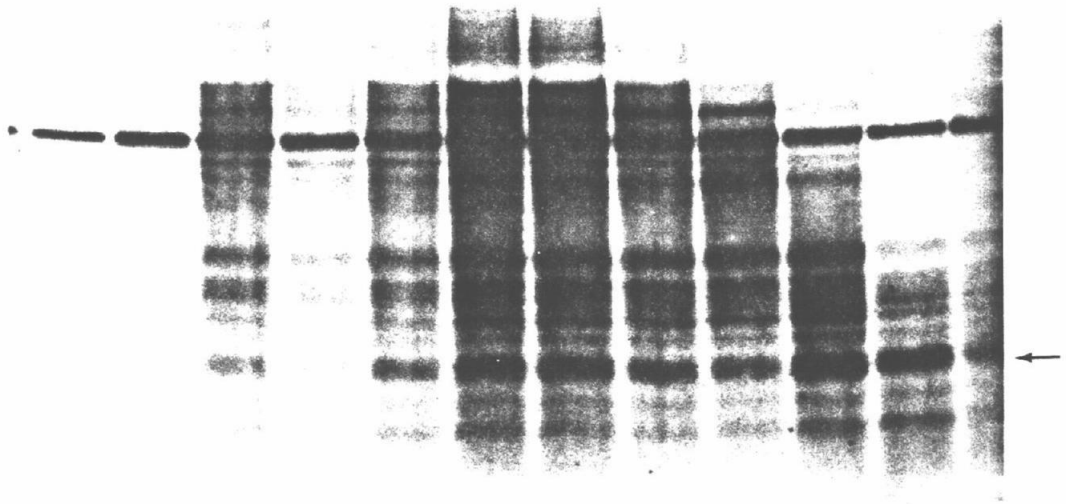
A. RNA from shoots of light-grown embryos.

B. RNA from shoots of dark-grown embryos.

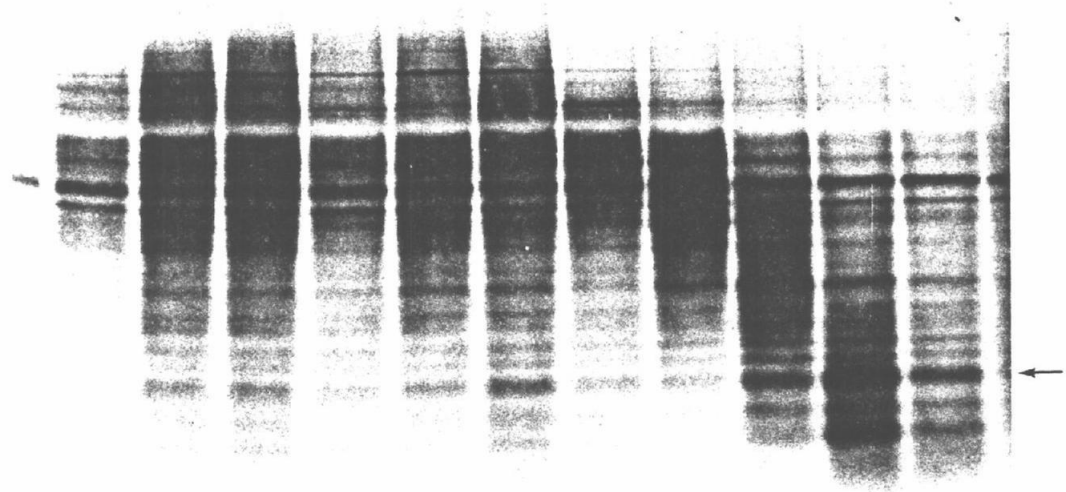
C. RNA from axes of ABA-treated embryos.

Figure 5

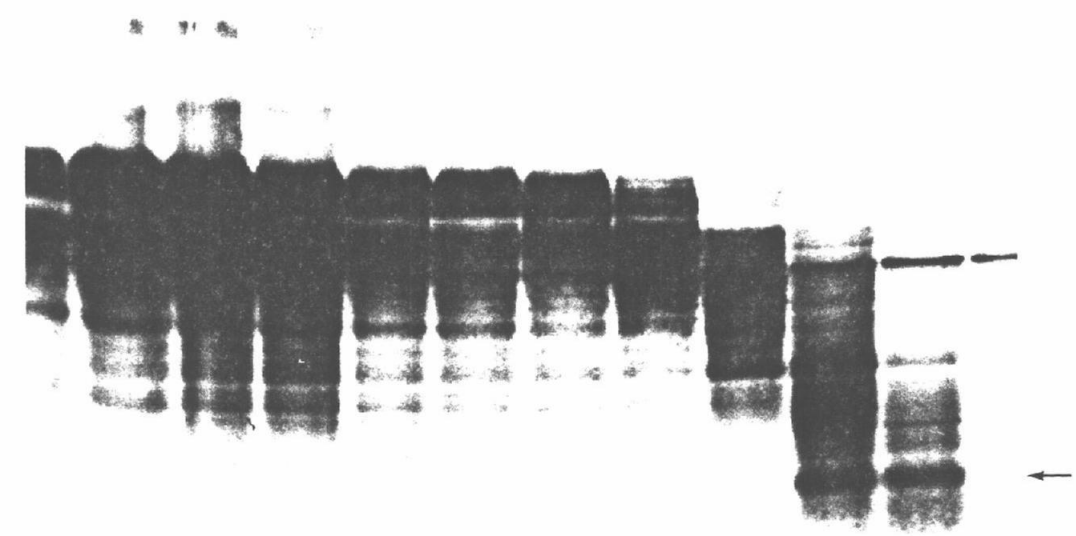
A.



B.



C.



indicated by arrows). With the exception of fractions 1, 2, and 4, which have very little template activity, P23 appears strongly in all fractions across the gradient of RNA from light-grown shoots (Fig. 5a). The proteins synthesized using RNA from dark-grown shoots are very similar to those of "light" RNA, but their distribution within the gradient is quite different (Fig. 5b). P23 is prominent in fraction 11, but quickly fades in the higher and lower regions of the gradient. RNA from dark-grown shoots also has much more activity in synthesis of higher molecular weight polypeptides. Likewise, ABA-treatment has yielded RNA which codes predominantly for higher molecular weight polypeptides (Fig. 5c). Fractions 10 and 11 contain a protein which seems to comigrate with P23, but which was subsequently shown to be a slightly smaller protein.

RNA from the same three total cellular RNA preparations described above was fractionated by oligo (dT)-cellulose chromatography to obtain a poly (A) fraction for each treatment. In addition, poly (A) RNA was obtained from total RNA of unimbibed embryos. Translation of these RNAs (Fig. 6) clarified the observations made using the gradient fractions described above. The P23 band from dark-grown material does indeed comigrate with the P23 band from light-grown material, but the major band in that size range coded for by ABA-treated poly (A) RNA is a different, smaller protein. There is a very weak band above it at the P23 position. Immunoprecipitation was used to determine whether these proteins were the authentic precursor to the small subunit of RuBPCase.

Immunoprecipitated translation products of these RNA fractions are shown in Figure 7. Whether poly (A) RNA or a fraction from the

Figure 6. Comparison of cell-free translations of poly (A) RNA (**A**) with those of enriched fractions of total cellular RNA (**B**).

A1. Poly (A) from unimbibed embryos.

A2. Poly (A) from shoots of light-grown embryos.

A3. Poly (A) from shoots of dark-grown embryos.

A4. Poly (A) from axes of ABA-treated embryos.

B1. Same as A2.

B2. Same as A3.

B3. Same as A4.

B4. Same as A1.

B5. Molecular weight standards: BSA - 66,000; β-lactoglobulin - 18,300; lysozyme - 13,400

B6. Purified small subunit of wheat RuBPCase.

B7. RNA from shoots of light-grown embryos, fraction 10.

B8. RNA from shoots of dark-grown embyros, fraction 11.

B9. RNA from axes of ABA-treated embryos, fraction 10.

Figure 6

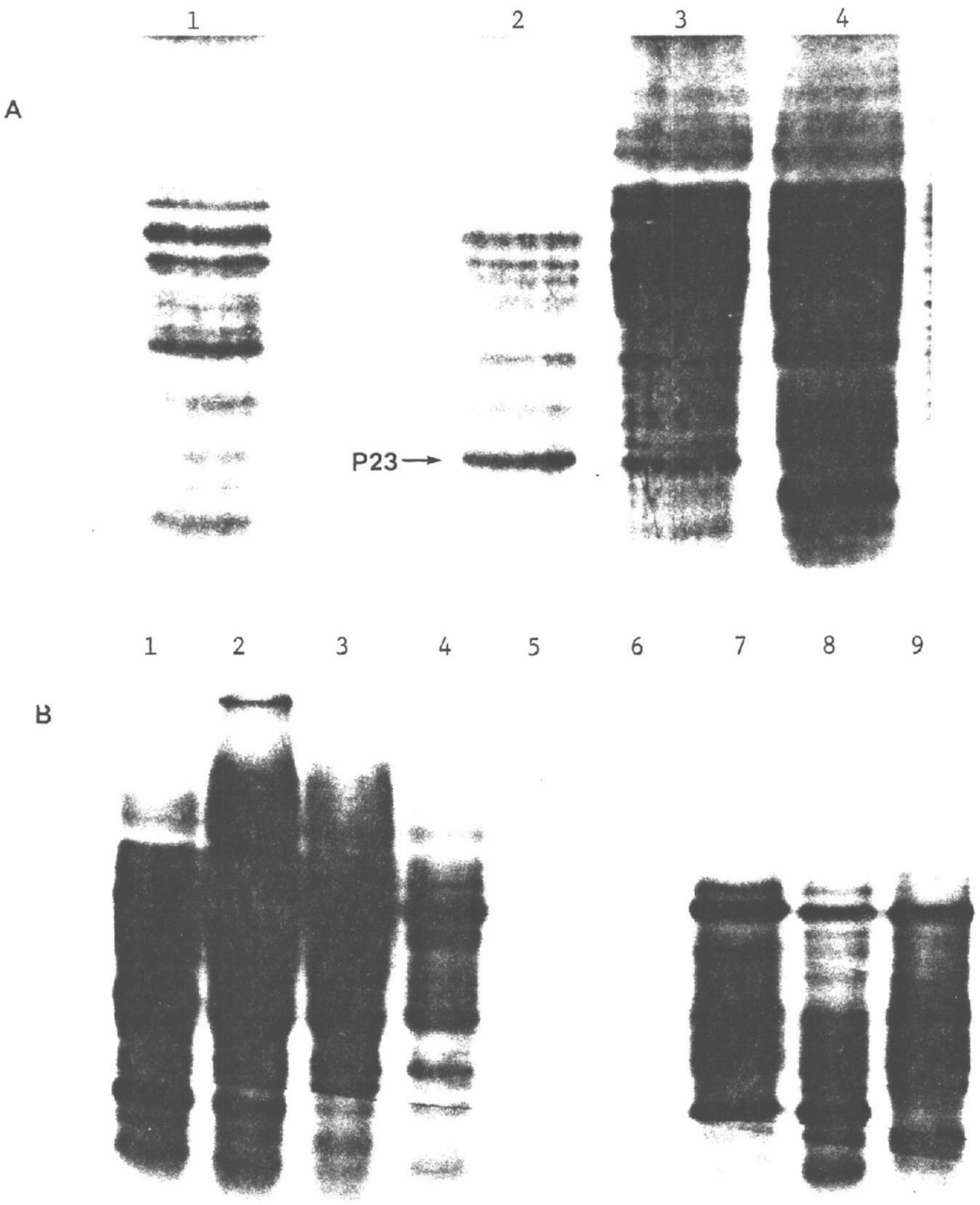


Figure 7. Immunoprecipitation of cell-free translation products of RNA from light-grown, dark-grown, and ABA-treated embryos.

1a. Total translation products, poly (A) from shoots of light-grown embryos.

1b. Immunoprecipitate of 1a.

2a. Total translation products, poly (A) from shoots of dark-grown embryos.

2b. Immunoprecipitate of 2a.

3a. Total translation products, poly (A) from axes of ABA-treated embryos.

3b. Immunoprecipitate of 3a.

1a. Total translation products, RNA from shoots of light-grown embryos, fraction 10.

1b. Immunoprecipitate of 1a.

2a. Total translation products, RNA from shoots of dark-grown embryos, fraction 11.

2b. Immunoprecipitate of 2a.

3a. Total translation products, RNA from axes of ABA-treated embryos, fraction 10.

3b. Immunoprecipitate of 3a.

Figure 7

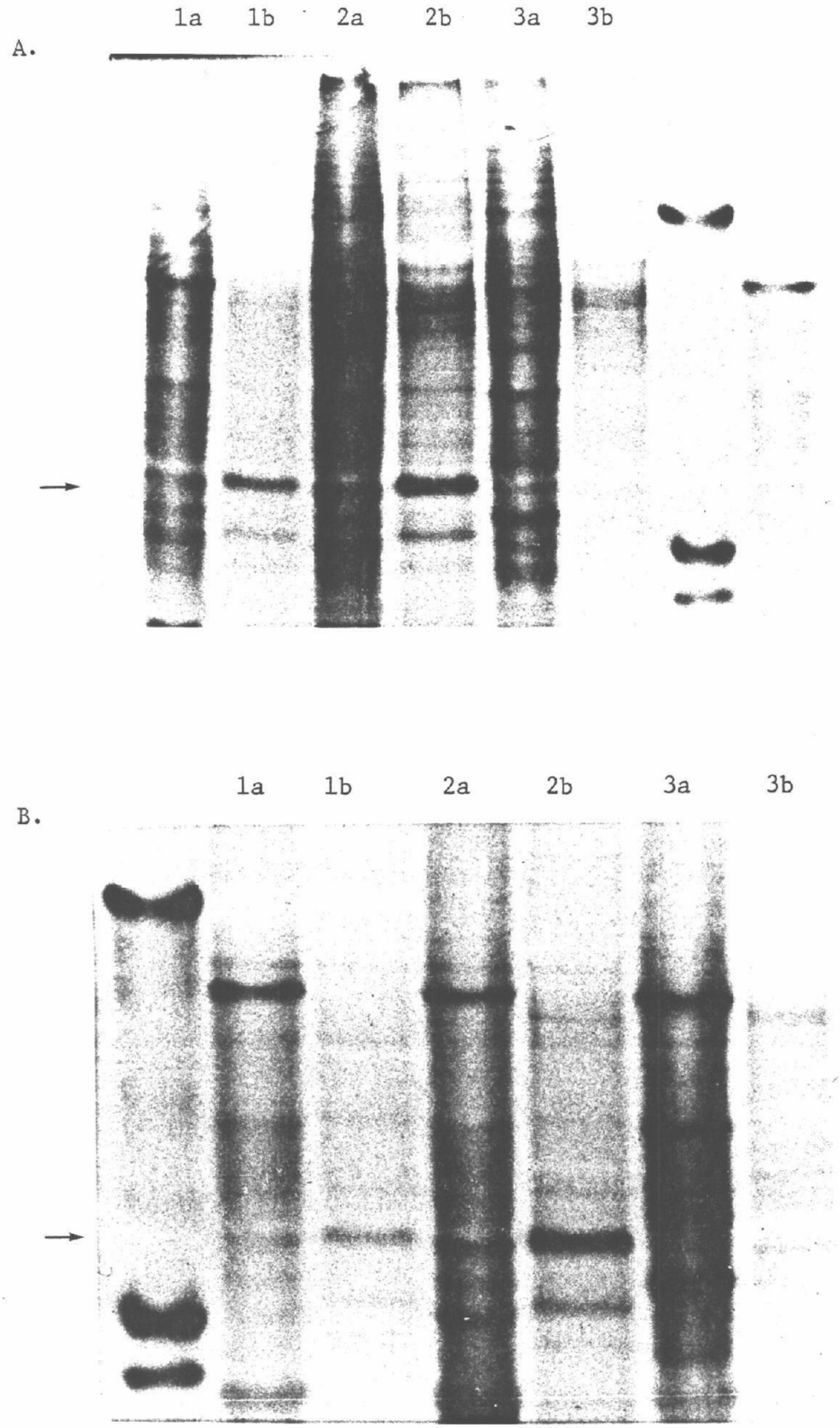


Figure 8. Immunoprecipitation of cell-free translation products of poly (A) RNA from light-grown, dark-grown, ABA-treated, and unimbibed embryos.

1a. Total translation products, poly (A) RNA from unimbibed embryos.

1b. Immunoprecipitation of 1a.

2a. Total translation products, poly (A) RNA from shoots of light-grown embryos.

2b. Immunoprecipitate of 2a.

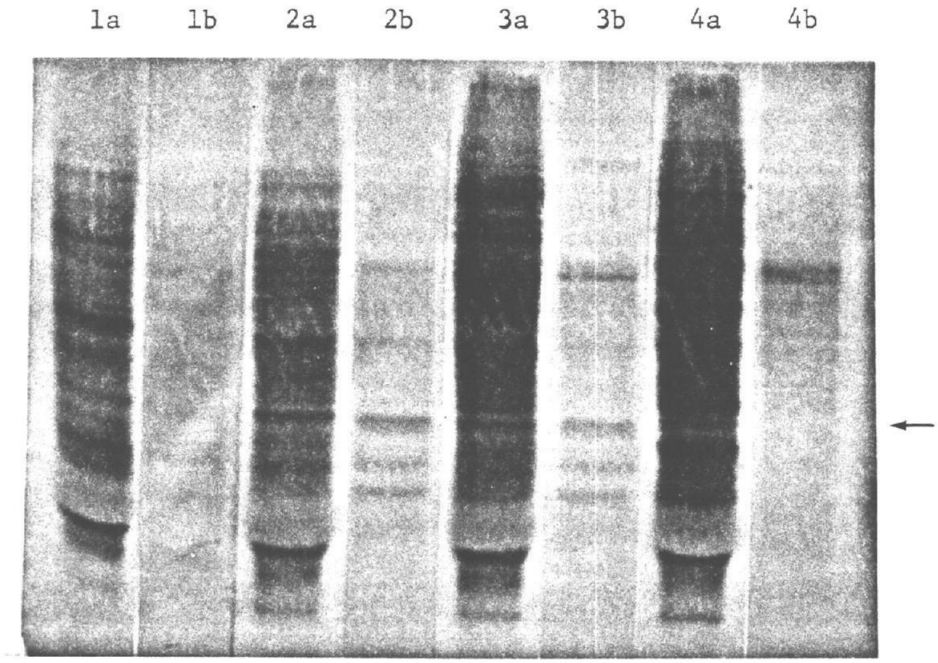
3a. Total translation products, poly (A) RNA from shoots of dark-grown embryos.

3b. Immunoprecipitate of 3a.

4a. Total translation products, poly (A) RNA from axes of ABA-treated embryos.

4b. Immunoprecipitate of 4a.

Figure 8



gradient was used as a template, P23 was immunoprecipitated from translation products when the RNA was from shoots grown without ABA, but was not immunoprecipitated when the RNA was from ABA-treated axes. The presence of ABA seems to eliminate the mRNA for P23 entirely. The absence of light has a less dramatic effect: P23 constitutes 9.5% of the total proteins made from poly (A) RNA of dark-grown shoots, as compared to 11.7% when shoots are from light-grown seedlings. However, due to the greater template activity of RNA from dark-grown shoots, P23 is synthesized in almost the same amount per μg poly (A) RNA in the two translation assays. Since mRNAs are translated with different degrees of efficiency, and will respond differently to changes in translation conditions (Lodish, 1976), it is best to use caution in making a correlation between the amount of a translation product and the amount of mRNA which must therefore be present. This is particularly true here since it was not established that the concentration of RNA used was in the linear response range of the translation system. Precise quantitation of mRNA levels must wait until a cDNA probe is available to directly detect the mRNA.

In a separate experiment, translation products of poly (A) RNA from unimbibed embryos were also immunoprecipitated (Fig. 8). Again, the characteristic pattern of three polypeptides is seen in immunoprecipitated translation products of RNA from shoots not treated with ABA (Lanes 2b and 3b), but is missing when RNA from unimbibed embryos (Lane 1b) or ABA-treated axes (Lane 4b) is used as the template.

## Synthesis of the Small Subunit of RuBPCase is Transcriptionally Controlled

The absence of immunoprecipitable P23 from cell-free translation products synthesized using poly (A) RNA from unimbibed embryos or ABA-treated axes is strong evidence that a functional message for the small subunit of RuBPCase is not present in these tissues. Nor is it present in total cellular RNA from ABA-treated axes. The extraction and fractionation methods employed exclude masking of the small subunit mRNA by protein or as double-stranded RNA, although the message could be present as an unprocessed, untranslatable primary transcript. Kamalay and Goldberg (1980) have shown by hybridization analysis that many rare mRNAs are post-transcriptionally controlled. Genes belonging to this class, whose protein products are not known, are constitutively transcribed in all tissues, but the transcripts leave the nucleus and become cytoplasmic mRNA only in those particular tissues in which the gene product is expressed. The mRNA for the small subunit of RuBPCase does not belong to this rare class of messages. According to Goldberg's model, abundant mRNAs are transcriptionally regulated, and the evidence presented here for regulation of an abundant mRNA appears to fit that model.

In the four types of plant material examined, presence of mRNA activity for P23 correlated with synthesis of the small subunit of RuBPCase as shown by *in vivo* labelling of the protein. This is true of embryos four days after imbibition. Examination of earlier time points could show that there is a lag period between appearance of mRNA activity and appearance of the protein, which would indicate that translational control, in addition to transcriptional control,

is operating in this system.

## The Small Subunit of RuBPCase as a Germination Marker

The postulated transcriptional control of the synthesis of the small subunit of RuBPCase seems to operate at two levels: the message is not present in the dormant embryo, but is transcribed and translated to a certain extent during the first days of germination in the absence of light and hence greening. This initial synthesis is part of the germination program and requires no additional environmental stimulus once germination has begun. Upon development of photosynthetic capability in the presence of light, the mRNA level increases and much greater amounts of the protein are synthesized.

ABA prevents normal germination, and may interfere with expression of germination-specific genes at different levels of control than those which operate during normal germination. The inhibition of α-amylase synthesis in barley aleurone layers treated with both gibberellic acid (GA) and ABA may involve such a change in control level. GA alone has been shown to induce the mRNA for α-amylase, an abundant protein, and to regulate synthesis at the transcriptional level (Ho and Varner, 1976). Simultaneous addition of ABA, however, prevents the appearance of α-amylase. Mozer (1980) has reported that transcription of α-amylase mRNA is unaffected by ABA, and that inhibition of enzyme synthesis is due to translational control. Subsequent work by Varner (1981) using cDNA hybridization has shown that GA stimulates a forty-fold increase in α-amylase mRNA sequences compared to untreated aleurone layers,

while treatment with GA and ABA stimulates a much smaller (three-fold) increase. This observation indicates that ABA may be affecting transcription in this system.

Whether or nor ABA plays a role in α-amylase regulation *in vivo* has not been established. There is no direct evidence that it does, but the ability of the tissue to respond to ABA suggests that it may be involved. Likewise, the work described in this thesis provides no evidence that ABA is directly involved in regulation of RuBPCase *in vivo*. ABA has been shown to prevent precocious germination *in vitro*, and the presence of high levels of ABA during the maturation phase of embryogenesis strongly suggests that it plays a major role at this stage. The mRNA for RuBPCase is not present in the developmentally arrested embryo, which precludes a role for ABA as a suppressor of translation of the mRNA during embryogenesis. Rather, it appears that ABA acts to prevent transcription of the RuBPCase gene, and presumably other germination-specific genes as well, during embryogenesis. The low ABA level which is attained in the mature seed allows for germination under favorable conditions, and initiation of a germination program which includes, at some point, new transcription of mRNA for RuBPCase. In cucumber cotyledon, the mRNA for the small subunit first appears two to three days after imbibition, fairly late in germination (Walden and Leaver, 1981). The time of appearance of the mRNA for RuBPCase in wheat will be important to determine, using a cDNA probe. Using cell-free translation, this study shows that the mRNA for RuBPCase is present at high levels four days after imbibition, and that its transcription is prevented by the continuous presence of ABA. It would be interesting to

germinate 20-day-old embryos without ABA for various lengths of time, then add ABA, to see how late one could add ABA and still see development in the embryogenic pathway; i.e., when do embryos become committed to germination? Also, does commitment to germination always result in RuBPCase synthesis? Can the two processes be uncoupled? In this way we could learn more about the place of RuBPCase in the germination pathway.

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