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Title: THE EFFECTS OF ABSCISIC ACID ON PROTEIN SYNTHESIS IN MATURE AND IMMATURE EMBRYOS OF WHEAT

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Abscisic acid (ABA) causes different growth responses in 20 day-old (immature) and 60 day-old (mature) embryos of the wheat Triticum aestivum L. var. Chinese Spring, Yamhill, and Twin. Immature embryos cultured on a nutrient medium containing L-glutamine (150 mM) and sucrose (2%) do not germinate when ABA is present in concentrations higher than 5 X 10^{-7} M. For mature embryos, 10^{-4} M ABA is the lowest concentration effective in completely inhibiting germination. Decreased sensitivity of mature embryos appears to be dependent, in part,on physiological changes which occur during dessication. Although germination is inhibited, ABA promotes embryogenesis in immature embryos resulting in a three to five-fold increase in fresh weight and acid-soluble protein during a five day culture period. Although a stimulation in fresh weight is not observed in mature embryos treated with ABA, a similar increase in acid-soluble proteins occurs.

Two-dimensional polyacrylamide gel electrophoresis of the acid-soluble proteins demonstrates that mature embryos grown in culture at

10^{-4} M ABA synthesize proteins which are clearly different from those extracted from embryos grown in the absence of ABA. They are unique to the embryo and with a few exceptions are synthesized equally in the embryo axis and the scutellum. In immature embryos the protein pattern observed is more similar between treated and untreated embryos. Certain proteins synthesized in response to ABA in mature embryos are translated from conserved mRNAs while others are synthesized as a result of *de novo* RNA synthesis. These conclusions are based on differential synthesis of these acid-soluble proteins when ABA-treated embryos are cultured in the presence of α-amanitin. When immature embryos are treated with 10^{-4} M ABA and $H_2{}^{35}SO_4$ at various times after isolation from the grain, a unique group of acid-soluble, high mobility proteins (HMP) is detected in culture within 72 hours of isolation. Synthesis of the same HMP is first detectable in embryos which developed on the intact plant at 28 days post-anthesis. Accumulation continues until 40 days post-anthesis. The time of HMP accumulation corresponds to the reported period when ABA levels are highest in the developing grain. These results indicate that ABA may play a key role in controlling protein synthesis during dormancy. With the identification of unique gene products, a molecular approach to the mechanism of ABA action and dormancy is possible.

THE EFFECTS OF ABSCISIC ACID ON PROTEIN SYNTHESIS IN MATURE AND IMMATURE EMBRYOS OF WHEAT

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LIST OF ABBREVIATIONS

ABA	abscisic acid
^{o}C	degrees Centigrade
cm	centimeter
cpm	counts per minute
DNA	deoxyribonucleic acid
DPA	days post-anthesis
EMB	embryo axis
gm	gram
HCl	hydrochloric acid
HMP	high mobility protein(s)
L.	Linnaeus
LMP	low mobility protein(s)
μCi	microcurie
μg	microgram
μl	microliter
mCi	millicurie
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
mmole	millimole
mRNA	messenger RNA
N	normal
Na_2EDTA	disodium ethylenediaminetetraacetic acid
nm	nanometer

RNA	ribonucleic acid
SCUT	scutellum
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminoethane
V	volt
var.	variety
v/v	volume/volume
WGA	wheat germ agglutinin
wt.	weight
w/v	weight/volume

THE EFFECTS OF ABSCISIC ACID ON PROTEIN SYNTHESIS IN MATURE AND IMMATURE EMBRYOS OF WHEAT

I. INTRODUCTION

Seed Development

Seed formation in angiosperms represents a developmental process whereby a plant provides a means to ensure its survival during adverse environmental conditions. Following fertilization of the polar nuclei and the egg (which are contained within the ovule), several distinct tissues arise which have specific functions during dormancy and germination (Dure, 1975). Most cereal grains contain a large proportion of endosperm which is a triploid tissue that serves as a nutritional source for the embryo during germination. The outermost layer of the endosperm of cereals is composed of one or more layers of cells termed the aleurone. During germination the aleurone synthesizes a number of hydrolytic enzymes which digest food reserves within the endosperm. The embryo arises from the zygote and gives rise to the new plant upon germination. The exterior of the seed is covered with maternal tissue, i.e. a seed coat and/or pericarp, which serves as a physical barrier between the embryo and the environment.

Normally, embryos of seed plants pass into a period of developmental arrest as the seed matures. Under certain conditions, however, the developmental arrest can be circumvented. For instance, viviparous mutants of corn have been isolated which do not require any dormancy period (Smith et al, 1977). Immature embryos of barley

(Umbeck and Norstog, 1979), *Brassica napus* (Crouch and Sussex, 1981), and cotton (Dure, 1975) can be cultured and germinated *in vitro* without any period of dormancy analogous to that found in the intact seed. Embryo formation in tissue culture proceeds to the seedling stage without any intervening arrest (Raghavan, 1976). In wheat and other cereals, a serious agronomic problem is the precocious germination of grains in the head as a result of preharvest rainfall. The affected grains are of little agronomic value at this point due to a loss of the proteins essential for proper baking (Derera *et al*, 1977). These exceptions to the characteristic pathway of seed development may provide investigators a tool to study the mechanisms of dormancy in seeds.

During embryogenesis many physiological changes occur in preparation for dormancy (Walbot *et al*, 1972; Dure, 1975; Bennett *et al*, 1975; Madison *et al*, 1976; Walbot, 1978). Several generalizations can be made about seed/grain development in both monocots and dicots. Embryogenesis takes place during the first half of embryo development and is characterized by rapid cell division (15-20 days post-anthesis). Although there is little increase in the average volume per cell, all of the organs that are recognizable in the mature embryo are detectable at this stage. Both fresh weight and dry weight increase dramatically as the cells begin to expand and food reserves in the form of storage proteins and starch are accumulated in the embryo and endosperm respectively. In *B. napus*, Crouch and Sussex (1981) have shown that storage protein accumulation occurs only in the cotyledons. Concurrently with a levelling off of the increase in dry weight, the onset of dessication occurs. Although fresh weight declines to a

point where as little as 10% of the total seed weight is water, the dry weight remains relatively constant. The metabolism of the seed declines, and the cellular machinery involved in macromolecule synthesis breaks down. In monocots, the RNA and DNA content of the endosperm decreases during dessication whereas protein, RNA, and DNA accumulate in the embryo. In contrast, the RNA content per cell in the dicot Phaseolus vulgaris remains constant once the maximum level is attained.

The first 20 days post-anthesis in wheat are characterized by cellular division of the zygote leading to formation of the embryo (Dure, 1975). RNA and protein content per cell increase up to 20 days post-anthesis (Zhi-ping et al, 1980). At this point the embryo is fully formed. After 20 days post-anthesis, cell enlargement takes place. The protein content per cell increases, but the total RNA content declines (Zhi-ping et al, 1980). There is an overall increase in size, fresh weight, and dry weight of the embryo (Dure, 1975). Between 15 and 20 days post-anthesis, the size of the embryo (1-2 mm) allows easy isolation from the grain and manipulation in culture. Several developmental pathways are possible for the embryo at this stage (Fig. 1). If left intact in the head, the embryo will continue to increase in size and weight. After an additional 20 days, developmental arrest occurs as the seed begins to dessicate (Pathway III). When imbibition takes place, the dormant embryo will germinate into a seedling with active meristems, functional roots, an emerging coleoptile, and a photosynthetically active leaf. As seen in Pathway I (Fig. 1), developmental arrest can

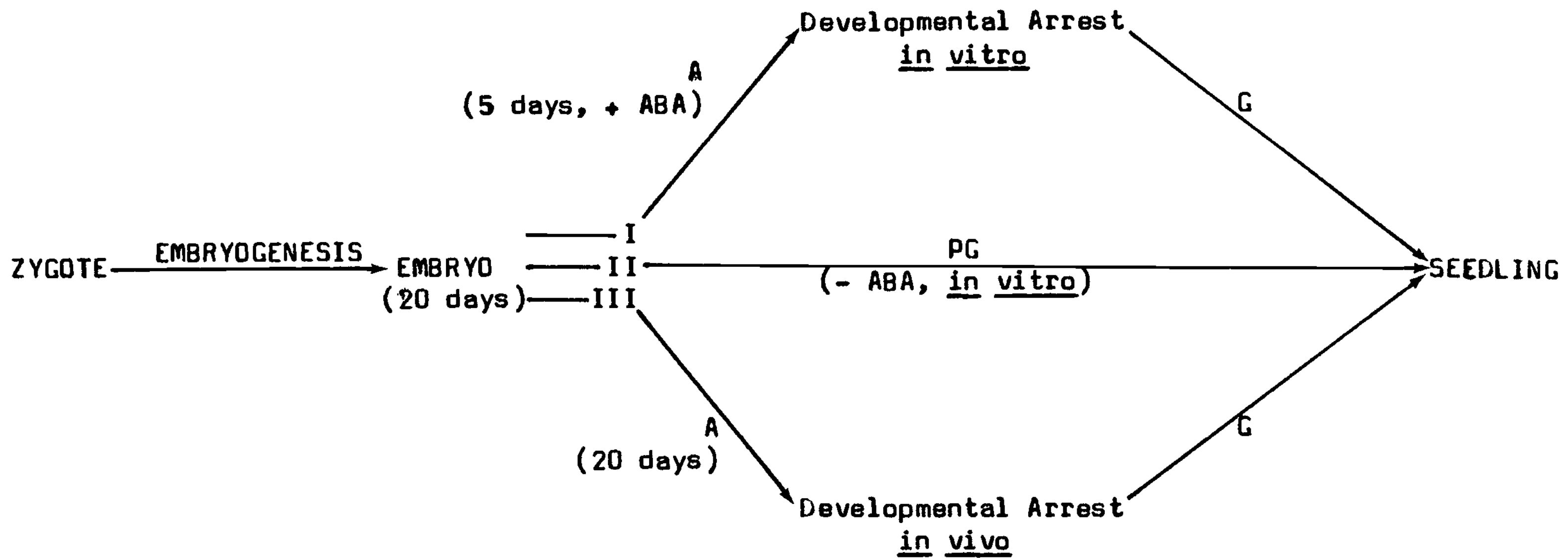

Figure 1. Development of the wheat embryo.
A- Arrest Pathway
PG- Precocious Germination Pathway
G- Germination Pathway

also be caused by culturing the isolated embryos in the presence of 10^{-4} M abscisic acid (ABA) for five days (see Results). The increases in size and weight are comparable to that observed in the embryo *in situ*. If the embryo is removed from the ABA, it can be germinated if cultured for an additional five days. Developmental arrest and the changes occurring from 20 days post-anthesis are not essential for development of the embryo into a seedling. At 15-20 days post-anthesis, the embryo can be cultured *in vitro* in the absence of ABA. As shown in Pathway II (Fig. 1), five days later, a small seedling, as previously described, will develop (see Results).

Although little is known about nucleic acid and protein synthesis in the precocious germination pathway, considerable information is available about the normal germination pathway in wheat. *De novo* mRNA synthesis is not required for protein synthesis after imbibition (Spiegel and Marcus, 1975). However, it is apparent that *de novo* mRNA synthesis is required for germination. Jendrisak (1980) has shown that concentrations of α-amanitin which inhibit the activities of wheat RNA polymerases *in vitro* are also effective in blocking germination. It was also found that the effects of α-amanitin on RNA polymerase activity *in vitro* correlated with the reduction in synthesis of the various classes of RNA observed in treated embryos. Thompson and Lane (1980) have also shown that α-amanitin blocks the synthesis of proteins which are detectable soon after imbibition when the embryos are allowed to germinate normally.

In vivo polysome formation and protein synthesis from pre-existing mRNAs have been detected in the presence of actinomycin D

and α-amanitin (Spiegel and Marcus, 1975; Cheung et al, 1979). This indicates that a conservation of some of the RNA synthesized during embryogenesis is taking place. During germination 25-40% of the mRNA becomes polysomal. Preliminary evidence by Brooker et al (1978) has shown that the complexity of the polysomal mRNA is not different from the complexity of the nonpolysomal population. Thus, there does not appear to be any selective synthesis from the conserved mRNAs. In the normal course of events, significant amounts of RNA synthesis occur soon after imbibition (Spiegel et al, 1975; Thompson and Lane, 1980; Huang et al, 1980) with 25-30% of the total RNA synthesis during the first seven hours being mRNA. After seven hours and coincidentally with the onset of cell expansion, the rate of ribosomal RNA synthesis increases to a level where it represents 92-96% of the total RNA transcribed. This is a proportional increase in the amount of ribosomal RNA transcribed and is not due to an overall increase in the amount of RNA transcribed (Huang et al, 1980). Brooker et al (1978) have shown that within two days of imbibition the mRNA population of the embryo changes dramatically. They concluded that the more prevalent classes of mRNA found in the imbibing embryo are not involved in germination but that they allow for a rapid resumption of growth after imbibition. Another interpretation of this data is that the more prevlent classes of mRNA may represent unused templates from dessication.

A question of considerable interest to researchers is what mechanisms are involved in seed dormancy and are they dependent upon specific hormonal responses. There are many examples of specific

classes of proteins being synthesized in response to hormonal treatments (e.g., Mozer, 1980; Zurfluh and Guilfoyle, 1980; and others). The classic example of this phenomenon is induction of α-amylase activity in barley aleurones by gibberellic acid (Chrispeels and Varner, 1967). During seed development several broad categories of hormone activity cam be generalized (Eeuwens and Schwabe,1975). Early in embryogenesis, hormonal activity is gibberellin-like and auxin-like in nature. Late in embryogenesis and during the progression of the embryo toward developmental arrest, ABA is the prevalent hormone with inhibitors of gibberellin activity also found. At maturity and in the dry seed, hormone levels are negligible. Upon germination higher levels of gibberellin and auxin activity are restored.

If a substance is to have a role in dormancy, it should have a number of characteristics which allow it to promote developmental arrest while blocking germination. It would be present in the embryo in maximum concentrations at the time that the embryo is capable of germination. Hormone levels would remain high until environmental conditions are no longer favorable for germination. As dessication occurs, the hormone level would be reduced to levels which would not block germination of the embryo when hydration takes place.

ABA concentrations in wheat generally fit this pattern (Radley, 1979). The changes in the level of ABA in developing wheat seeds have been characterized (McWha, 1975; King, 1976; King, 1979; King, et al, 1979; Radley, 1979). In cultivars Aotea, Arawa (McWha, 1975), and WW15 (King, 1976), ABA content per gram dry weight increased up to

40 days post-anthesis and, thereafter, rapidly declined to one tenth of the maximum concentration. In the cultivar 6A190 (King et al, 1979), the ABA concentration reached a maximum value at 22 days post-anthesis and did not show a decline until after 34 days post-anthesis. In all of these cultivars, the ABA peaks corresponded to the intervals of maximum dry weight increase. Because of this similarity between the predicted properties of a dormancy inducing substance and ABA, it can be considered a possible controlling hormone in wheat dormancy. Although the levels of ABA in wheat vary as one would predict for a hormone involved in dormancy, one must also consider another component in the possible role of ABA in dormancy: Does the sensitivity of the embryo to ABA change during grain development?

ABA is associated with a number of physiological responses including H^+/K^+ movements in guard cells in response to water stress, ion fluxes in other plant tissues, geotropic responses in roots, induction of dormancy in buds, and inhibition of certain enzyme activities (Walton, 1980). In P. vulgaris, ABA levels are maximal at 22 and 28 days post-anthesis, coinciding with the period of maximum storage protein synthesis (Hsu, 1979). This pattern is seen in many other species as well (Walton, 1980). The involvement of ABA in seed dormancy is also suggested by studies of viviparous mutants of corn which exhibit reduced levels of endogenous ABA and reduced sensitivity to exogenously supplied ABA (McDaniel et al, 1977). Furthermore, germination of seeds which are nondormant has been shown to be correlated with a reduction in the endogenous

levels of ABA. As the ABA level drops, the percentage of seeds capable of germination increases.

Dewdney and McWha (1979) have demonstrated that ABA, when applied to the embryos of developing grains, results in an increase in the rate of photosynthate translocation from the flag leaf to the ear. King (1976) determined the ABA concentration to be 464 ng/gm dry weight in 33 day-old embryos, more than four times that found in the testa and endosperm. ABA may be acting to cause the embryo to be a nutrient sink, though it is apparently having other effects. This is evident from the observation that application of ABA to the ear had no effect on the grain growth rate yet caused premature cessation of grain growth and dessication of the grain.

Although much is known about the general effects of ABA on embryo dormancy and its metabolism in the seed, little information is available on its physiological effects at the molecular level. In wheat embryos, Triplett (1979) found that wheat germ agglutinin (WGA), an acid-soluble protein, is synthesized in the embryo only from 25-35 days post-anthesis. It accounts for 6-10% of the total protein synthesis during this period. When 20 day-old embryos are precociously germinated <u>in vitro</u>, WGA synthesis is not detected. When the embryos are treated with 10^{-4} M ABA, germination is prevented and WGA synthesis is stimulated. WGA seems to be a tissue and stage-specific marker synthesized in the embryo only when the embryo goes through developmental arrest.

It is also apparent that ABA inhibits the activity of certain enzymes associated with germination. In barley aleurones, Mozer

(1980) found that the simultaneous addition of ABA and gibberellic acid caused a reduction of α-amylase synthesis compared to aleurone layers treated with gibberellic acid alone. The effect was at the translational level because *in vitro* translation of total poly(A)-RNA from ABA/gibberellic acid-treated aleurones resulted in the synthesis of native α-amylase. Van Onckelen *et al* (1980) have also found that ABA reduces α-amylase activity in germinating seeds of *P. vulgaris*. In wheat, King *et al* (1979) found that exogenously supplied gibberellic acid did not result in α-amylase activity in developing seeds until they were almost mature and only after they had been dried. This correlates with the period of ABA loss in the maturing grain (Radley, 1979). α-amylase activity was inducible when the ABA levels had declined. Thus, ABA allowed for the continuous accumulation of food reserves by blocking the synthesis of enzymes leading to their hydrolysis. One question that has not been answered, though, is how ABA mediates the blockage of transcription of the α-amylase mRNA.

Purpose of Study

The main goal of this study is to initiate an experimental approach to analyze the role of ABA in dormancy of wheat grains. As discussed in the Introduction, previous work has shown that:

A) The effects of ABA on cotton, bean, and barley embryos *in vitro* are similar to embryo development *in vivo*, i.e. embryo growth and development are promoted by ABA while precocious germination is inhibited.

B) ABA concentrations in developing wheat grains fit the predicted

model for a regulatory substance involved in preventing precocious germination, but other parameters, such as embryo sensitivity to ABA during development, have not been measured. C) With the exception of WGA, the effects of ABA on wheat embryo development have not been extended to identify gene products that are characteristic of the arrest pathway. D) The effect of ABA in preventing germination appears to be at the translational level, i.e. preventing synthesis of germination-specific proteins from pre-existing templates.

In view of these findings and a literature suggesting a role for ABA in controlling dormancy in cereals, I asked:

1) Can ABA promote embryogenesis and prevent precocious germination of wheat embryos _in vitro_?
2) If so, does the sensitivity of the embryo to ABA vary during development and how does this response compare to embryo development _in vivo_?
3) Can one identify a set of embryo-specific proteins that are induced by ABA _in vitro_?
4) If so, is this set of proteins synthesized in immature as well as in mature embryos and does ABA exert its effects at the translational or transcriptional level?
5) Are these ABA-induced proteins unique to the arrest pathway both in culture and in the whole plant?

II. MATERIALS AND METHODS

Plant Material

Mature grains of Triticum aestivum L. var. Yamhill, Chinese Spring, and Twin were surface-sterilized with 10% Clorox for ten minutes with occasional swirling. After washing the grains with three to five changes of glass-distilled water, they were placed equidistance apart in 9.0 cm Petri dishes containing three pieces of Whatman #1 filter paper and 10 ml of glass-distilled water. For immature embryos, heads were harvested from the varieties Chinese Spring and Twin which had been grown in the greenhouse under a 16 hour photoperiod maintained at 18°C during the day and 13°C during the night. The grains were separated from the chaff and sterilized as above. Individual embryos were isolated by squeezing the grains with forceps and forcing the embryos through the pericarp. The immature embryos were incubated in germination medium (GM) consisting of Linsmaier and Skoog salts (1965) supplemented with 2% (w/v) sucrose and 150 mM L-glutamine. All transfers were made in a laminar flow hood and all glassware and solutions were autoclaved or Millipore-filtered to ensure sterility.

Throughout this report the terms 20 day-old embryo and immature embryo will be used interchangeably. Twenty day-old grains are green and 5-7 mm in length while the enclosed embryos are 1-2 mm in length and have a pale, yellowish-white color. Similarly, the terms 60 day-old embryo and mature embryo will be used interchangeably. Sixty day grains are dry and yellow and 5 mm in length while the enclosed

embryos are 3-4 mm in length after imbibition and have a light yellow color.

Incubation Conditions

The isolated grains/embryos were incubated at 27°C in a growth chamber with a 16 hour photoperiod. In radioactive tracer experiments, 10 μl of $H_2{}^{35}SO_4$ (~10 μCi) at a specific activity of 1 mCi/mmole were added to 10 ml of medium (1μCi/ml) 24 hours after the initial plating. The plates were swirled to mix the label with the medium. Additions to dark-grown seedlings were made in the dark. The length of incubation in label as well as in abscisic acid (ABA- Sigma) is given in the legends.

For incubation in α-amanitin, embryos from mature grains of the variety Yamhill were excised, sterilized in 10% Clorox, and imbibed in 100 μg/ml of α-amanitin for one hour. The embryos were subsequently incubated in 0.1 μg/ml of α-amanitin for three days. In general agreement with the data of Jendrisak (1980), this sequential treatment with α-amanitin was shown to be the minimal concentrations necessary to prevent germination. Embryos imbibed in 100 μg/ml of α-amanitin and subsequently incubated in distilled water germinated normally compared to controls, as did embryos incubated and imbibed in 0.1 μg/ml.

Extraction of Acid-Soluble Proteins

The embryos/seedlings were harvested for each treatment and homogenized in 50 mM HCl. In mature grains the endosperm and

pericarp were separated from the seedlings prior to homogenization. The homogenate was stirred for 30 minutes and then dialyzed against 10 mM ammonium carbonate (changed once) in Spectrapor #3 dialysis tubing (molecular weight cutoff: 3,500) for two hours. The dialysate was centrifuged at 10,000 x g to remove the insoluble material. This entire procedure was carried out at 4°C. The supernatant was then frozen and lyophilized to dryness. The acid-soluble material was stored at -20°C.

Protein Determination

Protein determinations were made according to the procedure of Schaffner and Weissman (1978). Dried samples of the acid-soluble material were dissolved in glass-distilled water at a concentration of 1 mg/ml. Aliquots of these solutions were diluted to 0.27 ml with glass-distilled water and mixed with 0.03 ml of 1 M Tris-HCl (pH 7.5) containing 1% (w/v) SDS and 0.05 ml of 60% (w/v) trichloroacetic acid. The mixture was vortexed, allowed to stand for two minutes, and then filtered on a Millipore filter (4 HAWP, 025 CO). Each sample tube was washed with 0.30 ml of 6% (w/v) trichloroacetic acid, and the rinse was added to the same filter. The entire filter was then flooded with two aliquots of 6% (w/v) trichloroacetic acid followed by staining in 0.1% (w/v) amido black 10B in methanol: glacial acetic acid:distilled water (45:10:45) for 30 seconds. The filter was then destained in methanol:glacial acetic acid:distilled water (90:2:8). The destaining solution was changed twice at one minute intervals. Finally, the filter was rinsed for two minutes in

distilled water. Each blue spot was cut out, and the stain was eluted in 0.6 ml of 50% (v/v) ethanol containing 25 mM NaOH and 0.05 M Na_2EDTA. After ten minutes the absorbance of the eluate was measured at 630 nm. Bovine serum albumin was used as the standard.

One-Dimensional Polyacrylamide Gel Electrophoresis

Portions of the dried extracts were dissolved in equal volumes of glass-distilled water and sample buffer (0.125 M Tris-HCl, pH 6.8; 2.0% (w/v) SDS; 20% (w/v) glycerol; 10% (v/v) 2-mercaptoethanol; 0.001% bromphenol blue). After vortexing the samples, they were placed in an 80°C water bath for three to five minutes. The samples were electrophoresed for five hours at 100 V on a 1.5 mm wide polyacrylamide gel. The stacking gel contained 6.0% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.125 M Tris-HCl (pH 6.8), 0.048% (w/v) ammonium persulfate, and 0.1% (v/v) TEMED. The The running gel contained 18% (w/v) acrylamide, 0.12% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.75 M Tris-HCl (pH 8.8), 0.24% (w/v) ammonium persulfate, and 0.045% (v/v) TEMED. The running buffer contained 0.05 M Tris base, 0.38 M glycine, and 0.01% (w/v) SDS at a final pH of 8.3. Following electrophoresis, the gels were stained in 0.1% (w/v) Coomassie brilliant blue R 250 in methanol:distilled water: glacial acetic acid (5:5:1) for at least five hours. The gels were destained in ethanol:glacial acetic acid:distilled water (20:7:73).

Two-Dimensional Polyacrylamide Gel Electrophoresis

Protein samples were dissolved in equal volumes of glass-distilled water and sample buffer (30% (w/v) sucrose, 1.8 N acetic acid, and 0.05% (w/v) pyronin Y). The first dimension tube gels were cast in 200 μl capillary pipets which had been washed with boiling 95% ethanol and rinsed with acetone. The gels contained 15% (w/v) acrylamide, 0.1% (w/v) bisacrylamide, 5.4% (w/v) acetic acid, 2.5 M urea, and 0.005%(v/v) TEMED. The running buffer was 0.9 N acetic acid. Prior to the addition of the samples, the gels were electrophoresed for two hours at 150 V to remove the ammonium persulfate. At the end of the two hour pre-electrophoresis period, the running buffer was changed and the samples were loaded. Maximum sample volume was 20 μl. The samples were electrophoresed at 130 V to a distance of 8 cm as measured from the top of the tube gel to the leading edge of the tracking dye front. Some variation was observed in the time taken for given samples to enter the gel, but the time was usually four hours ± 15 minutes.

At the end of the electrophoresis period, the gels were removed from the tubes and soaked for two hours in a solution of 0.125 M Tris-HCl (pH 6.8), 2.0% (w/v) SDS, 20% (w/v) glycerol, and 10% (v/v) 2-mercaptoethanol. The tube gels were then placed on top of 1.5 mm wide slab gels containing 18% (w/v) acrylamide, 0.16% (w/v) bisacrylamide, 0.1% (w/v) SDS, 0.75 M Tris-HCl (pH 8.8), 0.024% (w/v) ammonium persulfate, and 0.045% (v/v) TEMED. The running buffer contained 0.05 M Tris base, 0.38 M glycine, and 0.1% (w/v) SDS at a

final pH of 8.3. The gels were electrophoresed for 16 hours at 50 V. Following electrophoresis, the gels were stained and destained in the same manner as the one-dimensional gels. After destaining, no visible bands were detected in the tube gels indicating that all of the proteins had moved into the second dimension slab.

Fluorography

Radioactive proteins were detected by fluorography according to the procedure of Chamberlain (1979). Destained gels were agitated in distilled water for 30 minutes followed by agitation in 1 M sodium salicylate for 30 minutes. The gels were then dried onto Whatman 3MM filter paper under acetate sheets on a Biorad gel drier for two hours. The dried gels were exposed to Kodak XR-5 X-ray film at $-70^{o}C$.

III. RESULTS

Morphological Development of Embryos In the Arrest and Germination Pathways

Striking differences are apparent when immature embryos of the variety Chinese Spring are removed from the grain and cultured on GM in the presence or absence of ABA (Fig. 2A). During the five day incubation period in 10^{-4} to 10^{-6} M ABA, the embryo axis and scutellum increase 2-2.5 fold in size and fresh weight (Fig. 3), but germination is infrequently observed at concentrations of 10^{-6} M or greater. All immature embryos of the varieties Chinese Spring and Twin cultured in the absence of ABA germinate and increase in fresh weight 8-10 fold during the five days in culture (Fig. 3). The seedlings have one main root, two tap roots, and an emerging coleoptile. Chlorophyll development is also observed in light-grown seedlings.

Differences are also found in the germination pattern of mature embryos from intact grains when cultured in the presence or absence of ABA (Fig. 2B). Unlike immature embryos, mature grains incubated in 10^{-6} M ABA are similar to those grown in the absence of ABA although the size and fresh weight are reduced. Seedlings from grains grown in 10^{-5} M ABA are green and appear to be at an intermediate developmental stage between seedlings grown in 10^{-4} and 10^{-6} M ABA. Although overall development into a seedling is inhibited at 10^{-4} M ABA, the fresh weight of mature embryos increases 4-7 fold (Fig. 4). The coleoptile and root are enlarged and root hairs are evident, but there

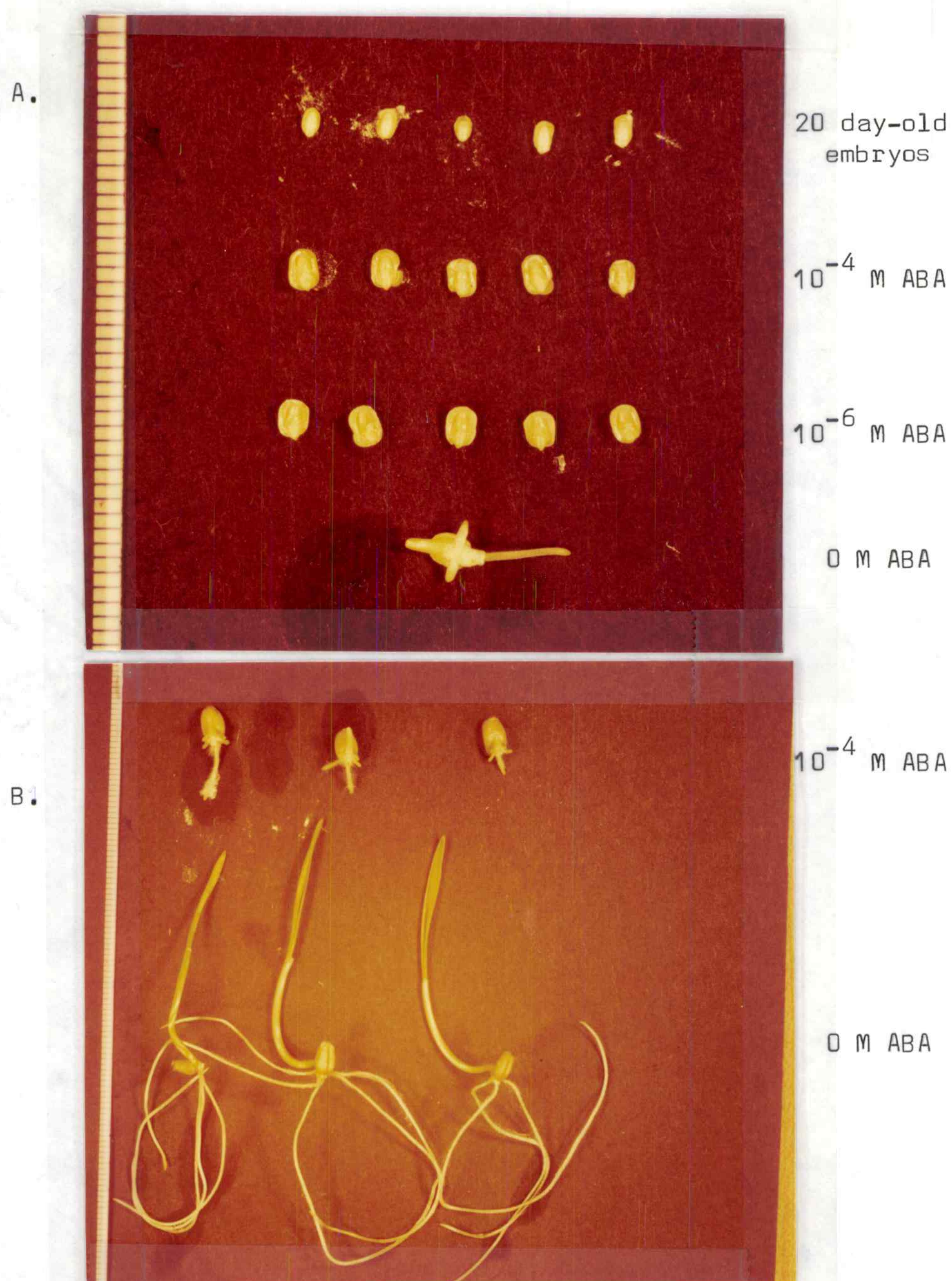

Figure 2. Morphological development of immature and mature embryos in the presence and absence of ABA. Immature embryos (A) and mature embryos in the intact grain (B) were incubated for five days in the presence or absence of ABA. The scale on the left is in millimeters

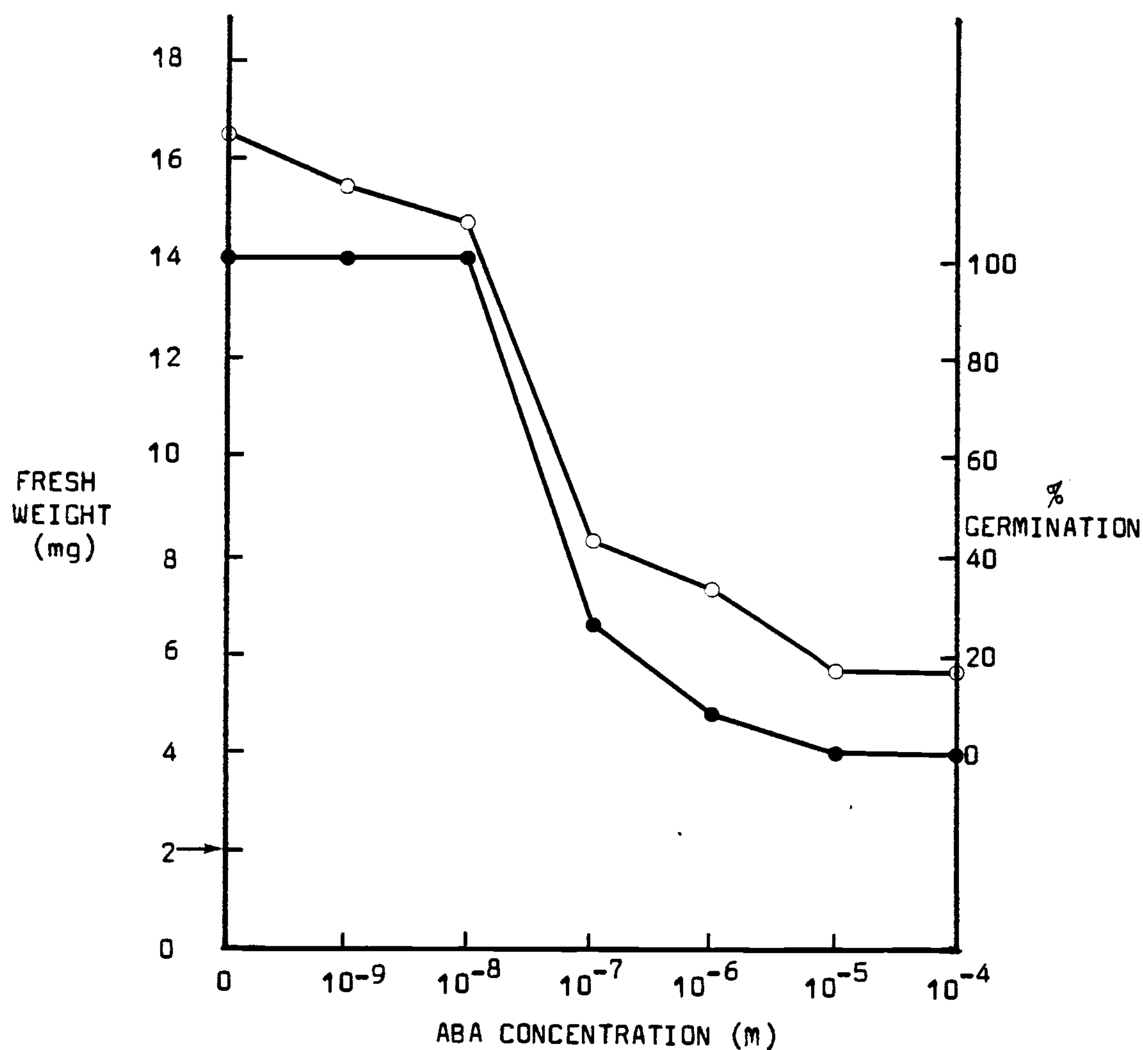

Figure 3. Measurement of fresh weight (open circles) and germination frequency (closed circles). Twenty day-old embryos of the variety Twin were isolated from grains and cultured in the dark for five days in GM with or without ABA. The arrow on the y-axis indicates the initial weight of an immature embryo (2.0 mg).

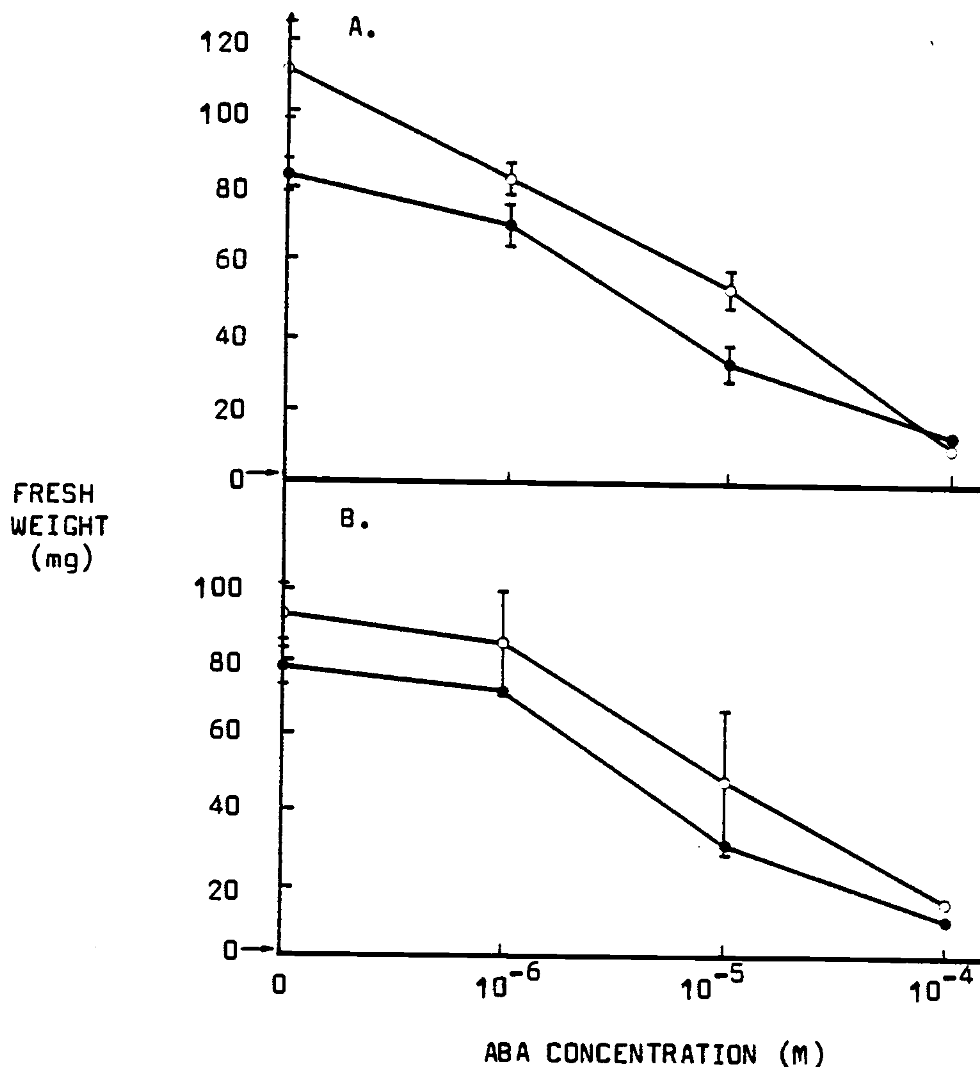

Figure 4. Fresh weight measurements in mature embryos treated with ABA. A. Mature seeds of the variety Yamhill (open circles) were incubated in the presence or absence of ABA for five days under a 16 hour photoperiod. Mature seeds of the variety Twin (closed circles) were treated similarly except that they were kept in the dark. B. Mature seeds of Chinese Spring were incubated as described above: light-grown (closed circles) and dark-grown (open circles). The arrows on the y-axis represent the initial weight of an imbibed embryo (1.88 mg). The bars indicate one standard deviation of the mean.

is no visual evidence of chlorophyll synthesis. This is in marked contrast to mature embryos germinated in the absence of ABA. The seedlings formed without ABA are green with a fully developed coleoptile, leaf, and several long roots. Fresh weight increases of 10 fold over ABA-treated embryos are observed during the five day incubation period (Fig. 4).

As seen in Figures 3 and 4, immature embryos appear to be more sensitive to ABA than 60 day-old embryos. At 5×10^{-6} M ABA, mature embryos in the grain of the varieties Chinese Spring, Yamhill, and Twin germinate but only attain a fresh weight that is 50% of the control after five days in culture. Essentially the same results are obtained with mature embryos excised from the grain before incubation. Twenty day-old Twin embryos, however, are observed to reach the 50% level of controls at 5×10^{-8} M ABA and do not germinate at 10^{-6} M. The high sensitivity of immature embryos to ABA is reduced when they are dried for seven days prior to incubation in ABA. Although growth is irregular in about 20% of these embryos (callus formed), 20 day-old embryos that have been dried show 40% germination at 10^{-6} M ABA compared to 6% germination of normal 20 day-old embryos at this concentration. Hence, the reduced sensitivity of mature embryos to ABA can be induced in highly sensitive 20 day-old embryos by premature drying.

The Acid-Soluble Protein Fraction

With the exception of immature Twin embryos, the amount of acid-soluble material per embryo is greater in embryos incubated in the

absence of ABA. However, when this value is expressed as the % of fresh weight, immature embryos treated with 10^{-4} M ABA contain from 2 to 5 times more total acid-soluble material than untreated seedlings. (Table I). ABA-treated embryos also contain 5-12 times more acid-soluble protein per milligram of fresh weight. Hence, ABA treatment not only prevents germination but leads to large increases in the acid-soluble proteins.

Is this increase in the acid-soluble protein localized in any region of the embryo, e.g. the scutellum? After five days in ABA, there are few differences between the acid-soluble proteins present in the scutellum and the embryo axis (Fig. 5). However, one band appears unique to the scutellum (upper arrow-Fig. 5) while a large staining intensity is apparent in another band (lower arrow-Fig. 5). The lower band may also be unique to the scutellum since complete separation of this tissue from the embryo axis is very difficult. The reduced amount of stain present in the lower band may be the result of the scutellum still attached, or possibly the absence of other proteins of a similar molecular weight. It is apparent that the proteins found in the embryo axis and the scutellum are similar, but the interesting possibility arises that ABA is causing a differential response in these two regions of the embryo as observed in the qualitative and quantitative differences in these two bands.

Are these proteins found only in the embryo or are they found in the endosperm? By dissecting away the endosperm in mature grains, contamination of the embryo extracts with acid-soluble endosperm proteins is reduced to negligible levels. One-dimensional SDS

TABLE I- THE QUANTITIES OF ACID-SOLUBLE MATERIAL IN EMBRYOS OF DIFFERENT VARIETIES OF WHEAT CULTURED IN THE PRESENCE AND ABSENCE OF ABA.

Variety	ABA Concentration (M)	Age of Embryos (DPA)*	Incubation Conditions**	Acid-Soluble Material: mg per seedling or embryo	Acid-Soluble Material: % of fresh wt.	Acid-Soluble Material: μg protein per mg fresh wt.
Yamhill	0	60	light	3.15	2.84	3.43
	10^{-4}			0.49	5.16	16.84
Chinese Spring	0	60	light	1.82	2.30	1.00
	10^{-4}			0.56	7.09	7.01
Chinese Spring	0	60	dark	0.94	1.02	0.92
	10^{-4}			0.50	3.52	4.90
Twin	0	60	dark	0.93	1.12	0.30
	10^{-4}			0.40	3.32	3.00
Chinese Spring	0	20	dark	0.53	2.50	2.06
	10^{-4}			0.33	13.20	17.56
Twin	0	20	dark	0.09	1.90	1.12
	10^{-4}			0.10	4.08	14.49

*DPA- days post-anthesis

**All seeds and embryos were incubated for five days at 27°C.

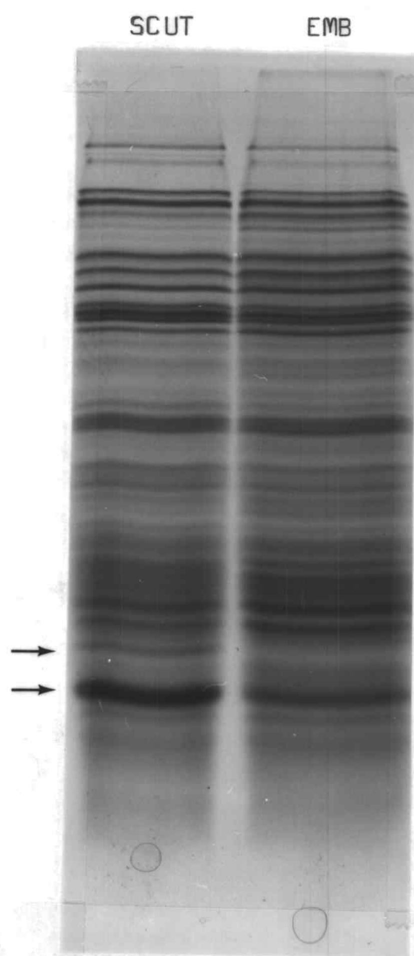

Figure 5. A comparison of the acid-soluble proteins from the scutellum and embryo axis. Immature embryos of Chinese Spring were isolated from 20 day-old grains and incubated for five days in GM with 10^{-4} M ABA in the dark. At the end of the incubation period, the scutellum tissue was separated from the embryo axis prior to acid-extraction. The acid-soluble proteins were separated in a one-dimensional SDS polyacrylamide gel. (SCUT: scutellum; EMB: embryo axis).

electrophoresis of equal amounts of acid-extractable material from embryo and endosperm tissue incubated with and without ABA reveals a completely different staining pattern. In addition, the overall staining intensity is greater for endosperm extracts indicating a higher protein content per mg of dry weight. In spite of the greater protein concentration on the gel, the amount of ^{35}S incorporated into the endosperm protein is less than 10% of that seen in the embryo proteins. Fluorography of the gels gives only faint bands for the endosperm proteins and, to be observed, requires long exposure times which result in very intense banding patterns for the embryo patterns. This result was not totally unexpected since the endosperm is terminally differentiated in the mature grain and completes cell division by 20 days post-anthesis (Dure, 1975). In immature embryos endosperm contamination is not a problem since the embryos are cleanly removed from the seeds prior to incubation and no endosperm is transferred during the culturing procedure. Hence, the acid-soluble proteins found in the embryo are unique, both in localization and synthesis.

In Figure 6, the staining patterns of the acid soluble proteins from ABA-treated embryos of Chinese Spring are shown. In the first dimension (acetic acid-urea polyacrylamide gel), all of the lysine and arginine residues in the proteins should have a positive charge due to the low pH of the gel system. The charged nature of the proteins in this system allows for separation both on the basis of molecular weight and on the basis of positive charge. This system also has the advantage over isoelectric-focusing in that proteins

differing by only a single post-translational modification such as acetylation or phosphorylation will have similar mobilities. Hence, in this system different protein bands or spots are not the result of post-translational modification of proteins. In the second dimension the proteins are separated on the basis of molecular weight in SDS polyacrylamide gels. The observed pattern is highly reproducible for the acid-soluble proteins from embryos treated with 10^{-4} M ABA.

A representative fluorogram of a two-dimensional separation of newly synthesized acid-soluble proteins from immature embryos of Chinese Spring is shown in Figure 6B. A large number of proteins containing ^{35}S can be resolved using this electrophoretic system. In the subsequent discussion, specific regions of this pattern will be analyzed. The region falling within bracket I contains those proteins with a low mobility in the acetic acid-urea gel and will be referred to as the low mobility proteins (LMP). Bracket II contains the high mobility proteins (HMP). In order to make direct

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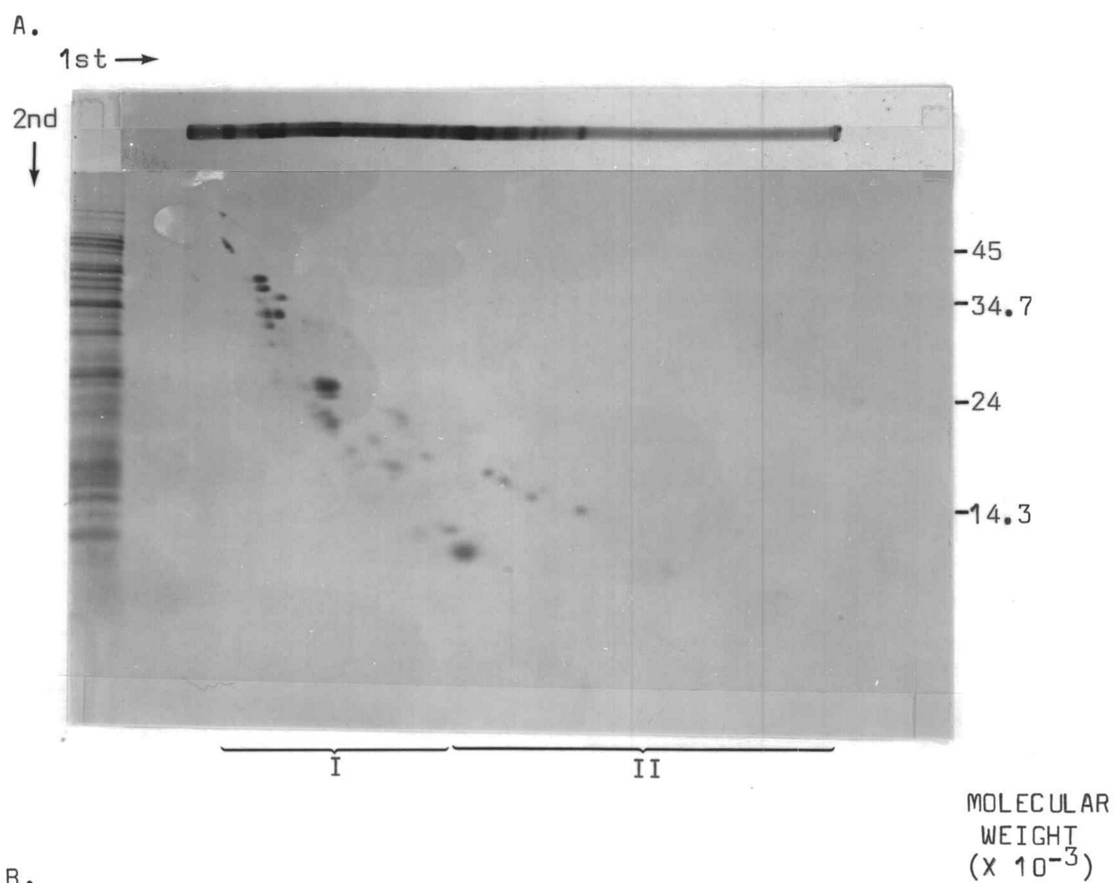
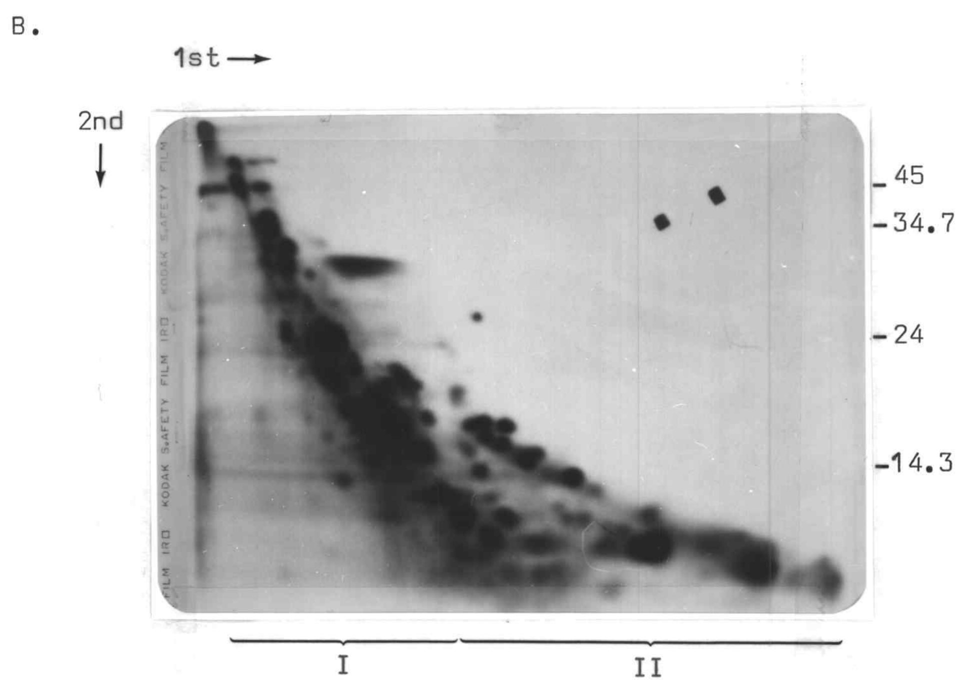
Figure 6. Two-dimensional electrophoresis of the acid-soluble proteins. Bracket I corresponds to the low mobility proteins (LMP), and bracket II corresponds to the high mobility proteins (HMP). A. Immature embryos of Chinese Spring were incubated for five days in GM in the presence of 10^{-4} M ABA. The acid-soluble proteins were extracted and separated electrophoretically. Across the top of the figure is the staining pattern seen in the acetic acid-urea tube gel. The separation obtained in a one-dimensional SDS gel is shown on the left-hand side. During staining and destaining, the slab gel expands more than the tube gel. The tube gel is actually smaller than shown. The molecular weight markers on the right-hand side are ovalbumin (45,000), pepsin (34,700), trypsinogen (24,000), and lysozyme (14,300). B. A fluorogram of the ^{35}S proteins synthesized during the five day incubation.



MOLECULAR WEIGHT ($X 10^{-3}$)



comparisons of the effects of ABA and α-amanitin on the LMP fraction, the upper halves of two tube gels were placed on the same slab gel for electrophoresis in the second dimension. This step eliminates potential variations that may occur as a result of slightly different conditions between slab gels.

The LMP Fraction

A. The Effects of ABA on Protein Synthesis in Immature and Mature Embryos

Diagrammatic representaions of the newly synthesized proteins in immature and mature embryos of different varieties in the presence and absence of ABA are presented in Figure 7. In both mature embryos of Yamhill (A and B) and Chinese Spring (C and D), there are numerous differences in the proteins synthesized in the presence and absence of ABA. Within region 1, all of the proteins synthesized in the absence of ABA (A) are also synthesized in the presence of ABA (B). A number of unique proteins are synthesized in the presence of ABA (B) and are indicated by arrows. In Chinese Spring, one unique protein (indicated by the arrow) is synthesized in the absence of ABA (C) and

Figure 7. Tracings of fluorograms showing synthesis of the LMP fraction in the presence and absence of ABA by embryos of different ages and different varieties. A and B. Mature embryos of Yamhill. C and D. Mature embryos of Chinese Spring. E and F. Immature embryos of Chinese Spring. ABA was present at a concentration of 10^{-4} M. The scale on the bottom of the figure represents the relative distance that the proteins moved in the acetic acid-urea gel. The scale on the left-hand side is the molecular weight range for each pane. Refer to the text for descriptions of the arrows and shading.

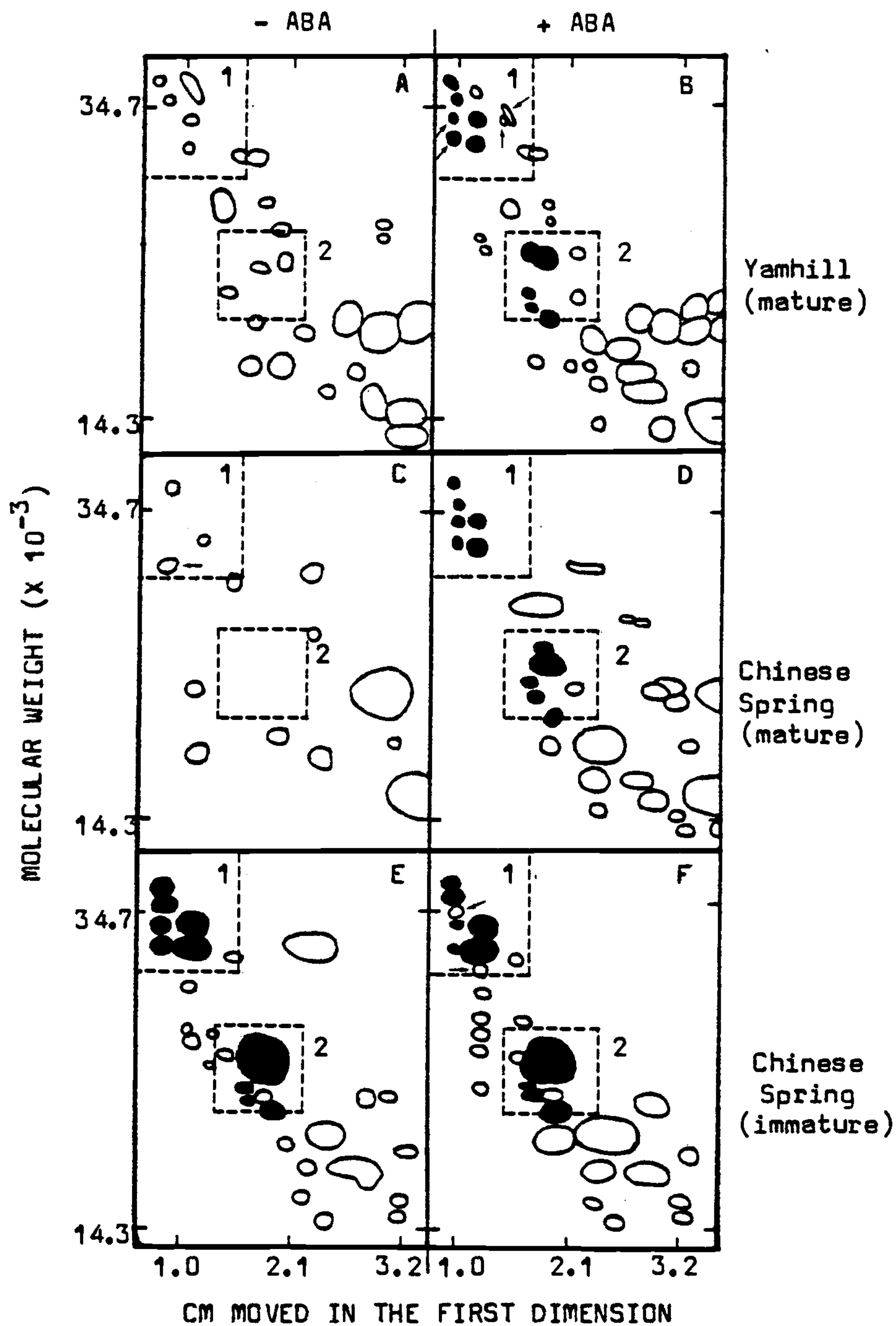


Figure 7.

four are produced when it is present (D). In Yamhill (A and B) there are only two common proteins within region 2. In Chinese Spring (C and D) there are no common proteins within region 2. Although this analysis could be extended further, these two regions serve to exemplify the differences in the proteins synthesized in the presence and absence of ABA. It should be noted that the overall intensity of the spots (i.e. the radioactivity present) in the untreated embryos is greatly reduced compared to treated embryos. Delineation of the radioactive regions is also more diffuse for the untreated embryos.

In immature embryos of Chinese Spring (E and F), the proteins found within regions 1 and 2 are the same except for the two unique proteins found within region F-1 (indicated by the arrows). Examination of the entire pane shows that there are not as many differences between treated and untreated embryos as there are in mature embryos. Also, the spots are of the same clarity and intensity for both treatments. The proteins synthesized by immature embryos in response to ABA do not appear to be as different as the response by mature embryos.

Also shown in Figure 7 are several proteins within regions 1 and 2 that are common to mature and immature embryos treated with ABA (B, D, and F), as well as to untreated immature embryos (E). These proteins have been indicated by shading. Although a few of these proteins are also found in untreated mature embryos (A and C), the intensity of the spots is much reduced. The synthesis of these proteins in untreated mature embryos may be a result of mRNA species which are retained through dessication and the first 24 hours after

imbibition. The experimental design does not allow one to distinguish between proteins synthesized early in the incubation period from those synthesized later. Hence, clear differences in the types of proteins synthesized in the presence and absence of ABA are observed, with mature embryos showing greater differences than immature embryos. Also, the proteins synthesized by either mature or immature embryos in response to ABA are very similar and almost identical to untreated immature embryos.

B. The Effects of α-Amanitin on Synthesis of the LMP Fraction

A comparison of the labelled LMPs from ABA-treated embryos incubated with and without α-amanitin is shown in Figures 8A and B. α-amanitin inhibits the synthesis of a number of proteins (indicated by arrows in B) while not affecting others. Those proteins not synthesized in the presence of α-amanitin must be dependent on *de novo* mRNA synthesis after imbibition. Conversely, those proteins which are synthesized in the presence of α-amanitin must be transcribed from mRNAs which are conserved through dessication of the embryo (Jendrisak, 1980). It also appears that α-amanitin is having a general effect of reducing protein synthesis, as the amount of radioactivity incorporated into the acid-soluble material per embryo is 69% lower in α-amanitin-treated embryos (1.13×10^6 cpm per treated embryo as compared to 3.64×10^6 cpm per untreated embryo). There is also an overall reduction in the intensity of the spots on the fluorograms.

A similar effect of α-amanitin is observed in immature embryos of Chinese Spring treated in the same way (Fig. 8C and D). Not only

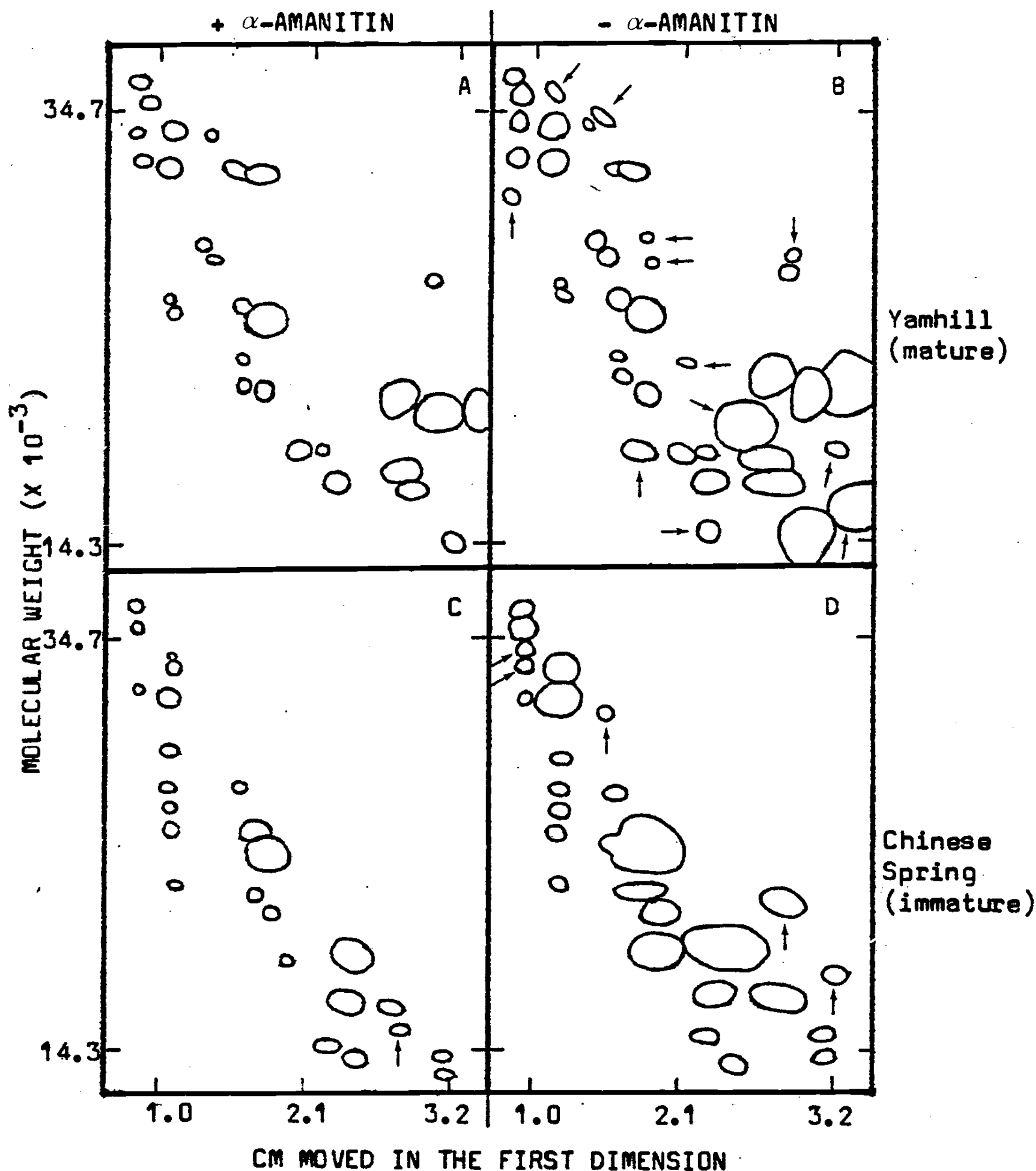


Figure 8. The effects of α-amanitin on synthesis of the LMP fraction. A and B. Mature embryos of Yamhill excised from the grains. C and D. Immature embryos of Chinese Spring. In A and C, the embryos were imbibed for one hour in 100 μg/ml of α-amanitin and subsequently incubated for three days in 0.1 μg/ml of α-amanitin. In B and D, the embryos were imbibed in distilled water then incubated in water (B) or GM (D) for three days.

is there a loss of fewer proteins compared to mature embryos, but the overall amount of radioactivity incorporated into the acid-soluble material per embryo is reduced by 25%. Five proteins were not synthesized in the presence of α-amanitin (indicated by arrows in D), and a protein was detected in α-amanitin treated embryos which was not detected in untreated embryos (indicated by the arrow in C). It is apparent that the mRNAs coding for acid-soluble proteins in the LMP fraction are, for the most part, already formed when the 20 day-old embryo is isolated from the grain. Different concentrations of α-amanitin were not tried. It is possible that the uptake of α-amanitin was less in immature embryos because the embryos were hydrated prior to incubation in 100 μg/ml of α-amanitin.

The HMP Fraction

A. Characterization of a Group of HMP Synthesized in Specific Response to ABA

Initial attempts to detect a specific group of proteins which were synthesized in response to ABA were complicated by the fact that the synthetic patterns examined were the result of total protein synthesis during the entire four day incubation period. When the embryos are pulsed with ABA and ^{35}S at different times after isolation from the grains (Fig. 9), a specific group of HMP accumulates label in increasing amounts (Fig. 10A, B, and C). These proteins are present in a characteristic line as shown in Figure 10. As the length of the incubation period without ABA is increased, the relative degree of labelling of the HMP line also increases. The only

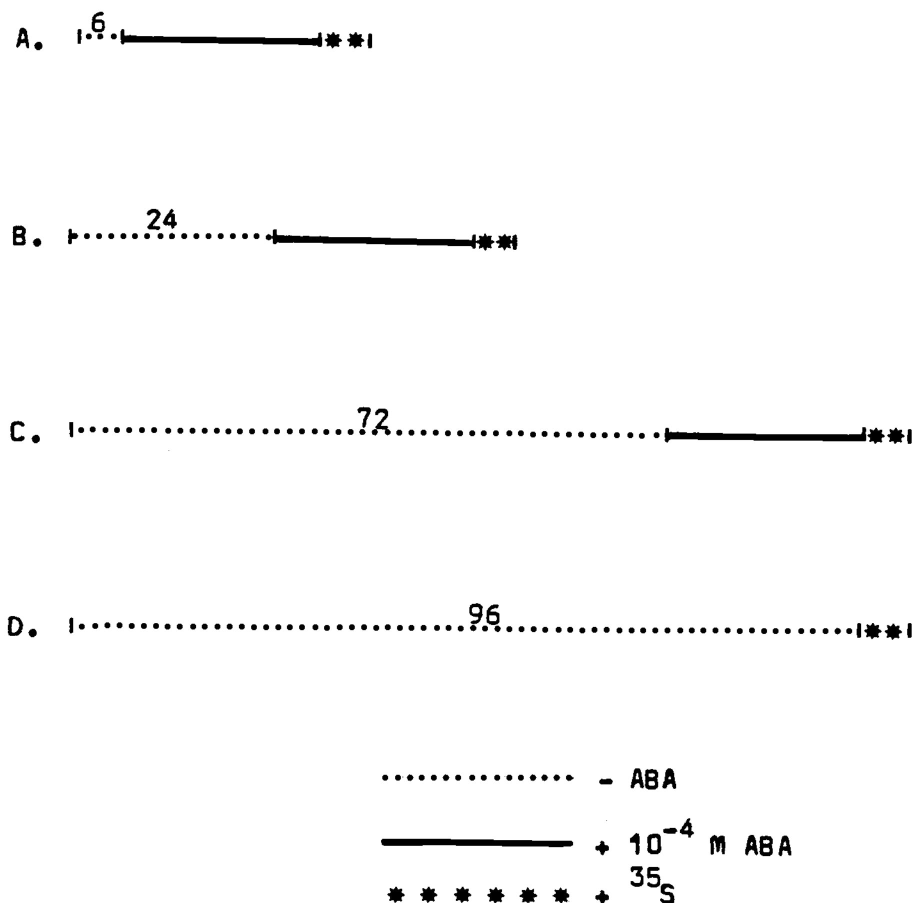


Figure 9. Incubation protocol for immature embryos. Immature embryos were isolated from 20 day-old grains of Chinese Spring and incubated according to each of the protocols above. The number above the dotted line indicates the number of hours without ABA. Embryos in A, B, and C were then incubated for 24 hours in 10^{-4} M ABA. All embryos were pulsed with $H_2{}^{35}SO_4$ for the last six hours of the incubation period. The letter designations for each protocol correspond to the appropriate fluorograms shown in Fig. 10.

difference between these treatments is the length of time that the embryos are without ABA. When immature embryos are incubated for 96 hours without ABA prior to the addition of radioactive label, the only proteins in this line which incorporate label are the two uppermost proteins and the lowermost protein (Fig. 10D). The quantity of label incorporated into these proteins is also reduced compared to ABA-treated embryos (Fig. 10C) and is similar to embryos incubated without ABA for only six hours (Fig. 10A). Hence, most of the newly synthesized proteins appear as a result of ABA addition.

It is possible that the ABA-induced HMP line might represent degradation products. This seems unlikely because of the following experiment. When embryos are removed from the label after a five day incubation period and transferred to germination medium without any label or ABA, the LMP fraction gradually becomes more diffuse over a five-day chase period. The ABA-induced HMP fraction, however, persists at the same intensity indicating that it is comprised of relatively long-lived proteins and not degradation products.

Figure 10. Synthesis of the HMP line in response to ABA. A. Immature embryos of Chinese Spring were isolated from 20 day-old grains, incubated for 6 hours without ABA, the next 24 hours with 10^{-4} M ABA, and finally pulsed for 6 hours with $H_2{}^{35}SO_4$. B. Immature embryos were treated as above except that they were incubated without ABA for 24 hours. C. Immature embryos were treated as above except that they were incubated for 72 hours without ABA. D. Immature embryos were treated as above except that they were incubated for 96 hours without ABA then pulsed with $H_2{}^{35}SO_4$ for 6 hours. For reference purposes, the arrows on the left and right of each pane indicate the line along which the ABA-induced proteins fall. The protein indicated by the triangle is in the same relative position in all of the treatments.

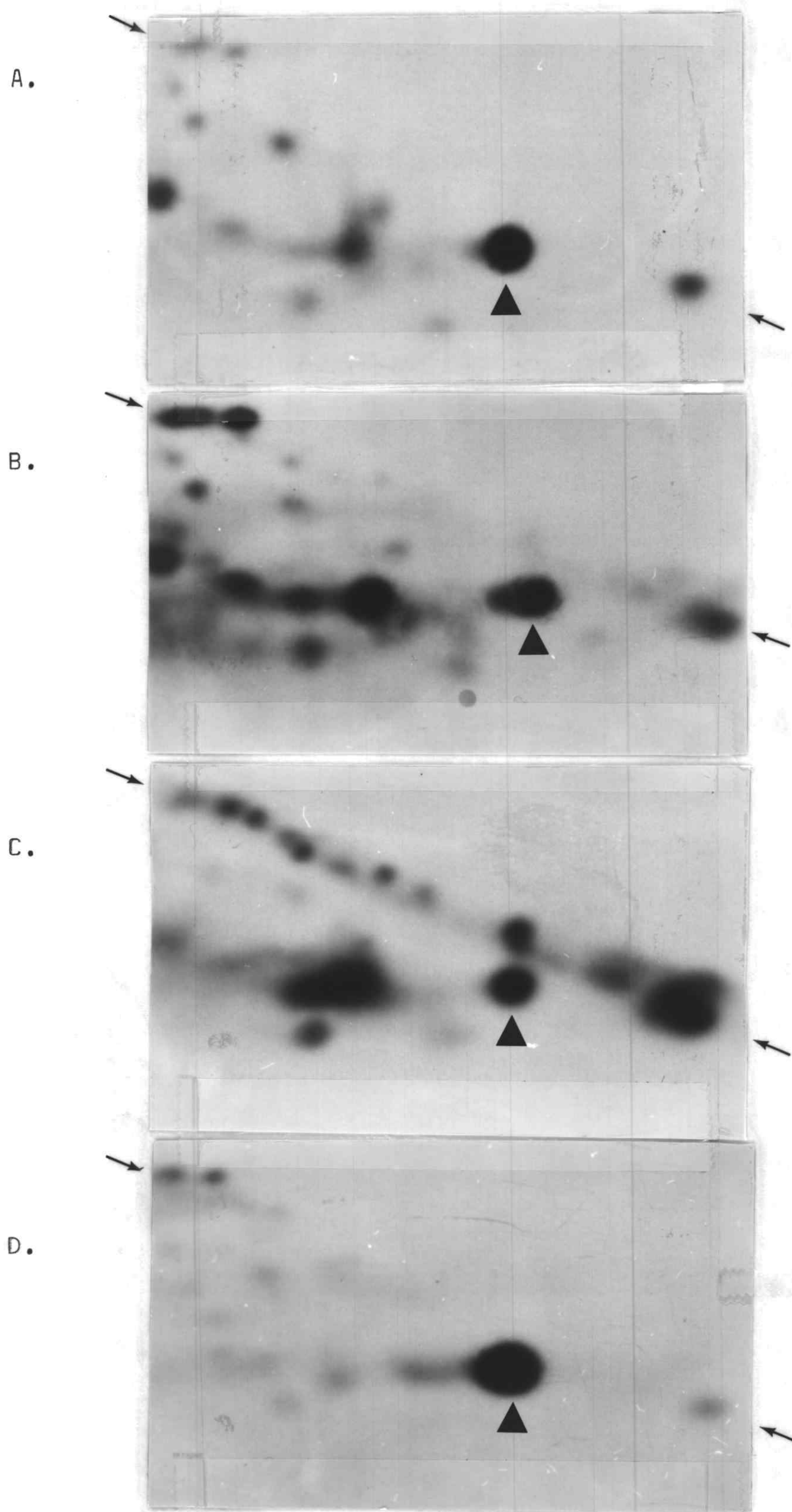


Figure 10.

B. Synthesis of the HMP Fraction in the Developing Grain

Evidence from the previous set of experiments indicates that ABA triggers the synthesis of HMP in cultured embryos. Are these same proteins synthesized by embryos in the developing grain and if so, how long after anthesis?

Grains of various ages were removed from the head and incubated in radioactive label for 24 hours. When the acid-soluble proteins from the embryos are electrophoresed, the HMP fraction is not detectable in 20 day-old embryos. The first visible staining in the HMP line is in extracts from 28 day-old embryos. The staining intensity increases up to 40 days post-anthesis (Fig. 11), but only the higher molecular weight proteins within this group are detected. It appears that the ABA-induced HMP fraction synthesized in isolated embryos is identical with a fraction of proteins which appear normally in embryos between 28-40 days after anthesis. The synthesis of this HMP fraction can be precociously induced in 20 day-old embryos in culture by ABA addition.

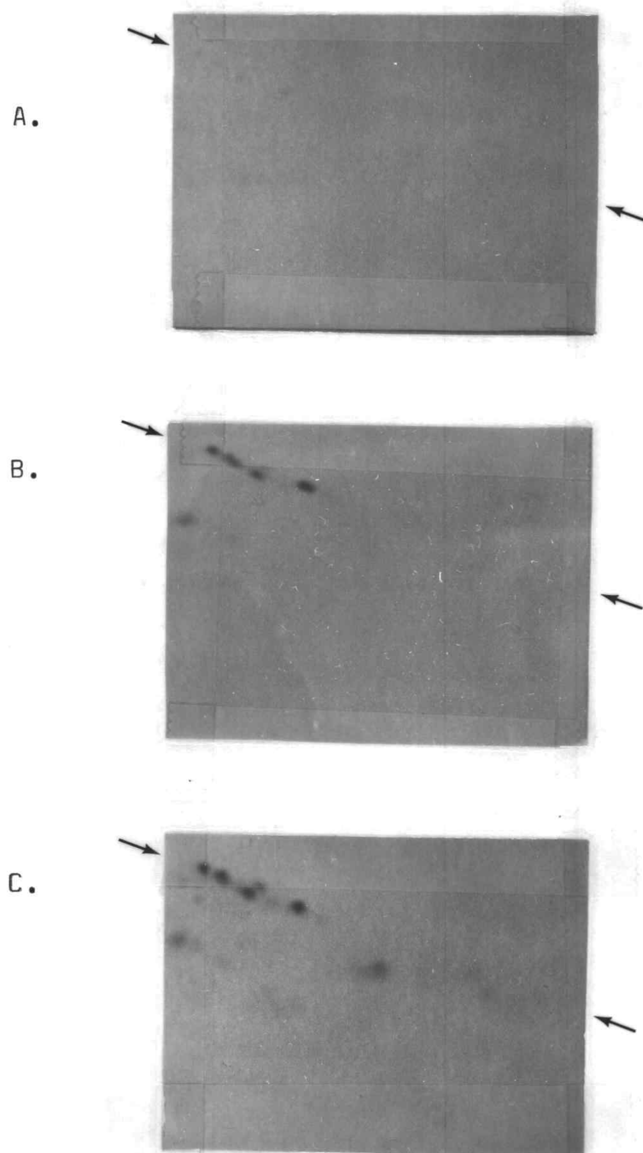


Figure 11. *In situ* synthesis of the HMP line (arrows). A. Acid-soluble proteins from embryos 28 days old. B. Acid-soluble proteins from embryos 40 days old. C. Acid-soluble proteins from 20 day-old embryos incubated for 72 hours without ABA, 24 hours with 10^{-4} M ABA, and then pulsed with $H_2{}^{35}SO_4$.

IV. DISCUSSION

The Effects of ABA on Embryo Development

The results of the experiments presented in this study indicate that ABA prevents the precocious germination of 20 day-old embryos of the varieties Twin and Chinese Spring. In both varieties, the germination response of immature embryos is 50 times more sensitive to exogenously supplied ABA than the germination response of mature embryos. The physiological cause of this change may be a result of dessication. Twenty day-old embryos which are dried for seven days prior to incubation in GM exhibit a higher percentage of germination than undried embryos at corresponding ABA concentrations. This raises the question of what physiological changes are occurring during dessication that reduce the sensitivity of the embryo to ABA.

Immature embryos may exhibit different rates of uptake, different rates of degradation, different sensitivities of ABA receptors and/or different levels of endogenous ABA when compared to mature embryos. For example, if the immature embryos are more efficient than mature embryos at accumulating ABA in culture (all other factors being equal), they would be expected to show a much greater reduction in growth in the presence of lower concentrations of exogenous ABA. It is also possible that one of the degradation products of ABA is the physiologically active component, e.g. phaseic acid. If this is the case, different degradative characteristics of immature and mature embryos would account for the differences in

the germination response. Finally, it is possible that endogenous levels of ABA are higher in immature embryos when they are isolated from the grains at 20 days post-anthesis. Lower levels of ABA in the growth medium may cause the intracellular concentration of ABA to rise to the critical,inhibitory level. If there is a loss of ABA during dessication and dormancy, higher levels of exogenous ABA would be required to reach this level in mature embryos and, thus, inhibit germination upon hydration. Radley (1979) found that ABA levels were highest from 28 to 40 days post-anthesis and declined rapidly as the embryos dried. Perhaps this mechanism to reduce ABA levels in drying grains is still active when the grain is rehydrated for germination. A greater exogenous ABA concentration would be required to offset this mechanism in mature grains, which is absent in immature embryos. Although this study cannot distinguish between these alternatives, further work is needed to determine the levels of ABA in the embryo under these conditions as well as the possible mechanisms involved in the uptake,synthesis, and degradation of ABA.

Protein Synthesis in the LMP Region

One of the goals of this study is to determine if specific proteins are being synthesized in the embryo in response to ABA. Such results would not be totally unexpected as Mozer (1980) has demonstrated the synthesis of two unique proteins in barley aleurones in response to ABA. Within the LMP region, there are a number of proteins which are synthesized only in the presence of ABA. These

can be the result of transcription of new templates or the translation of conserved templates. These two alternatives were distinguished on the basis of α-amanitin inhibition of their synthesis (Jendrisak, 1980).

Evidence from the α-amanitin experiments in this study indicates that synthesis of ABA-specific proteins is controlled at both the translational and transcriptional levels. In mature embryos, 26 proteins in the LMP region are synthesized in both the presence and absence of α-amanitin. Conservation of these mRNAs explains the observed synthesis of some of these proteins in mature embryos not treated with ABA. Since the mRNAs for these proteins are conserved and many of them are not synthesized in the absence of ABA, ABA appears to be controlling their synthesis at the translational level.

The results presented here also indicate that ABA controls the synthesis of some proteins at the transcriptional level. In mature and immature embryos, twelve proteins and five proteins, respectively, in the LMP region are not synthesized in the presence of α-amanitin. Messenger RNAs for these proteins are not present prior to α-amanitin treatment and are not synthesized when it is added simultaneously with ABA.

What is the nature of the proteins and mRNA that are conserved and what is known of the α-amanitin effect? Cuming and Lane (1979) have demonstrated that mRNAs for basic proteins (ribosomal?) are translated for the first several hours after imbibition. By 48 hours after imbibition, however, the mRNA population changes almost

completely. There is no preference for conserved or newly synthesized mRNAs by the protein synthetic apparatus in germinating wheat embryos (Caers _et al_, 1979; Brooker _et al_, 1978). In this study, however, proteins being synthesized from 24 hours and later were analyzed. It is not surprising then that some of the proteins synthesized used conserved templates. By not adding the label until 48 hours, one might be able to distinguish the nature of the proteins derived from new transcripts.

At concentrations of α-amanitin which inhibit RNA polymerase II activity _in vitro_, germination is also blocked (Jendrisak, 1980). But at the lower germination-inhibiting concentrations, there is no effect on _in vivo_ protein synthesis and polysome formation with pre-existing mRNA (Spiegel and Marcus, 1975; Cheung _et al_, 1979). Although higher concentrations inhibit overall protein synthesis (Thompson and Lane, 1980), the inhibitory effect probably involves reduced synthesis of ribosomal and transfer RNA (Jendrisak, 1980). Thompson and Lane (1980) have shown that α-amanitin blocks the synthesis of proteins synthesized within six hours of imbibition and that the translation of conserved mRNAs persists for a longer period of time. Caers _et al_ (1979) have estimated the half-life of conserved mRNAs at about two hours. The lower intensity and poor delineation observed in the newly synthesized proteins of untreated embryos also suggests that digestion of the conserved mRNAs is partially completed at the time of label addition. By blocking _de novo_ transcription of mRNA, the synthesis of enzymes involved in degradation of conserved mRNA in mature embryos may be blocked. For

example, Mozer (1980) has conclusively shown that ABA inhibits the synthesis of α-amylase at the translational level in barley aleurones. He showed that while ABA blocks the appearance of α-amylase *in vivo*, *in vitro* translation of total poly(A)-RNA isolated from ABA-treated aleurones results in the synthesis of functional α-amylase.

Protein Synthesis in the HMP Region

Within the HMP region, a specific group of proteins is synthesized in response to ABA. Incubation of immature embryos for 72 hours without ABA followed by 24 hours with ABA results in the synthesis of a unique group of proteins induced only in the presence of ABA. Chase experiments indicate that these proteins are relatively long-lived compared to most of the proteins in the LMP region. In fact, synthesis of proteins in the LMP region gradually decreases as the length of the incubation period without ABA is prolonged.

ABA at 10^{-4} M stimulated the synthesis of the HMP as they are detectable in the embryo within 72 hours of isolation (23 days post-anthesis) from the grain. At 20 days post-anthesis, the only detectable proteins in the acid-soluble fraction are the highest molecular weight proteins in the LMP region. When embryos are assayed directly from the plant without any culturing, there is no detectable synthesis in the HMP region until the embryos are 28 days old. Accumulation of the acid-soluble fraction persists until 40 days post-anthesis when maximum staining intensity is observed.

This pattern is similar to that seen for WGA in the wheat embryo (Triplett, 1979). Twenty-eight to forty days post-anthesis also corresponds to the period of maximum ABA concentration in the embryo (Radley, 1979). Hence, one can precociously synthesize the HMPs in embryos by treating them in culture with ABA.

Although the ABA-inducible proteins in the HMP region are detected in immature embryos when they are treated with 10^{-4} M ABA, the remaining proteins in the LMP region are insensitive to exogenous ABA after incubation for 72 hours without ABA. In accumulation experiments, they are detected regardless of whether ABA is present or absent. In pulse experiments, they are only detected when the ABA pulse comes within 48 hours after isolation from the grain. At the time of isolation of the immature embryos, the mRNAs coding for most of the proteins in the LMP region are probably already present as α-amanitin reduces, but does not block, their synthesis. (One can argue that α-amanitin is not penetrating the immature embryo. It is already hydrated at the time of isolation and does not go through a true imbibition phase. Jendrisak (1980) has found that imbibition is essential if the inhibitory effect of α-amanitin on germination is to be observed.) Thus, it appears that ABA prevents degradation of the mRNAs for proteins in the LMP region and stimulates synthesis of proteins in the HMP region.

The Role of ABA in Dormancy

None of the data presented in this study allow for the assignment of a physiological role for ABA-inducible proteins in the embryo.

However, there are a couple of possibilities that may be speculated upon based on the research of other investigators. A complex of mRNA and ribonucleoproteins can be isolated from plant embryos. These complexes represent a stored form of mRNA called informosomes which are conserved during dessication (Ajtkhozhin et al, 1976). If these stored mRNAs code for proteins involved in germination, ABA may be stimulating the synthesis of proteins which block their translation. This seems unlikely since de novo RNA synthesis is essential for germination (Jendrisak, 1980). Another possibility is that these mRNAs may represent stabilized forms of the mRNAs which are retained through developmental arrest and dessication of the embryo. This seems to be a more likely possibility as a large number of proteins stop being synthesized when immature embryos are removed from the grain and incubated without ABA. When ABA is added, these proteins are synthesized for extended periods of time. Iskakov and Aitkhozhin (1979) have tentatively isolated proteins from polysome-bound informosomes which range in molecular weight from 56,000 to 82,000 daltons. This range is three to five times greater than the molecular weight of the largest ABA-inducible HMP.

Another possible role for the ABA-inducible proteins is a structural one, i.e. ribosomal proteins. Cuming and Lane (1979) have characterized a group of basic proteins which are products of early protein synthesis in wheat embryos. Synthesis of these proteins is independent of de novo RNA synthesis but rapidly decreases within several hours of imbibition. That these proteins are synthesized only for a short period in imbibed embryos and only from conserved

mRNAs suggests that they are involved in translational capabilities of the ribosomes during developmental arrest. It would be interesting to characterize the synthesis of ribosomal proteins in response to ABA.

Conclusions

Several conclusions can be drawn from the data presented in this study concerning the effects of ABA on protein synthesis and development in wheat embryos.

1) ABA prevents precocious germination in immature embryos and normal germination in mature embryos.

2) Immature embryos are about 50 times more sensitive to ABA than mature embryos. Immature embryos will not germinate in culture unless the ABA concentration is lower than 5×10^{-7} M; mature embryos germinate at concentrations up to 10^{-5} M. The greater sensitivity of immature embryos to ABA is partially overcome by premature drying.

3) On a percentage fresh weight basis (for both mature and immature embryos), ABA-treated embryos contain two to five times more acid-soluble material than untreated embryos. ABA-treated embryos also contain 5-12 times more acid-soluble protein per milligram fresh weight than untreated embryos.

4) Using one-dimensional SDS polyacrylamide electrophoresis, the acid-soluble proteins found in the embryo are unique and are not found elsewhere in the grain. When equal quantities of acid-soluble proteins from the embryo axis and scutellum are compared under the same conditions, only two bands are different, both more prevalent

in the scutellum.

5) A two-dimensional electrophoretic system has been developed that resolves a large number of acid-soluble proteins.

6) Using this two-dimensional system, ABA induces the synthesis of proteins in mature embryos which are not detected in untreated embryos. In immature embryos, however, the differences between the proteins synthesized in ABA-treated and untreated embryos are not as dramatic.

7) The newly synthesized proteins induced by ABA in mature embryos are similar to the ABA response in immature embryos.

8) Since the appearance of some ABA-induced proteins is blocked by α-amanitin, their synthesis appears to be under transcriptional control. Some of the ABA-induced proteins continue to be synthesized in the presence of α-amanitin suggesting that their control is at the translational level.

9) In immature embryos, ABA induces the synthesis of a unique group of proteins with a high mobiblity in acetic acid-urea gels. These proteins appear three days after the 20 day-old embryos are exposed to ABA in culture. They are not evident in untreated embryos.

10) These same proteins do not appear in the embryo developing on the plant until 28 days post-anthesis. By 40 days post-anthesis, the amount is roughly equivalent to 20 day-old embryos treated with ABA for three days in culture. This coincides almost precisely to the time that ABA concentrations are highest in the developing grain.

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