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A set of genes which show developmental and tissue
specificity are expressed late in embryo maturation during
grain formation in wheat (*Triticum aestivum*). Two members
of this maturation gene set (MGS) are Em and triticin, a 7S
globulin. Results presented here show that during
embryogenesis, the expression of Em mRNA and polypeptide is
temporally associated with increasing levels of the plant
growth regulator abscisic acid (ABA). In the absence of
elevated ABA levels, neither the mRNA nor the polypeptide
for Em or triticin are detected at other developmental
stages or in non-embryonic tissues. *In situ* hybridization
shows that Em mRNA is localized in the embryo axis of the
developing grain with high amounts concentrated in the shoot
and root apices. Absence of Em mRNA in the scutellum and
endosperm, the embryo storage organs, suggests that the
function of Em in not one of a storage protein. However,
mRNA levels of Em and triticin increase in response to
higher levels of ABA at other stages of vegetative

development. When ABA is supplied to germinating seedlings, mRNA for both Em and triticin accumulate. Some seed globulin proteins also accumulate in germinating seedlings after exposure to ABA. Em mRNA accumulation coincides with the pattern of increasing endogenous ABA concentrations in dehydrating seedling shoots. However, neither Em nor seed globulin polypeptides are detected in dehydrating tissue. These results suggest that ABA promotes MGS transcription or mRNA stability, in tissues other than embryos, but other factors control tissue specific expression at the polypeptide level. Desiccation of the embryo during normal grain development does not appear to be a trigger to prevent expression of MGS mRNA in response to ABA in non-embryonic tissue. However, desiccation may serve to alter the metabolic environment, preventing MGS polypeptides from accumulating. The implications of these findings on the proposed function of the MGS gene products and their regulation during development are discussed.

THE EXPRESSION OF EMBRYO MATURATION GENES IN WHEAT
AND THE ROLE OF ABSCISIC ACID

by

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INTRODUCTION

In the life cycle of plants, the seed has an
important role. It is the link between one generation and
the next. The following work was undertaken in an effort to
better understand how developmental events unfold during
embryogenesis and maturation in plants and how the
expression of genes involved in embryo maturation may be
influenced by plant growth regulators.

A set of genes expressed preferentially late in
embryo maturation was identified initially in cotton (Ihle
and Dure, 1972) and subsequently in a number of other plant
species (reviewed by Quatrano, 1986). The products of this
maturation gene set (MGS) accumulated in developing seeds at
the time of increasing endogenous levels of the plant growth
regulator abscisic acid (ABA). They persisted in dry
embryos but were lost on imbibition and not reaccumulated at
other stages of the normal plant life cycle. They were not
essential for successful germination. They accumulated
precociously in early embryos in culture by application of
osmoticum or ABA.

The usefulness of genes comprising the MGS for
further genetic and developmental studies rested on two

criteria governing their expression: responsiveness to the plant growth regulator ABA, and the apparently strict tissue and time specificity. These two characteristics allowed a number of interesting questions to be addressed. What is the role played by ABA in this expression? What is the association of the presence of ABA and the expression of a responsive gene? Are there limitations for gene expression in tissues other than the normal target tissues? What limitations do the preceding developmental stages in the plant life cycle place on genes belonging to sets that are specific to a single developmental stage? Can the elements of hormone responsiveness and tissue/time specificity be separated or are they mutually dependent? These and other questions were the basis for the work presented here.

In Chapter I the normal expression pattern of Em, a gene product identified as part of the MGS in wheat, was investigated. Investigations into the expression of Em messenger RNA (mRNA) and protein and the relationship of this expression to endogenous levels of the plant growth regulator ABA are presented. Localization of the mRNA corresponding to this gene within the developing embryo was shown. Chapter II addressed the response of Em and triticin, a second member of the wheat MGS, to ABA at times other than embryo maturation. Throughout both chapters the influence and role of ABA was examined in light of its regulatory effect on the MGS in wheat.

REVIEW OF LITERATURE

Purpose of Literature Review

The embryo maturation phase of the higher plant life cycle is part of an overall "seed strategy" (Quatrano, 1987). Seeds must meet a number of criteria, meant to ensure the survival of a young seedling in the next generation. These requirements include an accumulation of nutrient reserves, arrest of tissue development and growth, acquisition of desiccation tolerance and, in some instances, dormancy. The latter characteristics have evolved to allow survival over long periods of time in a dry state until favorable environmental conditions occur. Fulfilling these requirements necessitates the switching between sets of genes, the products of which are required at spatially and temporally different stages throughout the development of the seed. As this developmental expression is complex, the regulation of gene expression and the interplay of factors regulating this expression is expected to be equally complex and intriguing. This fact is attested to by a number of reviews published in recent years dealing with the regulation of embryo and seed development, and the regulation of gene expression during this time in the life cycle of higher plants (Goldberg et al., 1989; Quatrano, 1986, 1987; Simmons, 1987; Pharis and King, 1985). This review of relevant literature is intended to give (1) an overview of the processes occurring during embryo and seed development in higher plants (with an emphasis on the later

stages of embryo development), (2) the nature of the agents affecting these processes, specifically plant growth regulators (with an emphasis on ABA), and (3) how these factors might be influencing gene expression, during seed development and at other times in the plant life cycle.

Overview of Seed Development

Double fertilization is unique to angiosperms and sets into motion a chain of complex and interrelated events. This first step initiates the formation of two cell lineages: endosperm, and embryo.

The role of the endosperm is thought to be chiefly one of supplying carbon and nitrogen to the developing embryo and growing seedling (review of endosperm development in Vijayraghavan and Probhakar, 1984; Simmons, 1987). That normal development of the endosperm plays some role in ensuring normal development of the associated embryo is evident from studies using developmental mutants of maize in which normal embryos are surrounded by mutant endosperm (Sheridan and Neuffer, 1986). Neuffer and Sheridan (1980), using the *defective kernel* class of mutants in maize, found that crosses between wild type and mutant plants sometimes resulted in the pairing of a wild type embryo with a mutant endosperm. In the majority of these cases (17/19), the mutant endosperm had no observable effect on the resultant embryos, however, one resulted in a dead embryo, and the other in a viable embryo that gave rise to a weak seedling. Another line of evidence for the influence of endosperm on

embryo development comes from the ability to rescue embryos from interspecific hybrid crosses that result in the formation of unrelated and sometimes toxic endosperm. Work done in pasture legumes showed that when the embryo is removed from the toxic endosperm and placed into culture, normal embryos may develop (Williams and deLatour, 1980).

The specific role of the endosperm as a seed tissue may not be very well understood, but there is a wealth of information about genes which are expressed in storage organs and the aleurone cells of persistent endosperm, especially in cereals. The primary reason for this is the superabundance of both storage protein messenger RNAs (mRNAs) and polypeptides in developing and mature endosperm. Some examples of the abundance of these proteins: in maize, zein polypeptides account for 60% of protein in the endosperm (Sorenson, 1984), in soybean, two major storage proteins glycinin and β-conglycinin make up about 70% of the protein found in the dry seed (Sorenson, 1984), and in *Phaseolus vulgaris*, 40-50% of the total seed protein is composed of phaseolin (Ma and Bliss, 1978). By 25 days after pollination (dap) in wheat, nearly 60% of all mRNA's being translated in developing endosperm are for storage proteins (Pernollet and Vaillant, 1984).

Seed storage proteins have a tissue and time specificity to their expression, they often undergo lengthy post-translational modification, and are often compartmentalized within vacuoles or protein bodies

(reviewed by Higgins, 1984). Also, there may be a differential distribution of seed storage proteins within the seed (Goldberg et al., 1989). Prolamins, the major endosperm storage protein in maize, are restricted to the starchy endosperm but globulins are found in both the endosperm and embryo (Dierks-Ventling and Ventling, 1982). How this tight spatial and temporal regulation is maintained is not altogether clear. It is likely that plant growth regulators have a role, although evidence for a direct effect is conflicting (Higgins, 1984).

Much use has been made of transgenic plants for analyzing the expression of genes coding for seed storage proteins, both in transient expression and stable incorporation systems (Goldberg et al., 1989). Sequences coding for phaseolin (a bean seed protein) are properly expressed after transfer to sunflower cells (Murai et al., 1983). Tobacco plants transformed with a gene for the β-subunit of β-conglycinin, a soybean storage protein, show expression of mRNA for this gene only within the embryo, with preferential localization within cotyledon and upper axis cells (Barker et al., 1988). This shows that the transferred DNA includes all the information required for correct temporal and spatial expression in a heterologous host. Hypocotyl explants of oilseed rape transformed with *Agrobacterium tumefaciens* strains carrying an engineered napin storage protein gene resulted in developmentally regulated expression of the re-introduced gene (Radke et

al., 1988). Information gained from experiments such as these has led to many excellent reviews being published on the nature and regulation of seed storage protein genes of both endosperm and embryo in recent years (Casey and Domoney, 1987; Heidecker and Messing, 1986; Higgins, 1984; Sorenson, 1984).

Embryo development in the majority of plants may be divided into three stages: early, mid, and late (Steeves and Sussex, 1972). During the early stages of embryo development the formation of a bipolar root-shoot axis and the establishment of epidermal, vascular, and ground tissue takes place. The mid stage of embryo development is characterized by the synthesis of storage reserves in the cotyledons and/or embryonic axis. Desiccation, leading to developmental arrest or dormancy, occurs during the late stage.

Because of the inaccessibility of the egg cell within the ovary of the plant, much of the information about the early events in embryo development has come from mutants and studies of somatic embryo formation in culture. The development of the embryo in some of the embryo lethal mutants of *Arabidopsis* is blocked at very early stages (Meinke, 1985). Further investigations using these mutants may shed some light on what processes are involved very early in embryogenesis. The most extensively studied system for somatic embryogenesis is that of *Daucus carota* (wild carrot) in which suspension cultures of various tissues will

form embryos when given the correct sequence of culture conditions (Street, 1976). Comparison of mRNA and protein from embryogenic cultures showed a difference from non-embryogenic cultures, however, both culture lines differed from the original explant (Sung and Okimoto, 1983). This showed that changes in expression from original material had preceded any initiation of embryo formation. It indicated that some of the cells in the carrot culture had already been re-programmed, as a result of the somaclonal variation inherent in the system, to begin embryogenesis before any treatment had started. This is in agreement with the descriptions of cytological changes occurring in tissue cultured plants (somaclonal variation reviewed by Lee and Phillips, 1988).

After the basic systems comprised of shoot and root meristems and other tissues have been established, development of the embryo continues. During the mid stage of embryo development, the embryo continues to increase in length and dry weight, and seed proteins and lipids accumulate (Goldberg et al., 1989). The late stage of embryo development is marked by a browning of seed tissues, a decrease in RNA and protein synthesis, a tolerance of desiccation and the onset of maturation drying, and the induction of seed dormancy (Bewley and Black, 1986).

Extensive work with cotton, examining the *in vitro* translation products of embryo RNA isolated from differing stages of development, has shown that there are sets of

genes showing characteristic patterns of expression (Dure et al., 1981). Work with other species has confirmed the idea of sets of genes being expressed in concert during different stages of development (reviewed by Goldberg et al., 1989; Quatrano, 1986, 1987). Some of the proteins comprising these developmental sets have been identified and their function determined. Seed storage proteins fall into the category of genes whose expression begins during mid-embryogenesis (Higgins, 1984). Genes expressed later in embryo development include an α-amylase inhibitor in barley (Robertson et al., 1989; Mundy and Rogers, 1986), lectins in bean (Zavala and Sussex, 1989), rice (Stinissen et al., 1984), and wheat (Triplett and Quatrano, 1982) and the Em polypeptide in wheat (Quatrano et al., 1983a). The acquisition of desiccation tolerance also characterizes the late stages of embryo development (Bartels et al., 1988). Bartels et al. (1988) showed that barley embryos excised earlier than 16 dap were intolerant of a desiccation treatment which was non-lethal later in development. The ability to withstand low moisture content is essential for seed survival over long periods of time once it is separated from the mother plant.

Plant Growth Regulators Found in Seeds

Developing seeds are a rich source of many growth regulators (Gage et al., 1989; Jones and Brenner, 1987; King, 1976; Eeuwens and Schwabe, 1975; Ueda et al., 1970; Letham, 1963). In fact, immature seeds were the first

source identified for some plant growth regulators (Letham, 1963). Most evidence linking seed or embryo development to regulation by growth regulators comes from an observed correlation between embryo growth and regulator concentration. Cytokinins by definition are substances known to promote cell division in plant tissues (Horgan, 1984) and the concentration of cytokinins in lupin seeds reaches its highest level when active cell division and enlargement is occurring within the seed (Davey and Van Staden, 1979). The highest concentrations of active gibberellins (GA's) found in dwarf pea seeds occur between 18 and 22 days into development, during the maximum growth rate of the developing embryo (Bewley and Black, 1986). The suspensors of developing *Phaseolus* embryos contain GAs and likely supply them to the growing embryo; very young bean embryos deprived of suspensors in culture do not develop any further unless the culture medium is supplemented with GA_1 (Brady and Walthall, 1985). Likewise, indole acetic acid (IAA) is found in highest concentrations at the times of greatest growth in pea embryos (Eeuwens and Schwabe, 1975).

In contrast to the growth promoters listed above, ABA has generally been associated more with the arrest of embryo growth than with its promotion (Bewley and Black, 1986). This difference in proposed regulatory activity also correlates with endogenous levels of ABA; levels of ABA are low when growth is maximum and high when growth has slowed (Naumann and Dorffing, 1982; King et al., 1979; Hsu, 1979;

King 1976). The maximum level of ABA in seeds is within the embryo and these levels decline with the onset of desiccation (Jones and Brenner, 1987; Walker-Simmons, 1987; Bewley and Black, 1986; King, 1976). Despite this, there is a large body of evidence that ABA does not act solely as an inhibitor, but will actually promote activities involved with seed development and maturation while inhibiting activities involved with germination (reviewed by Zeevaart and Creelman, 1988: Quatrano, 1986,1987).

Events and Gene Regulation During Late Embryogenesis and Evidence of a Role for ABA

At some point near the mid stage of embryo development, the still immature embryo of many plant species is capable of germinating if removed from the seed. The observation of the ability to germinate precociously was first made in cotton (Ihle and Dure, 1972). Although this capacity has since been seen in a number of other angiosperms (reviewed by Quatrano, 1986), it is not necessarily the rule. Embryos of mangrove (*Rhizophora mangle*) are naturally viviparous, where seedlings form on the plant without developmental arrest or desiccation (Sussex, 1975). At the other end of the spectrum, embryos of clematis are immature at the time of seed dispersal, and further development and maturation must occur before germination (Atwater, 1980).

From experiments on cultured immature embryos it is evident that germination and growth into a viable seedling

is realisable without full seed maturity. This suggests two things: (1) the final stages of embryo and seed maturation are expendable, not obligatory for the formation of viable offspring, and (2) there is some endogenous factor preventing this precocious germination from occurring when the seed is intact. It also identifies characteristics of a set of "maturation genes" (Rosenberg and Rinne, 1988; Quatrano, 1987). Genes that form this set would have to meet the following requirements: 1) their products are not essential for germination, that is if embryos are germinated in culture, these polypeptides would not accumulate, and 2) their products are present in the mature, dry embryo, but are lost and do not reaccumulate on imbibition and germination.

Maturation gene sets have been identified in a number of plants including soybean (Ackerson, 1984), cotton (Dure et al., 1981), and wheat (Quatrano et al., 1983a, 1986). Most of the proteins that have been identified as part of this maturation set are storage proteins (Finkelstein et al., 1985; Bray and Beachy, 1985), but the set also includes some lectins (Raikhel and Wilkins, 1987; Stinissen et al., 1984), and an enzyme inhibitor (Mundy et al., 1986). In each of these cases the genes identified share two additional characteristics which may be added to the criteria of "maturation genes": 3) in normal development, their gene products accumulate in the mid to late stages, at the time of increasing and/or high endogenous levels of ABA,

and 4) their gene products will precociously accumulate in developing embryos in culture by application of osmoticum or ABA.

In wheat, a number of genes and gene products which meet these criteria have been identified and include some isoforms of the lectin wheat germ agglutinin (WGA), the Em polypeptide and triticin, a 7s globulin (Quatrano et al., 1983a).

Timing, localization and control of expression by ABA has been shown for WGA protein (Raikhel and Quatrano, 1986; Quatrano et al., 1983b; Triplett and Quatrano, 1982), and a cDNA for one of the three isoforms of the lectin found in wheat has been cloned (Raikhel and Wilkins, 1987). *In situ* hybridization using this WGA cDNA clone, localized the mRNA for WGA to the epidermal layers of the radicle and coleorhiza, while in three-day-old seedlings, it was found in the root-cap cells which led the authors to suggest that the specific isoform of WGA encoded by this clone is not a member of the embryo maturation set (Raikhel et al., 1988).

The Em polypeptide was initially identified and characterized by Lane and co-workers as a prominent, low molecular weight, acid soluble protein present in dry mature wheat embryos (Grzelczak et al., 1982; Cuming and Lane, 1979). Isolation of pWG432, a cDNA coding for nearly 30% of mature Em mRNA, was reported by Cuming (1984). Isolation of p1015, a second Em cDNA coding for nearly 88% of the mature mRNA, was reported by Williamson et al. (1985). The cDNA

insert of p1015 was sequenced and comparison of its deduced
amino acid sequence to direct amino acid sequencing and
previously reported amino acid composition showed it to code
for the Em polypeptide (Litts et al., 1987). Comparison of
the Em sequence to other known plant proteins showed that
mung beans contain a similar protein (Manickam and Carlier,
1980). Recent work in cotton shows that this dicot also has
a homologous gene, the *Lea* D-19 nucleotide sequence shows
considerable homology to Em (Dure et al., 1989; Baker et
al., 1988). Williamson and co-workers (1985) showed that
levels of both Em polypeptide and mRNA are modulated by ABA
during wheat embryogenesis and in culture by application of
exogenous ABA to developing embryos. A genomic clone for Em
has been isolated, and the upstream region of the gene used
to analyze DNA sequence requirements for ABA regulation
(Marcotte et al., 1989; Marcotte et al., 1988; Litts,
personal communication). It was found that 646 bp of the
promoter region of the Em gene was sufficient for ABA-
regulated expression of a linked reporter gene when
transformed into rice protoplasts (Marcotte et al., 1988)
and in transgenic tobacco plants (Marcotte et al., 1989).
Further characterization of this 5' promoter has shown it to
contain two sequence motifs nearly identical to sequences
found in the 5' regions of other ABA-responsive genes
expressed in rice embryos (Mundy and Chua, 1988), soybean
(Chen et al., 1986), and wheat (Litts, personal
communication).

A second clone identified in screening for ABA-inducible sequences in wheat embryos by Williamson and co-workers, (Quatrano et al., 1983a) was designated p511. This clone was tentatively identified as encoding a 7S storage globulin on the basis of amino acid homology to vicilin. In a stretch of 73 amino acids near the C-terminus of vicilin, there was homology with 58 amino acids (30 exact, 28 neutral substitutions) from a deduced amino acid translation of p511 (Quatrano et al., 1986). This globulin has been referred to as "triticin". That a cereal should contain a storage protein with a high degree of homology to the 7S storage proteins of legumes is not surprising, as it has been shown that there is a large degree of cross-reactivity between 3S and 7S globulins of oat, wheat, barley, rye, corn, rice and pea (Robert et al., 1985). More recent work has identified possible homology of triticin with two cotton vicilin genes, the β-subunit of soybean β-conglycinin, and pea convicilin (Quatrano, personal communication).

The regulation of this maturation set is of great interest from a developmental standpoint. One of the criteria for members of the maturation gene set is that their expression is limited to the mid and late stages of embryo development. During normal development, neither transcripts nor polypeptides are accumulated to a large degree at any other time. At the end of embryo and seed development, the expression of maturation genes is turned off, and the expression of genes whose products are involved

in germination and seedling growth is turned on (reviewed by Quatrano, 1987). Subsequent imbibition and germination are accompanied by the expression of a new and different set of genes (Misra and Bewley, 1985; Adams and Rinne, 1980). Factors involved in this developmental switch are not clearly understood.

The desiccation experienced by most seeds during the later stages of development has been proposed to trigger this switch from maturation to germination (Misra and Bewley, 1985; Dasgupta and Bewley, 1982). However, it has been shown that developing castor bean seeds, when detached from the mother plant and maintained under conditions of high relative humidity, will switch to the germination mode without the requirement of complete desiccation (Kermode and Bewley, 1989). The level of ABA declines in seeds during the later stages of seed development and natural desiccation (Kermode et al., 1989; King, 1976) and this may play a role in the switch from maturation genes which are responsive to ABA (loss of a promoter) to germination genes (loss of an inhibitor). On the other hand, the embryo may become less sensitive to ABA on dehydration. Following natural or artificially imposed drying, embryos of castor bean became relatively insensitive to application of concentrations of ABA which at other stages promoted developmental processes and inhibited germination and germinative processes (Kermode et al., 1989).

In addition to inhibiting germination, exogenous ABA

has been shown to have a role in promoting embryo maturation. The presence of ABA in the medium used to culture embryos of *Brassica napus* stimulates the accumulation of the 12S globulin protein, an embryo maturation protein (Crouch and Sussex, 1981). The same is observed for globulin proteins in French bean (Sussex and Dale, 1979), soybean (Eisenberg and Mascarenhas, 1985), and wheat (Berge et al., 1988; Williamson et al., 1985) all of which are considered embryo and maturation specific.

Carotenoids are believed to be precursors of ABA (reviewed by Zeevaart and Creelman, 1988). Inhibitors of carotenoid synthesis may be used to deplete the level of ABA in plant tissues. Fluridone is an inhibitor of carotenoid synthesis (Bartels and Watson, 1978) and has been used in a number of studies. Treatment of cultured soybean cotyledons with fluridone inhibits the accumulation of the -subunit of -conglycinin, an embryo and maturation specific ABA-inducible protein (Bray and Beachy, 1985). Treatment of immature maize embryos with fluridone will induce premature germination (Fong et al., 1983), and the subsequent application of ABA will partially reverse this chemically induced vivipary. Somatic embryogenic cultures have also been used to investigate a possible role for ABA in the initiation of embryo formation. The ability of tissue explants of *Pennisetum purpureum* (Napier or Elephant grass) to undergo somatic embryogenesis in culture appears to be associated with high levels of endogenous ABA (Rajasekaran

et al., 1987a), as does that of caraway (Ammirato, 1974) and pearl millet (Vasil and Vasil, 1982). It has been found that somatic embryogenesis in Napier grass leaf explant cultures was enhanced by application of exogenous ABA, and inhibited by application of fluridone, while inhibitors of GA and IAA synthesis did not show any reduction in embryogenic capacity (Rajasekaran et al., 1987b).

Recent findings using the ABA insensitive mutants of *Arabidopsis thaliana* (Koornneef et al., 1984) suggest that endogenous ABA has at least two different effects in developing seeds (Koornneef et al., 1989). Koornneef et al. (1989) compared seed development in recombinants of *aba* (ABA deficient mutant) with *abi*1 or *abi*3 (ABA response mutants) with wild type and single-mutant type parents. While embryo development appeared to be normal in the *aba, abi*1, *abi*3 and *aba.abi*1 seeds, it appeared abnormal in *aba.abi*3 seeds. Embryos of *aba.abi*3 seeds did not seem to enter the late stages of development, i.e. maintained a green color until maturity, did not accumulate the normal late abundant storage proteins, did not develop a toleration for desiccation, and often showed viviparous germination. If exogenous ABA was applied to the *aba.abi*3 mutant, these symptoms were partially alleviated. However, all single and double mutants had reduced dormancy, suggesting that the action of ABA on seed development differs from that on dormancy.

Role of ABA in Plants Other Than During the Late Stages of Embryogenesis and Seed Maturation

ABA has been termed a "stress hormone" because of its presence under conditions of various stresses, i.e. cold, drought, heat, salinity, (Walton, 1980). Under conditions of environmental stress, gene expression in the plant changes (reviewed by Matters and Scandalios, 1986; Sachs and Ho, 1986; Bewley, 1981). A role for ABA in response to stress has been implicated in plant adaptation to cold temperatures, high concentrations of salt, and changes in water availability (most recently reviewed by Zeevaart and Creelman, 1988, and Gusta and Chen, 1987).

Plants, being non-mobile organisms, should and do have a number of mechanisms for responding to changing availability of water in their immediate surroundings, be it an over-abundance or a drought (reviewed by Hsiao, 1973). Among the numerous responses of plants to water stress is a striking elevation of endogenous levels of ABA (Wright, 1969). Hiron and Wright (1973) subjected seedlings of bean, wheat, rice, and tomato to conditions of either drying by a continuous stream of warm air or waterlogging. In all species tested the warm air treatment caused an increase in ABA levels within seedling tissue. The same was observed following the waterlogging treatment in all species but rice (which normally grows under waterlogged conditions, and so was not exposed to any additional stress by this treatment).

The biosynthesis of this stress induced ABA has been

the focus of much research. The increase seen in endogenous levels of ABA during dehydration stress is due to *de novo* synthesis of the regulator (Zeevaart, 1980) and requires transcription (Guerrero and Mullet, 1986). In work of Guerrero and Mullet (1986), pea plants pretreated with actinomycin D or cordycepin (inhibitors of RNA polymerase II transcription in higher eukaryotes) prior to dehydration did not elevate endogenous levels of ABA. Recent work involving a molybdenum co-factor mutant of barley which is unable to elevate levels of ABA under conditions of water stress, indicates that the production of stress-induced ABA is also dependent on a molybdoenzyme (Walker-Simmons et al., 1989). Further evidence supporting *de novo* synthesis of ABA under stress comes from work using mutants which lack carotenoids or by treating normal plants with herbicides which inhibit carotenoid biosynthesis, such as fluridone or norflurazon (Bartels and Watson, 1978). *Albostrians* is a mutant of barley which carries a mutation in the nuclear genome that inhibits the formation of functional chloroplasts, resulting in seedlings with shoots which are either white, striped or green (Hagemann and Scholz, 1962). When seedlings of *albostrians* were subjected to dehydration stress, ABA accumulation in young shoots was severely inhibited in completely white mutant leaves, in white portions of striped leaves, and in norflurazon treated green leaves (Quarrie and Lister, 1984). Normal seedlings of pearl millet grown in the continual presence of norflurazon lacked chlorophyll and

failed to accumulate ABA when water stressed (Henson, 1984).
Fluridone treatment of dark grown barley inhibited both
carotenoid accumulation and ABA biosynthesis under
dehydration stress (Gamble and Mullett, 1986). Additional
support for synthesis of ABA from carotenoid precursors
during water stress comes from labelling studies. The
incorporation of ^{18}O from a labelled carotenoid,
violaxanthin, into ABA in water-stressed bean leaves showed
that a pre-formed carotenoid is a likely precursor for the
regulator in water stressed tissue (Li and Walton, 1987).

The influence that elevated levels of ABA exert on
the plant can be divided into two different classes, rapid
and slow. An example of a rapid response is the closure of
stomata (<5 min), while slow responses (>30 min) appear to
involve changes in RNA and protein synthesis (Zeevaart and
Creelman, 1988). ABA exists as two enantiomers, R- or "-",
and S- or "+" and the two affect the two classes of
responses to different extents thus suggesting that the mode
of action of the two responses is different or at least act
through different receptors (Milborrow, 1980).

Evidence that ABA plays a role in stomatal closure
comes from the presence of high affinity binding sites for
ABA on the plasmalemma of guard cells of *Vicia faba*
(Hornberg and Weiler, 1984), the rapid closure on response
to exogenous application of ABA (Zeiger, 1983), and from the
inability of ABA-deficient mutants of potato, tomato and
Arabidopsis thaliana (the so-called "wilty" mutants) to

close their stomata in response to water deficit (wilty mutants reviewed by Quarrie, 1987). However, ABA is not the only stomatal inhibitor found in plants (Munns and King, 1988). Munns and co-workers (1988; and Munns, personal communication) have been investigating the activity of a compound that is present in the transpiration stream of wheat plants. Xylem sap collected from plants exposed to drying soil conditions and applied to detached leaves of well-watered plants results in a lowered transpiration rate. Various treatments of the sap to remove ABA were not effective in reducing the drop in transpiration. Although this compound is not ABA, results do not rule out the possibility that it may operate via ABA, i.e. it may be an ABA precursor or may signal an increase in ABA levels.

Changes in the specific types of mRNA and proteins synthesized during times of water stress are seen in a variety of plants including *Brassica* (Vartanian et al., 1987), rice (Quarrie and Lister, 1988), barley (Chandler et al., 1988; Mundy and Chua, 1988), maize (Gomez et al., 1988; Heikkila et al., 1984), wheat (Berge et al., 1988) and rye (Cloutier, 1983). In cases where it has been tested, these changes may be mimicked by applying exogenous ABA (Chandler et al., 1988; Mundy and Chua, 1988; Gomez et al., 1988). Mutant corn seedlings which cannot accumulate ABA on water stress also cannot accumulate mRNA for dehydrins, a family of mRNA's which accumulate under water stress, identified initially in barley but later found in other cereal

seedlings (Chandler et al., 1988). On rehydration, levels of both the mRNA and polypeptides for the dehydrins decline rapidly, and this decline is closely related to the decline in ABA levels. The same pattern of accumulation of mRNA and protein with the accumulation of ABA on water stress, and decline in mRNA with decline of ABA on rehydration, is seen for a glycine-rich protein found in maize (Gomez et al., 1988). Investigating the changes in protein and mRNA synthesis under conditions of water stress is still a recent area of active research. The function of these water stress related gene products is unclear, but the reversibility seen in their expression on rehydration suggests that they might be involved in drought tolerance.

Proposed Mechanism of Action for ABA

The mechanism by which ABA is acting on cells to elicit these responses is as yet poorly understood (Zeevaart and Creelman, 1988). One way that ABA has been proposed to be acting is by affecting the ion flux into and out of cells (VanSteveninck and VanSteveninck, 1983). Hornberg and Weiler (1984) reported the presence of high-affinity binding sites for ABA on the plasmalemma of *Vicia faba* guard cells. Schauf and Wilson (1987) showed that the kinetic characteristics of voltage-sensitive K^+ channels in these same cells were stimulated by ABA to produce long bursts of channel opening. In large unilamellar liposomes, Harkers et al. (1986) demonstrated K^+ efflux mediated by ABA. Work done recently investigating Ca^{2+}-infux at the plasmalemma of

isolated guard cells of *Commelina communis*, showed that ABA did not appear to have a direct consistent effect on Ca^{2+} flux, sometimes stimulating, sometimes having no effect, and sometimes inhibiting the ion flux (MacRobbie, 1989).

A number of other studies showing a possible role for ABA in altering membrane transport and influencing a Ca^{2+} messenger system in plants have been reviewed (Owen, 1988). In his review, Owen proposes a model of ABA acting as a universal Ca^{2+} agonist, universality based on the finding of ABA in mammalian brain tissue (LePage-Degivry et al., 1986). Although direct evidence for Ca^{2+} as a second messenger in plant is sparse, there have been reports of Ca^{2+} channel activity (Andrejauskas et al., 1985), protein kinase C activity (Elliot and Skinner, 1986; Olah and Kiss, 1986), and calcium-calmodulin activity (Marme and Dieter, 1983). Further evidence of a Ca^{2+} mediated signal transduction pathway in plants came with the cloning of a cDNA for calmodulin (CaM) from barley (Ling and Zielinski, 1989) and potato (Jena et al., 1989). It was found that the barley CaM is encoded by a single copy gene, and that CaM mRNA is expressed in all tissues of vegetatively growing barley seedlings. All these activities are intimately related with the Ca^{2+}-cAMP second messenger system in animals (Rasmussen, 1970).

There is work reported recently which suggests that ABA may be involved in a Ca^{2+}-cAMP signal transduction system. Yamaguchi-Shinazaki et al. (1989) reported an

analysis of four ABA-responsive genes in rice that contain a conserved sequence (the core of which is also found in ABA-responsive cotton genes) that is reminiscent of the cAMP responsive DNA element reported by Deutsch and co-workers (1988). Work of Hernandez-Nistal et al. (1989) showed that chick pea seeds germinated in an ABA solution had a decrease in the level of CaM accumulated in the embryonic axis compared with control seeds. Furthermore, the distribution of CaM within subcellular organelles was altered. However, although these results may be suggestive, it remains to be seen what part ABA has to play in a Ca^{2+}-mediated second messenger system.

Summary of Literature Review

The processes leading to the formation of a viable seed are complex. The regulation of genes which are normally expressed late in embryo and seed maturation, as well as some genes induced by dehydration stress, can be modulated by the plant growth regulator ABA. High concentrations of ABA are present in plant tissues at two stages in the plant life cycle: the later stages of seed and embryo development, and under conditions of water stress. Given the fact that the later stages of seed development involve the loss of moisture, the conditions resulting in high endogenous levels of ABA are not considerably different. At each of these times the ABA appears to be having an effect on the regulation of specific mRNA and protein synthesis, with some of the same genes being

affected. Also, at times of water stress, there is an additional component of regulation by ABA in regard to stomatal closure. It is not clear by what mechanism ABA is elliciting the observed responses. As research progresses, and basic biochemical processes in plant tissues become more apparent, the specific role of ABA and other plant growth regulators in plant development and gene regulation will emerge.

CHAPTER I: THE NORMAL DEVELOPMENTAL EXPRESSION AND

LOCALIZATION OF Em mRNA

Introduction

The formation of seeds by most higher plants is a

highly successful adaptation.   During the developmental

stages of seed formation, expression of gene sets that

regulate this complex set of processes change in response to

various factors.   While the earlier developmental stages are

characterized by active cell division and enlargement, the

later stages of embryo maturation are marked by the

cessation of elongative growth, a tolerance of desiccation,

and in some cases the induction of dormancy (Quatrano,

1987).   Changes in specific protein expression during embryo

development have been characterized in a number of species

including barley (Bartels et al., 1988), maize (Sanchez-

Martinez et al., 1986), rapeseed (Crouch and Sussex, 1981),

cotton (Galau et al., 1986) and wheat (Quatrano et al.,

1983a).

One factor believed to be involved in some of these

changes is the plant growth regulator abscisic acid (ABA).

ABA content increases throughout seed development, reaches

its highest level late in development and then declines as

water content decreases and desiccation follows (Bewley and

Black, 1986).   This has been clearly documented for wheat

(Walker-Simmons, 1987; King, 1976).   The highest levels of

ABA are detected during embryogenesis, with the ABA content

remaining at a low basal level throughout other stages of

the normal wheat life cycle (Simmons, 1987). Hence, a temporal correlation exists between ABA and embryo maturation.

Additional evidence that ABA is playing a role in seed development comes from a number of studies involving mutants in ABA synthesis or sensitivity and the use of inhibitors of ABA biosynthesis. The viviparous-1 (*vp1*) mutant of maize shows an insensitivity to ABA in embryo tissue resulting in precocious germination, bypassing the normal maturation and dormancy phases (Robichaud and Sussex, 1986). In addition to vivipary, aleurone tissue of *vp1* seed shows a block in anthocyanin production and a number of other enzyme deficiencies (Dooner, 1985). These results suggest that the *vp1* gene product may be necessary for the ABA effects on embryo maturation in maize. The recent isolation and characterization of the *vp1* allele (McCarty et al., 1989) should allow investigations into the role of this product in the ABA-response of maize embryonic tissues. Additional ABA-insensitive mutants have been identified in *Arabidopsis thaliana* (Koornneef et al., 1984). The ABA-insensitive mutants *abi1* and *abi3* have normal or slightly elevated ABA levels during seed development and produce normal seed, but do not develop dormancy. When the *abi3* mutant was crossed with an ABA-deficient mutant (*aba*), the resulting double-recessive seeds (*aba.abi3*) did not appear to enter the late stages of development i.e. they maintained a green color at maturity, did not accumulate the normal

late abundant storage proteins, did not become desiccation tolerant, and often showed viviparous germination (Koornneef et al., 1989). Furthermore, reducing endogenous ABA levels of immature wild type maize embryos by treatment with fluridone (an inhibitor of carotenoid biosynthesis, Bartels and Watson, 1978) will chemically induce vivipary, a symptom partially relieved by the subsequent application of exogenous ABA (Fong et al., 1983). Fluridone treatment of cultured soybean cotyledons inhibits the accumulation of the β-subunit of β-conglycinin, an ABA-inducible embryo maturation specific protein (Bray and Beachy, 1985). This suggests that ABA levels, as well as tissue sensitivity to ABA, play an important role in the control of late seed development and embryo maturation.

A set of genes expressed abundantly during the later stages of embryo development, when ABA levels are high, was identified initially in cotton (Dure et al., 1981) and subsequently in other plant species (Dure et al., 1989; Quatrano, 1986, 1987). Members of this embryo maturation set have the following characteristics: (1) their expression occurs during the mid to late stages of embryo and seed development at the time of high or increasing ABA content; (2) their gene products are present in the mature seed, but disappear on imbibition and germination; (3) their gene products are not essential for germination; and (4) the precocious accumulation of their products can be promoted in culture by application of exogenous ABA (Quatrano, 1987).

In wheat, one member of this embryo maturation set is the Early-methionine polypeptide, Em (Quatrano et al., 1983a). Em was initially identified as one of the most prominent products labelled with ^{35}S-methionine when an *in vitro* translation system was programmed with mRNA from mature wheat embryos (Grzelczak et al., 1982; Cuming and Lane, 1979). On imbibition and germination, levels of both Em message and protein decline, and within days become undetectable (Williamson et al., 1985; Cuming, 1984). When isolated immature wheat embryos are cultured in the presence of ABA, there is an accumulation of both Em mRNA and protein (Williamson et al., 1985). In addition, if mature dry embryos are imbibed and germinate in culture with the continual presence of ABA, the level of Em remains at the level found in dry embryos for at least 48 hr (Williamson et al., 1985). Williamson and Quatrano (1988) reported that the level of Em mRNA induced by ABA in imbibed mature embryos was relatively insensitive to the RNA polymerase II inhibitor -amanitin when ABA was included in the culture medium. This indicates that ABA has an affect at a post-transcriptional level. Recent work includes the isolation and characterization of Em cDNA (Litts et al., 1987) and genomic clones (Litts, personal communication). A 646 base pair region of the 5' promoter has been shown to direct ABA-regulated expression of a linked reporter gene in rice protoplasts (Marcotte et al., 1988) and transgenic tobacco (Marcotte et al., 1989). Further characterization of this

promoter region by Litts, (personal communication) revealed the presence of two sequence motifs nearly identical to sequences found in the 5' regions of other ABA-responsive genes expressed in rice (Mundy and Chua, 1988), soybean (Chen et al., 1986), and wheat (Litts, personal communication).

Although molecular studies in progress will continue to clarify levels of regulation, the function of Em and other maturation proteins remains obscure. The fact that a protein accumulates during a given time in development does not define the function of that protein. However, its specific localization within the tissue may provide clues to this function. The technique of *in situ* hybridization has been used successfully to locate a number of gene products within a variety of plant tissues (Reviews of the technique as applied to plant tissues are by McFadden, 1989; Raikhel et al., 1989). Examples include localization of the mRNA for lectins in embryos of wheat (Raikhel et al., 1988) and rice (Wilkins and Raikhel, 1989), localization of a 12S storage globulin mRNA within the embryo and cotyledons of *Arabidopsis thaliana* (Meyerowitz, 1987), and localizing expression of (1-3, 1-4)-β-glucanase within germinating barley grains (McFadden et al., 1988). Success in these cases suggested that the technique would be useful for localizing expression of Em mRNA within developing wheat embryos and perhaps give clues as to its role during embryo development.

The first step in understanding the regulation and function of any gene or gene product is to clearly define the normal pattern of expression during development. What is the normal pattern of accumulation of Em polypeptide and mRNA during embryo development? What is the relationship between their accumulation and the level of ABA found in the same tissue? What is the localization of Em mRNA within developing embryos? Can this localization give a clue to the function of the protein? The work which follows was initiated to (1) describe the normal pattern of expression of both the mRNA and protein for Em, a member of the maturation gene set (MGS) in wheat, (2) determine the levels of ABA during the times when this gene is expressed throughout normal grain development, and (3) observe the localization of Em mRNA during embryo development.

Materials and Methods

Plant material: Wheat grains (*Triticum aestivum* (L.)

cv. Highbury) were seeded at a rate of three grains per pot

(1:1 mixture of perlite and vermiculite) and grown under

controlled greenhouse conditions of 16° C (night) and 21° C

(day) in the Phytotron (CSIRO Division of Plant Industry,

Canberra, Australia). Individual grains were harvested and

embryos isolated at Stage 3, Stage 4, late Stage 4, and

maturity (Rogers and Quatrano, 1983). Mature grains were

surface sterilized (30 minutes in 1% sodium hypochlorite,

washed 6 times in sterile distilled water, 10 minutes in 10

mM HCl, and washed an additional 6 times in sterile

distilled water), sown on filter paper wetted with sterile

distilled water in petri dishes, and placed in the light

(Crompton 18W white) at 26° C for 4, 8, or 24 hours in a

growth cabinet (Environ Air, SRJ Cabinets Sales, Greenacres

NSW, Australia). After incubation, grains were harvested

and embryos isolated. Embryos isolated from developing and

imbibing grains were either frozen individually in Beem

capsules (Polyscience Inc., Warrington, PA, USA) containing

OCT tissue embedding medium (Miles Scientific, Naperville,

IL, USA) for later sectioning or bulked and frozen in liquid

N_2 for ABA, ribonucleic acid (RNA) and protein extraction.

RNA extraction: Frozen tissue samples were ground to

a powder using a mortar and pestle with liquid N_2. A small

portion of this frozen powder was placed into pre-weighed

tubes for ABA assays and protein extraction and stored

frozen at -20° C. The remaining frozen powder was mixed with a 1:1 mixture of NTES (NTES: 0.1 molar (M) sodium chloride, 10 millimolar (mM) Tris(hydroxymethyl)amino methane (Tris), 1 mM ethylene-diaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS), pH 7.5) and phenol: chloroform: isoamyl alcohol (50:48:2, equilibrated against NTES and stored at 4° C). The resultant mixture was transferred to a centrifuge tube and centrifuged to separate the organic and aqueous phases. Sodium acetate (3 M, pH 5.8) was added to the aqueous phase to a final concentration of 0.2 M, two volumes of 95% ethanol added, and the nucleic acid precipitated by storage at -20° C overnight. The precipitated nucleic acids were collected by centrifugation, washed once with 70% ethanol, air dried briefly and dissolved in sterile distilled water.

A small amount (1.5 to 2 µg) of the isolated nucleic acid was run on an agarose gel containing methylmercuric hydroxide to determine size range and integrity of RNA before analysis of RNA by northern blotting (Gel: 1% agarose, 1X borate buffer, 5 mM methylmercuric hydroxide. Running buffer: 1X borate buffer. 10X borate buffer: 0.5 M boric acid, 0.05 M sodium borate, 0.1 M sodium sulphate, 1 mM EDTA, pH 8.2).

Northern analysis: RNA samples (5 to 10 µg) were heated at 70° C for 5 minutes in a solution of 50% formamide, 6.6% formaldehyde, and 1X MOPS buffer (10X MOPS buffer: 0.2 M 3-(N-morpholino)-propane sulfonic acid (MOPS),

0.05 M sodium acetate, 0.01 M EDTA, pH 7.0). After

denaturation, 1/4 volume of sample buffer dye (0.02%

bromophenol blue, 0.04% xylene cyanol, 8% Ficoll, 0.1X MOPS

buffer) was added and RNA samples were separated on agarose

gels (Gel: 1.5% agarose, 1X MOPS buffer, 6.5% formaldehyde.

Running buffer: 1X MOPS). Gels were blotted onto

nitrocellulose (Schleicher & Schuell, Keene, NH, USA) by

capillary action using 20X SSC (3 M sodium chloride, 3 M

tri-sodium citrate). The nitrocellulose membranes were

baked under vacuum at 80° C for 2 hours. Prior to

prehybridization, membranes were boiled for 5 minutes in 20

mM Tris (pH 8.0). Prehybridization of washed membranes was

in prehybridization buffer (50% formamide, 10X Denhardt's

(50X Denhardt's: 1% Ficoll, 1% polyvinylpyrrolidone, 1%

bovine serum albumin (BSA)), 5X SSC, 0.1% SDS, 100 μg/ml

sheared salmon sperm DNA, 10 μg/ml poly A) at 42° C for 2 to

16 hours. Prehybridization buffer was removed and

hybridization reactions were carried out using fresh

prehybridization buffer containing labelled complementary

deoxyribonucleic acid (cDNA) at a concentration of 2-5 x 10^5

counts per minute per milliliter (cpm/ml) and incubated at

42° C for 16 to 24 hours. Radioactive labelling of cDNA was

done using a BRESA oligolabelling kit (OLK-C Oligo-labelling

Kit, BRESA, Adelaide, South Australia, Australia) which uses

random hexamer primers, labelling newly made DNA with ^{32}P-α-

deoxycytosine (^{32}P-α-dCTP, 1500 Curies per millimole

(Ci/mmol), BRESA). Specific activities of probes achieved

with this labelling procedure ranged from 0.5-1.5 x 10^8 cpm
/μg. Post-hybridization washes were as follows: 5 minutes
at room temperature in 2X SSC, 0.1% SDS, 1 hour at 70° C in
2X SSC, 0.1% SDS, and 20 minutes at 55° C in 0.1X SSC, 0.1%
SDS. Membranes were air-dried and placed under X-ray film
(Fuji Film Co. Ltd., Japan) for exposures from 1 to 4 days
using DuPont Lightning-plus screens at -70° C. All exposed
X-ray film was developed using Kodak developer and fixer
(Eastman Kodak Co., Chicago, IL, USA). Autoradiographs were
quantified by densitometric scanning using an MK III CS
microdensitometer (Joyce Lobel & Co. Ltd., Gateshead-on-
Tynell, England).

ABA *assays*: Assays of free ABA were performed using
the method of Walker-Simmons (1987). In this procedure
sample wells of an ELISA plate are coated with an ABA
conjugate (carbon 4 of ABA is linked to BSA) and the sample
or ABA standard is allowed to react with a limited amount of
monoclonal antibody (Mab) to free *cis, trans* (+) ABA
(Mertens et al., 1983). The uncomplexed Mab reacts with the
conjugated ABA coated to the wells and is a site for rabbit
antimouse IgG phosphatase attachment. The amount of
phosphatase activity is inversely proportional to ABA
content in the sample. The method is sensitive to as low as
5 pg ABA per reaction well.

ABA was extracted from previously frozen samples by
incubation at 4° C overnight in glass centrifuge tubes with
an extractant solution (80% methanol, 10 mg/l butylated

hydroxy toluene, 10 ml/1 glacial acetic acid. 0.1 ml extractant per mg fresh weight of samplewas used). A spike of approximately 1000 cpm ^{3}H-ABA (DL-*cis,trans*-(G-^{3}H) ABA, 10-20 Ci/mmol, Amersham, Arlington Heights, IL, USA) was added to the sample to follow recovery rates. The supernatant was collected by centrifugation, poured into clean glass scintillation vials and concentrated using a Speed Vac Concentrator (Savant, Farmingdale, NY, USA). The sample was redissolved in 1 ml 50% methanol and applied to a pre-wetted Sep Pac C18 column (Waters Associates, Milford MA, USA). Columns were prewet with 10 ml chloroform, 5 ml methanol, 5 ml distilled water, and 5 ml of air forced through the columns to expel any excess water. The first 5 ml eluted from the column with 50% methanol were pooled and dried to completion in the same scintillation vials under vacuum. The resultant film was dissolved in 0.1 ml methanol and 3.9 ml TMSB (6.05 g Tris, 0.2 mg magnesium chloride, 8.8 g sodium chloride, 1 g BSA per liter, pH 7.8) to yield the final sample. 0.4 ml of this sample was used to assay the recovery of the tritiated ABA spike. Recovery rates between experiments ranged from 60 to 99% as measured by liquid scintillation counting (LS 6800, Beckman Instruments, Inc., Palo Alto, CA, USA)

The procedure of ELISA assay of ABA content followed the published procedure of Walker-Simmons (Walker-Simmons, 1987) except for the inclusion of 4% polyethylene glycol 8000 in the solution containing the secondary antibody.

This addition allows less rabbit antimouse IgG alkaline
phosphatase to be used. ELISA plates (MaxiSorp Nunc-
immunoplates, Medos Ltd., Burwood, Victoria, Australia) were
read on a Titertek Multiskan MC (Flow Laboratories, Sydney,
Australia). Replicate ABA standards were run on each plate.
Measurements of ABA in tissue samples were calculated from
the standard curve obtained for ABA standards on each plate.
All sample results are the numerical average of 2 or 3
dilutions read from the same plate.

Protein extraction and visualization of total protein
by silver-staining or specific antigens by Western blot
analysis: Frozen tissue was ground to a powder in a mortar
and pestle with liquid N_2. The ground tissue was extracted
in Laemmli sample buffer (Laemmli, 1970) by heating at 80-
90° C for 5 minutes. Protein concentration of samples was
determined using the method of Schaffner and Weissmann
(1973) and gels were run on the basis of equal g protein
per lane.

Total protein visualized by silver staining was
electrophoresed through a 12-25% (w/v) linear gradient SDS-
polyacrylamide gel after the method of Laemmli (1970). All
steps in the following procedure were performed at room
temperature. After electrophoresis, gels were shaken in 12%
TCA for 1 hour, rinsed briefly with fixer (ethanol:distilled
water:glacial acetic acid (8:7:5)) prior to staining with
Coomassie brilliant blue R-250 (0.5% (w/v) in fixer).
Destaining was done for up to 24 hours in ethanol:distilled

water:glacial acetic acid (10:27:3). Destained gels were
shaken for 10 minutes in 50% methanol followed by shaking
for 60 minutes in 10% glutaraldehyde (Fluka Chemicals,
Switzerland). Several washes in distilled water and an
overnight rinse in tap water were done to remove all
glutaraldehyde from gels. Well-washed gels were shaken for
15 minutes in a silver diammine solution. The diammine
solution was prepared by dissolving 2.48 g silver nitrate in
7 ml water, and adding this silver nitrate solution to a
mixture of sodium hydroxide (61.5 ml of a 0.39% solution)
and concentrated ammonium hydroxide (4.5 ml). Silver
diammine treated gels were washed briefly in distilled water
and placed into developer (0.05 g citric acid, 0.5 ml 37%
formaldehyde per liter). Color development of the gel was
monitored over a light box until complete and stopped by
placing the gel into 0.1% acetic acid. After 5 minutes in
the acid stop solution, gels were placed into distilled
water for storage.

Proteins used for analysis with the Em antibody were
electrophoresed through a 15% (w/v) SDS-polyacrylamide gel
according to the method of Laemmli (1970). The separated
proteins were electroblotted onto nitrocellulose. Prior to
treatment with antibodies, nitrocellulose membranes were
stained briefly with amido black 10B (Sigma) to visualize
pea storage proteins run as molecular weight standards
(Spencer et al., 1980). Blocking was at 37° C for 60
minutes in TBS containing 1% non-fat dry milk powder (TBS:

20 mM Tris, pH 7.5, 0.5 M sodium chloride), followed by 3 X

5 minute washes in TTBS (TTBS: TBS + 0.05% Tween 20 (v/v)).

Incubation with Em antibody was at 37° C for 60 minutes

(1:200 dilution in TTBS, 0.2% milk powder), followed by 3 X

5 minute washes in TTBS. Incubation with anti-rabbit IgG-

alkaline phosphatase (Promega Biotec, Madison, WI, USA) was

at 37° C for 60 minutes (1:7000 in TTBS, 1% milk powder),

followed by 3 X 5 minute washes in TTBS and 1 X 5 minute

wash in TBS. Identification of the Em polypeptide was via

alkaline phosphatase reaction using 5-bromo-4-chloro-

indolylphosphate (BCIP) and nitrobenetatrazolium (NBT)

purchased from Bethesda Research Labs (Gaithersburg, MD,

USA) in BCIP buffer (BCIP buffer: 100 mM Tris, pH 9.5, 100

mM sodium chloride, 5 mM magnesium chloride).

In situ hybridization: All solutions used in this

procedure were made with DEPC-treated water unless noted

otherwise. Previously frozen embryos were cut into 10 m

thick sections using a CTI refrigerated cryostat

(International Equipment Co., Needham Heights, MA, USA) and

collected on slides coated with poly-L-lysine (Sigma, St.

Louis, MO, USA). Clean microscope slides were coated with

poly-L-lysine according to the method of Raikhel et al.

(1989). Slides containing sections were placed on a 40-45°

C hot plate for 2 minutes. Heated tissue sections were

fixed at room temperature for 20 minutes in freshly prepared

fixative (PBS containing 4% paraformaldehyde. PBS: 140 mM

sodium chloride, 3 mM potassium chloride, 8 mM sodium mono-

hydrogen phospate, 1.5 mM potassium di-hydrogen phospate, pH 7.5). Fixing was followed by two 5 minute washes in PBS (without paraformaldehyde) and one 5 minute wash in distilled water. If slides were not to be prehybridized immediately they were dehydrated by passage through an ethanol series (2 minutes in each 30%, 70%, 95%, and 100% ethanol) and stored air-tight in an ethanol-saturated environment at 4° C. Prior to hybridization, slides containing sections were re-hydrated by passage through the ethanol series in reverse. Some of the fixed sections received an RNAse A (Boehringer-Mannheim, FRG) treatment prior to prehybridization (RNAse A treatment: 1.0 mg/ml RNAseA in 0.1 M Tris, 2 mM magnesium chloride, pH 7.5, at 37° C for 2-3 hours. Enzyme reaction was stopped by washing the sections with 0.1 M Tris, 5 mM EDTA, pH 7.5, and slides were dehydrated by passage through the ethanol series).

Dry slides containing fixed sections were washed in prehybridization buffer (McFadden et al., 1988) once at room temperature for 10-15 minutes, and once at 38° C for 40 minutes. Sections were washed briefly with 1X SSC and dehydrated in two ethanol washed (2 minutes each in 70% and 95% ethanol). Slides were either directly hybridized with probe, or stored in an ethanol-saturated environment at 4° C for no longer than 2 days before application of probe.

Probes used for *in situ* hybridization were Em (cDNA insert from the plasmid p1015, Williamson et al., 1985), wheat ribosomal DNA (wheat genomic rDNA from the plasmid

pTA71, Gerlach and Bedbrook, 1979) and pUC19 (plasmid vector, BRESA). Synthesis of ^{32}P-labelled probes was done as described above using the BRESA oligolabelling kit. Specific activity of probes used for *in situ* hybridization were in the range of 1-10 X 10^8 cpm/μg. Aliquots of ^{32}P-labelled probe (10 μl prehybridization buffer containing 5 X 10^5 cpm probe) were applied to clean dry coverslips and prehybridized sections were inverted onto the coverslips. This ensured that no air bubbles were trapped under the coverslips and that there was an even distribution of probe over the sections. Hybridization was in a sealed black slide container at 42° C for at least 20 hours.

After hybridization, coverslips were removed by soaking slides in 4X SSC at room temperature for 30 minutes. Two post hybridization washes were done: 2X SSC at room temperature for 5 minutes, and 1X SSC at 38° C for 60 minutes. Washes were followed by a brief rinse in 95% ethanol and air dried. Dry slides were coated with photographic emulsion (Ilford Nuclear Research Emulsion, Ilford Scientific, Cheshire, UK) allowed to dry, and then stored in a light tight box at 4° C for exposures of 3 to 10 days. Exposed slides were developed in Kodak developer (5 minutes), rinsed briefly in water, and fixed in Kodak fixer (5 minutes). Micrographs of developed slides were taken using a Leica M3 camera body (Leica, West Germany) attached to an Ortholux microscope (Leitz Wetzlar, West Germany). Black/white photographs were taken using Ilford FP4 film

(Ilford Scientific). Selected sections were stained with toluidine blue (0.5% toluidine blue in 1.0% sodium tetraborate) to enhance contrast. Data is presented as light micrographs. Using regular light microscopy the silver grains, exposed by the radioactive decay of the hybridized probe within the tissue, show up as black dots. The greater the density of dots, the greater the amount of hybridizable material found at that site.

Results

Accumulation patterns of Em protein, mRNA and ABA

Changes in protein composition in the developing

embryo reflect changes in metabolic requirements during

grain formation. Most of the proteins making up this

developmental program in wheat are uncharacterized and have

no known function. While changes in polypeptides are

numerous and mostly subtle, it is evident even on a one-

dimensional SDS-polyacrylamide gel, that there are a number

of bands which reflect major changes in accumulation during

different stages of development (Figure I.1, arrow, stars).

Based on earlier work involving the Em polypeptide,

including its predicted molecular weight and high abundance

in dry embryos (Williamson et al., 1985), it was possible to

assign one of these developmentally regulated bands to Em

(Figure I.1, arrow).

A larger quantity of these same protein extracts was

challenged with a polyclonal antibody to the Em polypeptide

to further verify that this designated band corresponds to

Em (Figure I.2). The level of Em found in Stage 3 embryos

is quite low, (Figure I.2, lane 1) but this level increases

during development and reaches a peak in late Stage 4

(Figure I.2, lane 3). Mature dry embryos contain Em (Figure

I.2, lane 4), and on imbibition and germination the level

decreases slowly (Figure I.2, lanes 5 and 6). Based on the

sensitivity of similar antibody reactions (Quatrano et al.,

Figure I.1

Developmental changes in proteins from isolated immature, mature and germinated wheat embryos. Lanes are as follow:

1 = Immature embryos from Stage 3 grains

2 = Immature embryos from Early Stage 4 grains

3 = Immature embryos from Late Stage 4 grains

4 = Mature embryos from dry Stage 5 grains

5 = Embryos from mature whole grains imbibed on water for 4 hr

6 = Embryos from mature whole grains imbibed on water for 8 hr

Proteins were separated on an SDS 12 - 25% polyacrylamide gel and visualized by silver-staining. Approximate molecular weight markers are given in kD. Arrow (▶) designates position of Em polypeptide. Stars (*) designate protein bands with maximum abundance at a specific developmental stage.
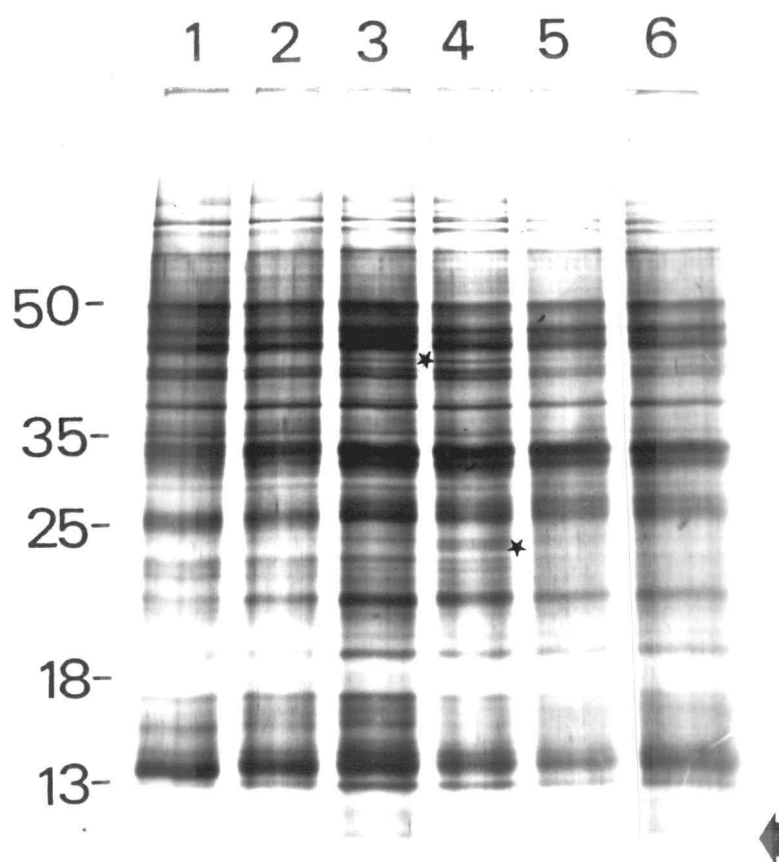
Figure I.1

Figure I.2

Western analysis showing developmental expression of the Em polypeptide from immature, mature and germinated wheat embryos. Lanes are as follow:

1 = Immature embryos from Stage 3 grains

2 = Immature embryos from Early Stage 4 grains

3 = Immature embryos from Late Stage 4 grains

4 = Mature embryos from dry Stage 5 grains

5 = Embryos from mature whole grains imbibed on water for 4 hr

6 = Embryos from mature whole grains imbibed on water for 8 hr

Nitrocellulose membrane was reacted with polyclonal antibodies raised against the Em polypeptide. Arrow (◀) designates position of Em polypeptide. Approximate molecular weight markers are given in kD.

Figure I.2
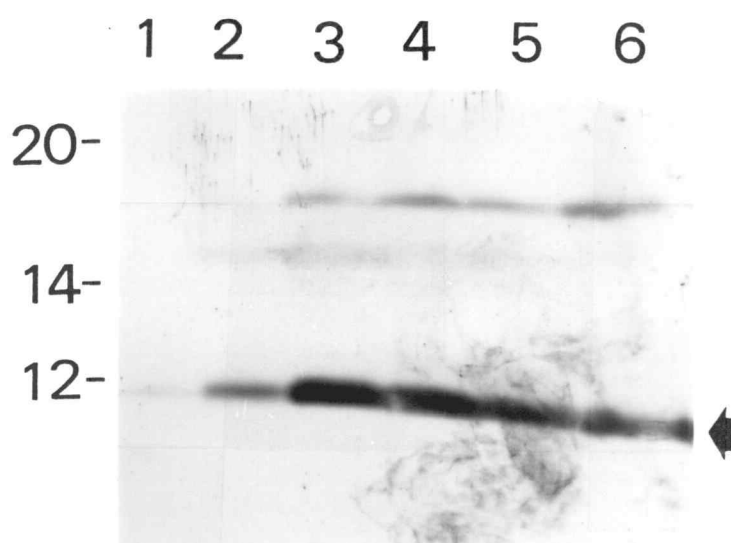
1983a), 10 - 100 pg of the Em polypeptide should be detectable by the corresponding antibody.

The pattern of Em mRNA accumulation during embryogenesis parallels that of the polypeptide (Figure I.3). During embryogenesis, the steady-state level of Em mRNA increases from a low level early in embryogenesis, to the highest level during the mid to late stages (Figure I.3, lanes 1-3). The abundance of Em mRNA found in dry embryos at the completion of embryogenesis and seed maturation is 55% of that found in late Stage 4 embryos (Figure I.3, lane 4). During imbibition and germination, the steady-state levels of the message decline rapidly (Figure I.3, lanes 5 and 6). The level of Em message in germinating embryos after 4 hours on water, is less than 25% of that found in dry embryos, and less than 12% of the level found at the peak of accumulation (Figure I.3, lane 5). This decrease is even more evident after 24 hours on water. The level of Em mRNA after 24 hr into germination is less than 10% that of dry embryos, and less than 5% that found at the peak of accumulation (Figure I.3, lane 6). Em mRNA is not detectable by Northern analysis beyond this stage.

The rate of accumulation of Em mRNA seems to parallel the rate of accumulation of ABA in the developing embryos (Figure I.3). The level of ABA found in the developing embryos is 92 pg/mg fresh weight during Stage 3, and reaches its highest level of 613 pg/mg fresh weight late in Stage 4. This 6.6-fold increase in ABA content during development is

Figure I.3

Changes in the levels of Em mRNA and ABA during

embryo development and early germination of wheat. Lanes

are as follow:

  1 = Immature embryos from Stage 3 grains

  2 = Immature embryos from Early Stage 4 grains

  3 = Immature embryos from Late Stage 4 grains

  4 = Mature embryos from dry Stage 5 grains

  5 = Embryos from mature whole grains imbibed on

      water for 4 hr

  6 = Embryos from mature whole grains imbibed on

      water for 24 hr

Graph showing the changes through embryo development in the

abundance of Em mRNA (bars), and ABA content (line) in the

same tissue sample. ABA measurements are given in pg ABA

per mg fresh weight (left axis), with the range of values

indicated. Values for Em mRNA abundance are given in

relative densitometric units (a value of "1" equal to the

peak of accumulation seen in late Stage 4 embryos) derived

from scanning the film of a northern analysis (pictured

above graph). Equal quantities of total RNA extracted from

isolated immature, mature or germinated wheat embryos were

separated on a 1% agarose gel and blotted onto

nitrocellulose. The membrane was probed with radioactively

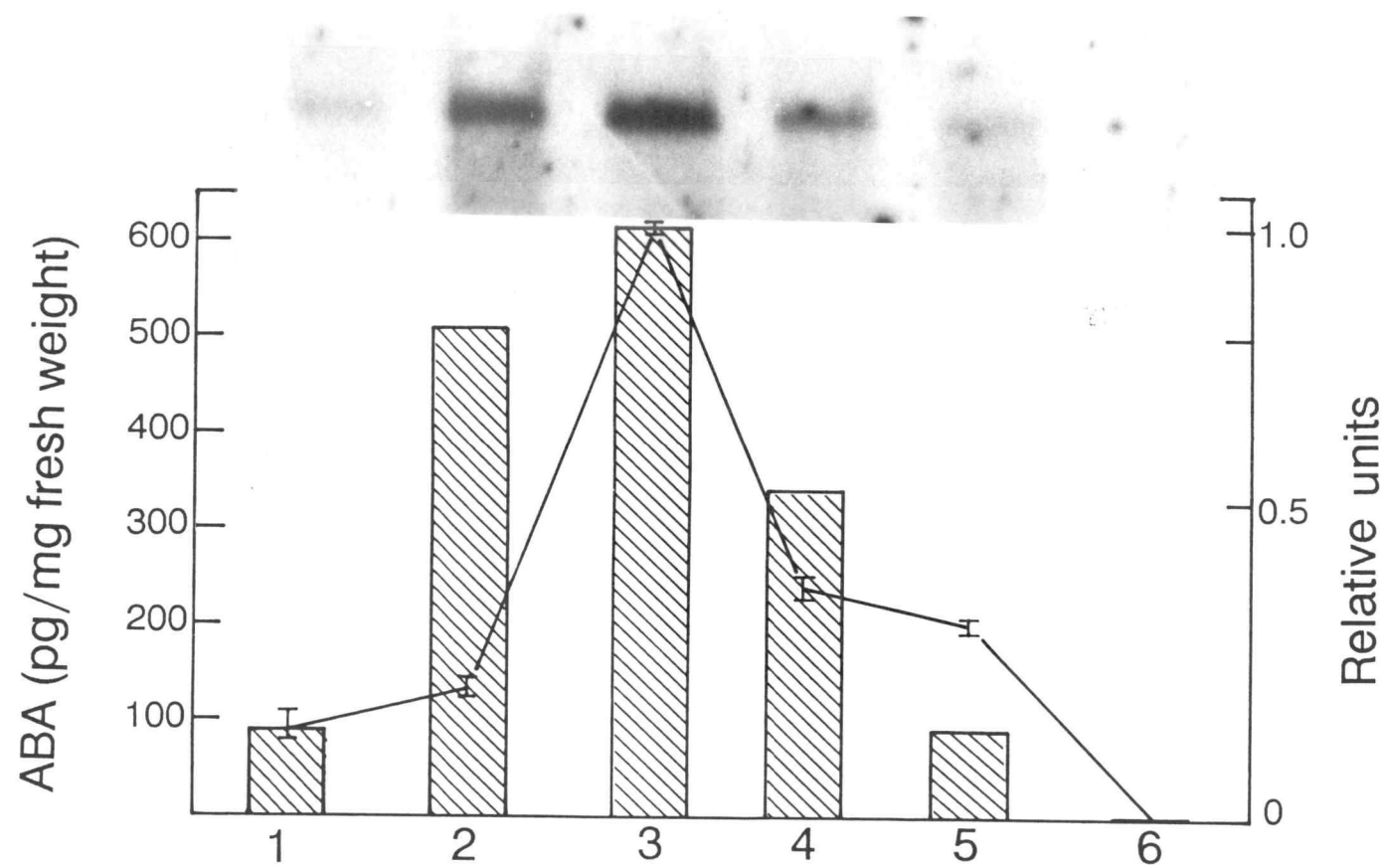labelled Em cDNA, revealing a single band of approximately

780 bp.

Figure I.3

accompanied by a 6.3-fold increase in abundance of Em mRNA (compare lanes 1 and 3 in Figure I.3). However, such an exact correlation between ABA content and Em mRNA level is not observed throughout this time of embryo development. For example, early in Stage 4 a relatively small increase in ABA content (44% above Stage 3 level) is accompanied by a large increase (550% above Stage 3 level) in Em mRNA abundance (compare lanes 2 and 3 in Figure I.3). Also, during early germination, a large decrease (75% below dry embryo level) in Em mRNA level is observed although there is only a slight decrease (16% below dry embryo level) in ABA level (compare lanes 4 and 5 in Figure I.3).

Localization of Em mRNA

In situ hybridization is the technique of hybridizing labelled nucleic acid probes to sections of fixed tissue to determine the spatial distribution of a particular mRNA. This technique has been successfully used with a variety of plant tissues (reviews by McFadden, 1989 and Raikhel et al., 1989). Using this technique it is possible to determine levels of Em mRNA in different regions of the wheat embryo.

Late Stage 4 embryos show a clear distinction between tissue types within the embryo (Figure 1.4). Within the developing embryo the plumule (P) is surrounded by the protective tissue of the coleoptile (Co), and together these tissues form the apex of the embryonic axis which will give rise to the seedling shoot. The root (R) is in turn surrounded by its protective tissue, the coleorhiza (Cr),

Figure I.4

Light micrograph showing late Stage 4 wheat embryo in transverse section. Labelled tissue types are as follow:
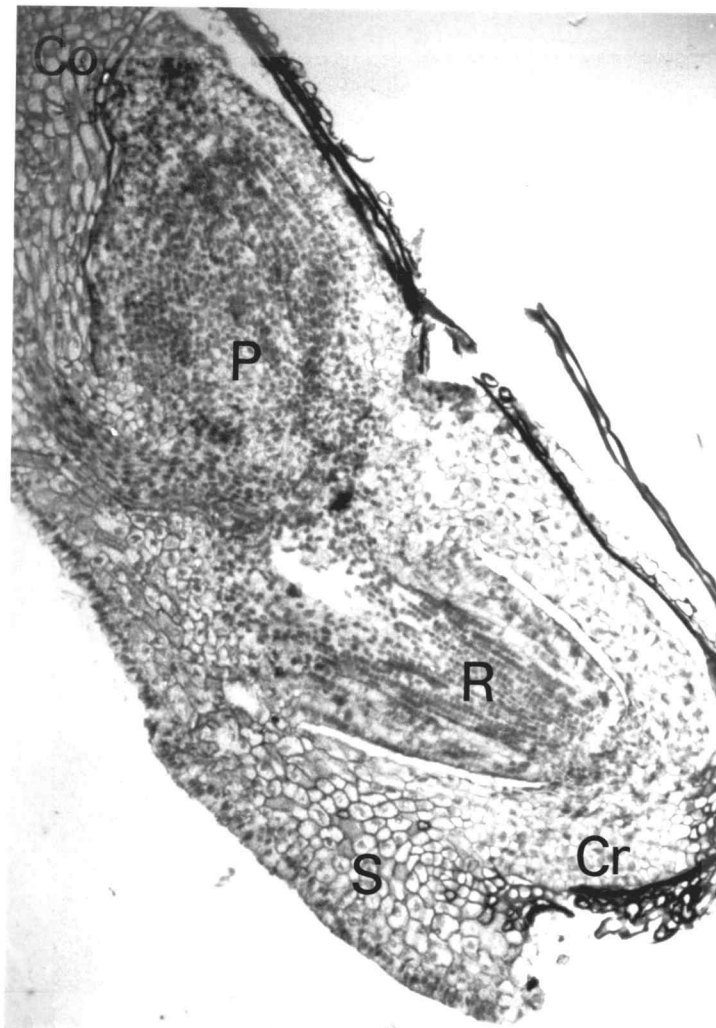
Co = Coleoptile

Cr = Coleorhiza

P = Plumule

R = Embryonic root

S = Scutellum

Bar = 200 μm.

Figure I.4

and together these tissues form the root apex of the embryo. The scutellum (S) is the modified single cotyledon of wheat and other cereals and acts both as a reservoir of nutrients for the embryo and a link between the large storage reserves of the endosperm and the embryo.

Figure I.5 shows the results of *in situ* hybridization of control and Em probes to coleorhizal tissue of late Stage 4 embryos. When labelled ribosomal DNA (rDNA) is used as a probe, a high level of hybridization is seen throughout the section (panel A). The distribution of rRNA is shown to be both diffuse over the cytoplasm and concentrated in the nucleoli. Pretreatment of sections with RNAse A (an endoribonuclease that specifically attacks pyrimidine nucleotides at the 3'-phosphate and cleaves the 5'-phosphate linkage to the adjacent nucleotide) results in a dramatic reduction in hybridization (panel B). When probed with DNA unrelated to mRNA in developing wheat embryos, radioactive pUC 19 DNA, hybridization is at a low background level (panel C). Hybridization to the Em probe appears to be diffuse throughout the coleorhizal tissue and clearly above background levels (panel D).

Hybridization of the Em probe to other tissues within the developing embryo is shown in Figures I.6 and I.7. Em mRNA is identified in the embryo proper, both shoot and root (Figure I.6, panels A and B, respectively). Hybridization to Em mRNA in the coleoptile and coleorhiza appears to be of lower intensity than the embryo proper (Figure I.6, panels A

Figure I.5

In situ hybridization of labelled probes to late

Stage 4 wheat embryo coleorhizal tissue. Probes used are as

follow:

   A = rRNA

   B = rRNA, after pretreatment with RNAse A

   C = pUC19

   D = Em

Bar = 30 μm.
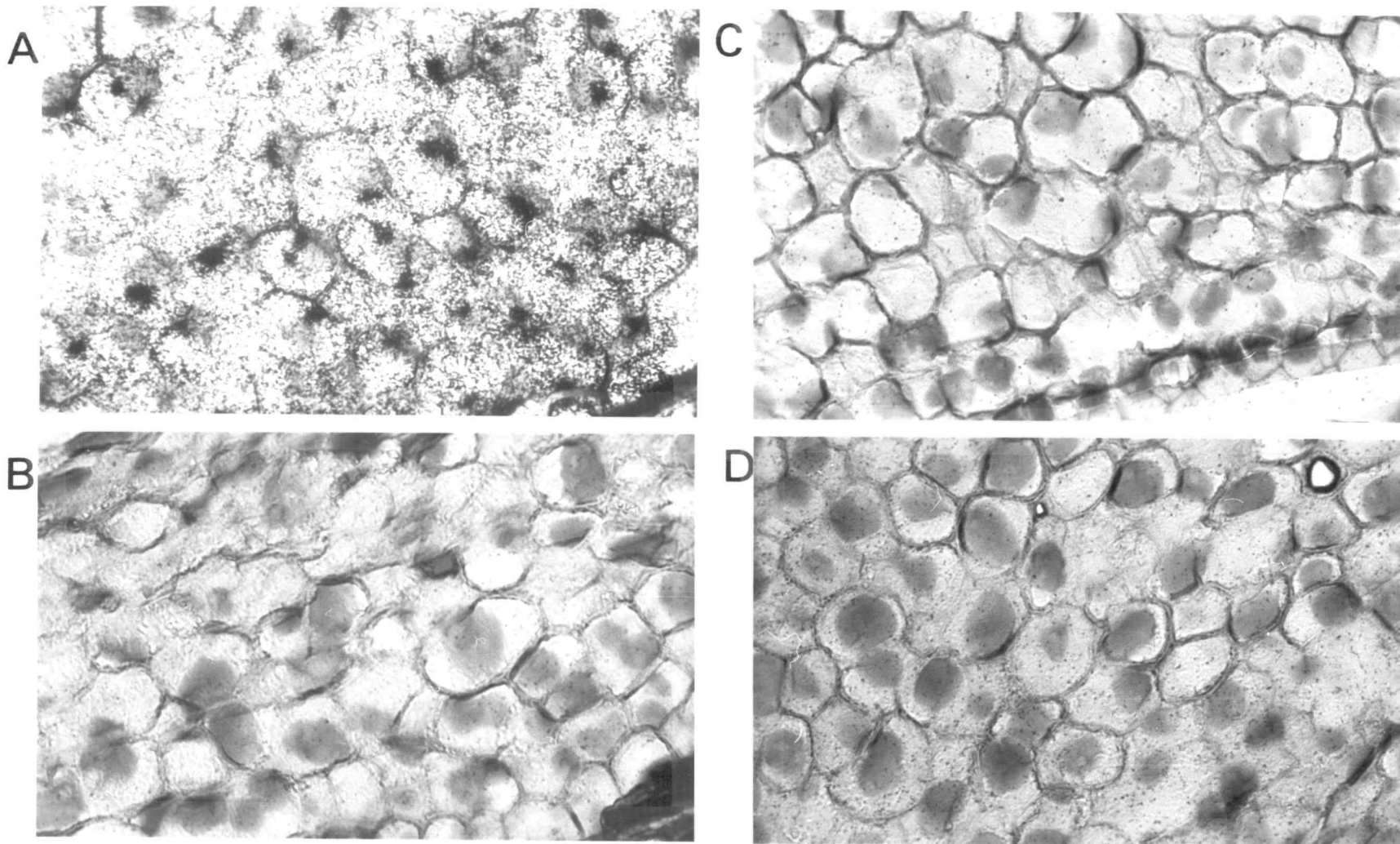
Figure I.5

Figure I.6

In situ hybridization of Em cDNA to late Stage 4
wheat embryo tissues. Panels are as follow:

  A = plumule and coleoptile

  B = root

  C = root cap and coleorhizal tissue

Labelled tissue types are as follow:

  Co = Coleoptile

  Cr = Coleorhiza

  Rc = Root cap

  P = Plumule

Bar = 70 μm.
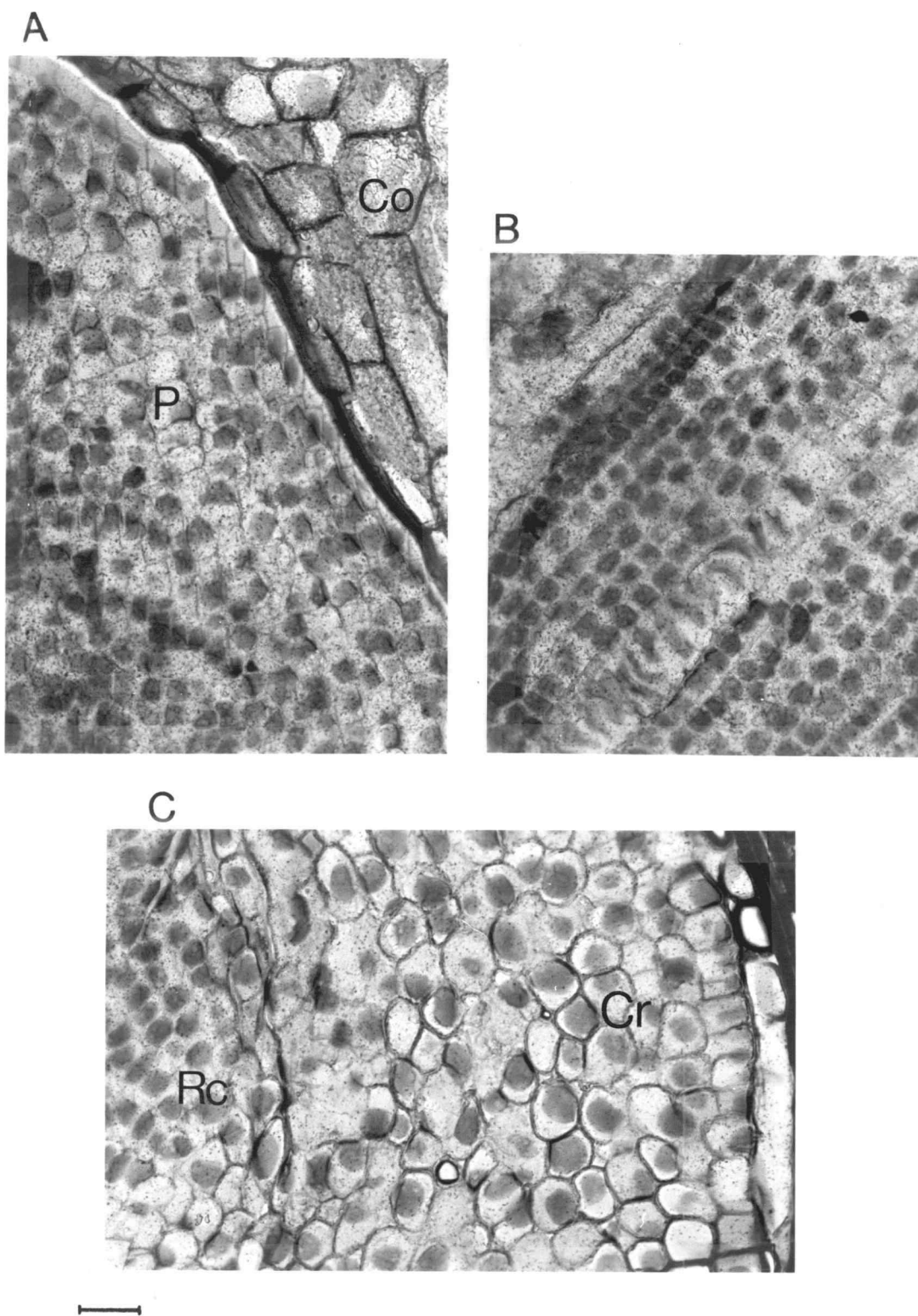
Figure I.6

Figure I.7

In situ hybridization of Em cDNA to late Stage 4 wheat embryo tissues shown at higher magnification. Panels are as follow:

A = root tip, root cap, and coleorhizal tissue

B = vascular tissue and coleoptile

Labelled tissue types are as follows:

Co = Coleoptile

Cr = Coleorhiza

Rc = Root cap

T = Root tip

V = Vascular tissue

Bar = 30 μm.
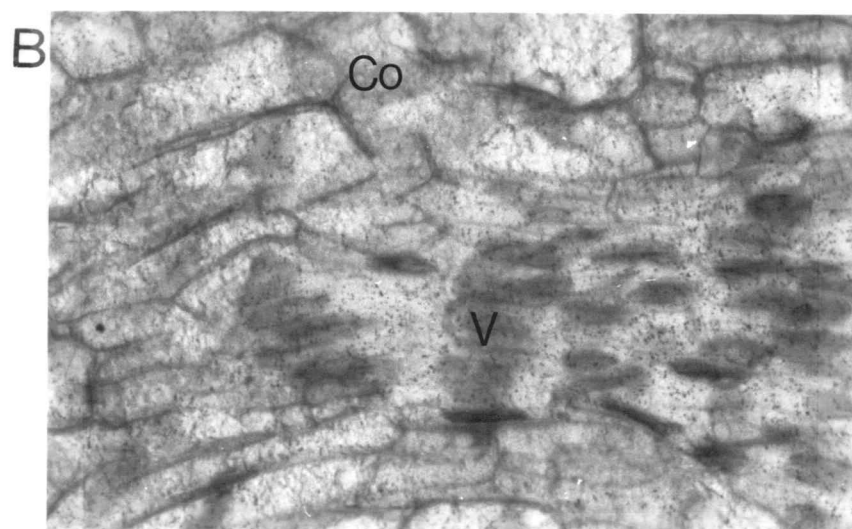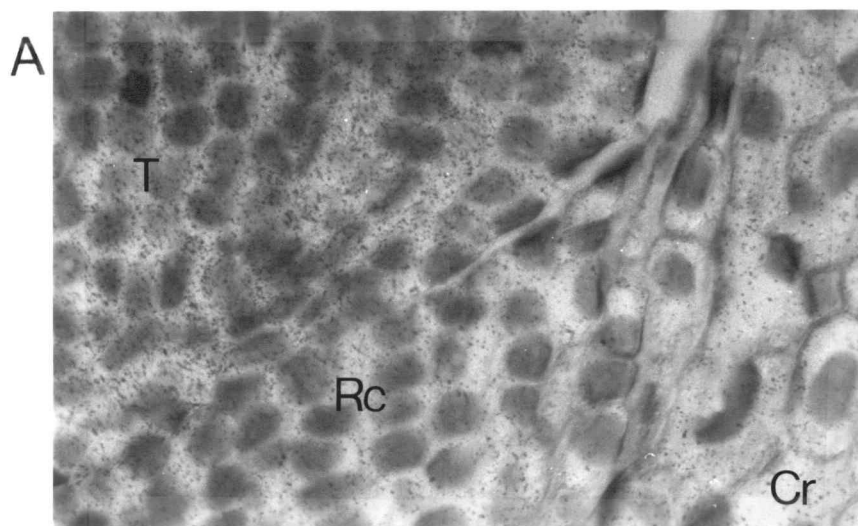
Figure I.7

and C, respectively). At higher magnification it is seen
that there is a greater intensity of hybridization to the
root tip than to either the root cap or the coleorhiza
(Figure I.7, panel A). Vascular tissue also contains a
relatively high level of hybridization to the Em probe
(Figure I.7, panel B). Hybridization of Em cDNA to extra-
embryonic and scutellar tissue does not appear to be above a
background level (data not shown).

Discussion

Results presented here demonstrated the following:

1) Silver-stained one-dimensional SDS-PAGE and use of a polyclonal antibody to the Em polypeptide indicated that accumulation of this embryonic protein began during Stage 3, reached its highest level late in Stage 4, and declined during seed desiccation. It persisted in dry seeds and declined on imbibition and germination. 2) The accumulation of a single 780 bp RNA species corresponding to the Em mRNA closely followed that of the polypeptide: present at low levels during Stage 3, reached its highest level late in Stage 4, declined during desiccation, and disappeared on imbibition and germination. 3) ABA content within the same tissue showed a pattern of accumulation qualitatively similar to that of Em mRNA. However, it was clear that the Em mRNA levels did not parallel ABA levels in a strictly quantitative manner throughout embryo development. 4) *In situ* hybridization indicated that Em mRNA was localized to the embryo proper in developing wheat embryos and was concentrated within the meristematic regions of both the root and shoot primordia.

The abundance of the Em polypeptide in developing grains was evident from the ability to identify it as a band on a one-dimensional SDS-polyacrylamide gel (Figure I.1, arrow). Williamson et al. (1985) showed that a protein of approximately 10 kD accumulated in immature embryos cultured in the presence of ABA and *in planta*. The accumulation of

Em polypeptide as monitored by use of a polyclonal antibody to this polypeptide confirmed this earlier work showing a single polypeptide of approximately 11 kd which reached its highest level during the late stages of embryo development and declined during seed desiccation, imbibition and germination (Figure I.2). Other seed proteins showing a similar pattern of accumulation have been observed in a number of different species (Casey and Domoney, 1987; Higgins, 1984).

The accumulation of Em mRNA during embryogenesis followed quite closely that of the Em polypeptide (Figure I.3). Although it was previously shown that ABA modulates Em mRNA levels in immature and mature embryos in culture (Williamson and Quatrano, 1988; Williamson et al., 1985), Figure I.3 presented a developmental time course showing changes in the level of Em mRNA *in planta*. The accumulation of a single species of Em mRNA of approximately 780 bp was at a basal level in Stage 3, reached its highest level late in Stage 4, declined during the time of seed drying to an intermediate level in the dry embryo, and was undetectable 24 hr after germination (Figure I.3). It was possible to measure ABA levels in the same tissue samples using a sensitive monoclonal antibody assay which detected free (+)-*cis,trans*-ABA (Walker-Simmons, 1987). This is one of the first examples showing an association between the changing levels of both ABA and a specific mRNA in the same tissue samples. The level of Em mRNA did not exactly mimic the

changing levels of ABA in the developing embryos, but did follow the same trend. For example, the level of ABA increased slightly between Stage 3 and Stage 4 (from 92 to 133 pg ABA/mg fresh weight) as did the level of Em mRNA, however, the magnitude of the mRNA increase was much greater. This could be explained by a change in the sensitivitiy of the tissue to ABA. An alteration in sensitivity has been observed in isolated immature embryos of rapeseed in culture (Finkelstein et al., 1985). They showed that both ABA levels and sensitivity declined during maturation. Also, following natural or imposed drying, isolated immature embryos of castor bean become relatively insensitive to levels of ABA which at earlier stages both suppressed germination and enhanced the synthesis of some developmentally regulated proteins (Kermode et al., 1989). Furthermore, Bartels et al. (1988) showed that the ability of ABA to induce desiccation tolerance in barley embryos depended on the particular developmental stage of the embryos. Clearly the relationship of gene expression in developing embryos to ABA is quite complex, involving both the levels of ABA and the tissue sensitivity to ABA.

The observed decline and disappearance of Em mRNA upon imbibition was in agreement with the work of Cuming (1984) who showed that Em mRNA, while present in mature dry embryos and maintained at a comparable level until 2 hr into imbibition, was detectable 6 hr after imbibition but not at 24 hr. Upon imbibition, the decrease in Em mRNA clearly

preceded the disappearance of polypeptide. The rapid disappearance of Em mRNA may be due to the rapid decline in ABA levels observed during germination (McWha and Hillman, 1974). ABA was shown to promote Em mRNA stability within imbibing mature wheat embryos since blocking mRNA synthesis with α-amanitin had little effect on Em mRNA accumulation (Williamson et al., 1988). Alternatively, the level of Em polypeptide may be maintained for a short time after imbibition by its continued synthesis from stored Em mRNA. Ample evidence exists for the presence of stored mRNA in wheat embryos. In imbibing wheat embryos there is a lag period of 5.5 hr preceding rapid growth (Huang et al., 1980). The prevention of RNA synthesis by cordycepin or α-amanitin during the first 40 min of imbibition has little effect on the increase in protein synthesis which occurs during this period (Cheung et al., 1979; Speigel and Marcus, 1975). This implies that stored mRNAs are acting as templates for protein synthesis during the earliest stages of imbibition.

While the temporal expression of genes during embryo development has been well characterized, and in many cases the sequence of both mRNA and polypeptide are known (as is the case for Em (Litts et al., 1987), the spatial expression of these genes within the embryo has not been reported for members of the MGS. To identify portions of the wheat embryo in which Em is expressed, transverse sections of late Stage 4 embryos were exposed to a series of ^{32}P-labelled

probes and observed under the light microscope. Late Stage
4 embryos were selected for this exercise because the
highest levels of both Em mRNA and protein occurred during
this stage.

Sections of late Stage 4 embryos were first
hybridized with a series of ^{32}P-labelled control probes to
establish specific conditions for *in situ* hybridization
using this tissue (Figure I.5). Every cell of the embryo
shows hybridization to a ^{32}P-labelled probe for ribosomal
DNA, with the signal concentrated in the nucleolus and
rather less dense throughout the cytoplasm (Figure I.5,
panel A). This was expected because the nucleolus is the
site of rRNA genes and free ribosomes are scattered
throughout the cellular cytoplasm. Treatment of fixed
embryo sections with RNAse A prior to hybridization resulted
in a dramatic reduction of hybridization to a ^{32}P-labelled
rRNA cDNA (Figure I.5, panel B). Sections of developing
embryos hybridized with ^{32}P-labelled pUC19 DNA, a DNA
sequence unrelated to any wheat DNA fragment, showed only a
low level of hybridization (Figure I.5, panel C). The
hybridization seen after RNAse A treatment and using the
pUC19 probe was taken to be the background level.

When sections of late Stage 4 embryos were hybridized
with ^{32}P-labelled Em cDNA, localization was found to be
diffuse throughout the embryo proper (Figure I.5, panel D)
and Figures I.6 and I.7). An increased density of
hybridization was observed in meristematic regions of both

root and shoot primordia. The levels of message found in the coleoptile and coleorhiza appear lower than that in the embryo proper but still above background levels (compare Figure I.5 control panels to Figure I.6, panels A and C). Hybridization of Em cDNA to extra-embryonic and scutellar tissue appears identical to background levels (data not shown).

This more general pattern of localization within the embryo is different from the specific expression exhibited in wheat embryos by mRNA for the B isoform of wheat germ agglutinin (WGA). A combination of *in situ* hybridization and transcription showed WGA-B mRNA to be expressed only in the epidermal layers of the radicle and coleorhiza of developing embryos and in the root cap of three-day-old seedlings (Raikhel et al., 1988). The pattern of WGA mRNA localization was identical to the localization of WGA protein (Triplett and Quatrano, 1982). Hybridization of a rice lectin mRNA to developing rice embryos showed expression confined to root caps, several cell layers at the periphery of the coleorhiza and radicle, and in all cell layers of the coleoptile (Wilkins and Raikhel, 1989). The expression of these lectins within the protective tissues of the embryo supports the proposed role of lectins as being part of a plant protective mechanism (Etzler, 1985).

Does this pattern of Em mRNA localization suggest what function Em may be performing within the developing embryo? Em has been proposed to function as a storage

protein (Hofmann et al., 1984; Grzelczak et al., 1982).
This would be in accordance with a broad distribution of
expression within the parts of the embryo which will need
nourishment upon germination. While Em may be atypical as a
storage protein from the stand point of amino acid
composition (proteins of this sort normally contain a large
number of glutamine residues where Em contains only 7 of
92), it does contain a large number of gluconeogenic amino
acids (50 of 92). The immediate requirements of the
developing embryo for sucrose could be met from the
breakdown of certain amino acid residues (i.e. glycine,
aspartate, glutamate, alanine and serine) until imports were
available from the stored reserves in the endosperm which
may not begin until after 2-3 days (Bewley and Black, 1986).
Other evidence that suggests Em is not a classic storage
protein comes from the observation that the polypeptide does
not contain a signal peptide (Litts et al., 1987) used by
most storage proteins for targeting to either the vacuole or
protein bodies. In addition protein bodies within the wheat
embryo show a pattern of localization primarily in the
scutellum (see Figure 3 of Quatrano et al., 1983a).
Assuming Em polypeptide localization coincides with the
localization of Em mRNA, as was seen for the example of WGA,
these results suggest that Em is not found in the storage
organelles, where other known storage proteins are normally
accumulated and stored.

A second proposed function of Em, and also in

agreement with a broad distribution of expression within a developing embryo is that of a cryptobiotic protein (McCubbin and Kay, 1985). This suggestion is based in part on the high degree of hydrophillicity predicted by the amino acid composition of Em and other related proteins (Dure et al., 1989). Such a cryptobiotic protein is found in *Artemia* (DeHerdt et al., 1981) and may confer a form of desiccation tolerance on the cytoplasm of the cells in which it resides. The extent of desiccation experienced by embryos during seed maturation would be lethal to other plant tissues. Thus, it seems likely that there would be some form of protection within the cells of the embryo that allows such a severe water loss to occur. The highest level of Em was found at the time when the grain was beginning to lose water (Figure I.3). If Em does perform a desiccation-protective function, this temporal association is consistent. In addition, the higher level of Em mRNA observed in the root and shoot meristematic regions would also be supportive. These meristems are the portions of the embryo that will give rise to the new seedling, and are thus the most crucial part of the embryo in terms of whole plant survival.

To truly understand the function of a gene or gene product in overall organism development, it is necessary first of all to know the normal pattern of both temporal and spatial expression for that specific gene. In addition, the biochemical and biophysical role of the polypeptide must be identified. The results presented here indicated that Em

mRNA and polypeptide show coordinated peaks of accumulation during the late stages of wheat embryo development and that these high levels corresponded to times of highest ABA content. In addition it was observed that expression of Em mRNA within developing embryos was localized to the embryo proper with a slightly higher level of expression in the meristematic regions of both root and shoot. While these observations do not in themselves lend support to any proposals of a specific regulation or function for Em, or indeed any members of the embryo maturation gene set, they do present a framework within which these genes must perform.

CHAPTER II: THE RESPONSE TO ELEVATED LEVELS OF ABA BY

MEMBERS OF THE MATURATION GENE SET IN WHEAT

Introduction

Plant growth regulators play an important role in many phases of the plant life cycle (Jacobsen and Chandler, 1987; Quatrano, 1986, 1987; Pharis and King, 1985). The exact nature of this influence and the sequence of events initiated by the presence of a plant growth regulator remains largely unknown. One of the best characterized responses is that of barley aleurone layers to gibberellic acid (GA) and ABA (Jacobsen and Chandler, 1987). The response to these regulators identified in this system include a number of factors (Jacobsen and Beach, 1985; Chandler et al., 1984; Higgins et al., 1976).

The concentration of plant growth regulators, such as GA and ABA found in plants is generally quite low, but can increase to high levels in specific tissues and stages of development. For example, levels of ABA are normally elevated only during the mid to late stages of seed development (Bewley and Black, 1986) and when the plant is placed under conditions of water stress (Davies and Mansfield, 1983; Walton, 1980). As shown in Chapter I, changes in the levels of ABA and tissue sensitivity to the regulator are correlated with changes in gene expression during grain maturation. What is the molecular mechanism underlying this regulatory effect, and what is the relationship between the molecular mechanism and the tissue

specificity of the response?

The induction or up-regulation of specific mRNAs and proteins by application of exogenous ABA has been shown in a number of plants other than wheat (Williamson and Quatrano, 1988) such as cotton (Galau et al., 1986), barley (Higgins et al., 1976), castor bean (Dommes and Northcote, 1985), rapeseed (Finkelstein et al., 1985) and soybean (Eisenberg and Mascarenhas, 1985). Many of the gene products identified in the above studies are normally expressed only when endogenous ABA levels are high, such as during embryo maturation, and thus constitute a "maturation gene set" (Quatrano, 1987).

A number of genes belonging to the maturation gene set (MGS) in wheat embryos have been identified (Quatrano et al., 1983a). One member of this set encodes the Em polypeptide and another codes for triticin, a protein that shows characteristics of a 7S globulin. The expression of this set of genes follows the accumulation of ABA in the embryo (Chapter I of this thesis). Isolated immature wheat embryos when cultured in the presence of ABA do not germinate but continue to accumulate the gene products of the maturation set (Quatrano et al., 1983a) and the same has been seen for members of embryo maturation gene sets in other species (reviewed by Quatrano, 1986). Thus it appears that ABA may be positively regulating expression of this set of genes and that their expression is specific to both a developmental stage and a certain tissue, i.e. developing

embryos.

In general, then, seed protein genes, although their products persist in mature dry seeds, appear to be expressed either exclusively during embryogenesis or at very low levels in mature plant organ systems (Goldberg et al., 1989; Higgins, 1984). On imbibition and germination, protein and RNA synthesis are reprogrammed from embryo maturation and development to begin production of enzymes involved with germination and seedling growth (Misra and Bewley, 1985; Adams and Rinne, 1980). Okamuro et al. (1986) and Walling et al. (1986) showed that mRNA for soybean lectin was present in mature plant root and leaf, but at levels several orders of magnitude lower than those of embryos, while soybean storage protein mRNA was undetectable in mature plant organ systems. What controls this limited expression is unclear.

There is some evidence for DNA methylation influencing the tissue specificity of zein storage proteins in maize (Bianchi and Viotti, 1988). However, Walling et al. (1986) showed that transcriptionally active and repressed seed protein genes in soybean embryos did not have any detectable difference in their methylation patterns. In addition, a recent study of embryogenic competence in leaves and callus of Napier grass showed that changes in this trait did not correlate with levels of DNA methylation (Morrish and Vasil, 1989).

The desiccation experienced late in development by

most seeds has been proposed to be the switch that changes the program of gene expression from one of maturation to one of germination and that this switch is irreversible (Misra and Bewley, 1985; Dasgupta and Bewley, 1982). However, studies with immature embryos suggest that complete embryo maturation and maturation drying is not essential for germination to occur. Immature embryos of castor bean kept under conditions of high relative humidity will switch into the germination mode without the requirement of desiccation (Kermode and Bewley, 1989). Immature embryos of many other plant species will precociously germinate if removed from the seed and placed in culture on nutrient medium (Finkelstein et al., 1985; Stinissen et al., 1984; Quatrano et al., 1983a; Ihle and Dure, 1972). Isolated immature wheat embryos in culture do not continue to make maturation proteins, but commence synthesis of germination proteins such as ribulose-1,5-bisphosphate carboxylase (Quatrano et al., 1983a). Immature wheat embryos respond to ABA in culture by accumulating gene products of the maturation set (Quatrano, 1987).

Genes that are members of the embryo maturation set in wheat, and that are also responsive to ABA, may be used to test the proposal that normal desiccation of the embryo *in planta* has altered the responsiveness of the genes involved in embryo and seed maturation and rendered them irreversibly "closed", or not inducible by ABA. If this set is indeed regulated by ABA only during embryo maturation, it

should not respond to ABA at later stages, i.e. after desiccation , and in non-embryonic tissues. Does the ABA response include an accumulation of mRNA for gene set members at these later stages and in non-embryonic tissue? If there is an accumulation of mRNA, is there an accumulation of the corresponding translation products? Is the cytoplasmic environment, now changed to a vegetative state after desiccation, imbibition, and germination no longer supportive of translation and/or maintenance of embryonic mRNAs and polypeptides? Is it possible to dissociate the developmental program from the hormonal regulation of these genes?

In addressing these questions it was necessary to increase the level of ABA in the plant at times other than embryo maturation. To do this two approaches were taken, both using mature grains of wheat that had undergone desiccation on the plant during normal plant development. The first approach involved the application of exogenous ABA to isolated embryos or whole grains after their germination in an ABA-free environment. The second approach involved taking advantage of the fact that wheat accumulates ABA when put under conditions of water stress (Gusta and Chen, 1987; Hiron and Wright, 1973; Wright, 1969). If the ABA regulation of these genes is tightly controlled by the developmental program of embryogenesis and maturation, and desiccation inactivates their subsequent expression during later stages in the plant life cycle, gene products from

the maturation gene set in wheat should not accumulate in
non-embryonic tissue in response to increased levels of ABA.

Materials and Methods

Plant material: Isolated embryos and whole grains of wheat (*Triticum aestivum* (L.) cv. Chinese Spring) were used throughout the experiment. Plants were grown in pots under greenhouse conditions at an average temperature of 20-25° C. Immature grains were collected at various stages through development (Rogers and Quatrano, 1983). Grains were surface sterilized (10 minutes in 0.5% sodium hypochlorite followed by 3 rinses with sterile distilled water), embryos were isolated, immediately frozen in liquid N_2, and stored at -20° C until ribonucleic acid (RNA) and protein were extracted. Embryos were detached from mature whole dry grains by mechanical shearing in a Waring blender followed by sieving the particles to separate embryos from other grain parts after the method described by Williamson et al. (1985).

The first experiment involved exposing both isolated embryos and whole grains to elevated ABA levels by exogenous application of the growth regulator. In the case of isolated embryos, the embryos were placed on sterile filter paper which had been wetted with ABA-free germination medium (GM, Triplett and Quatrano, 1982) and placed in sterile sealed petri dishes. The sealed petri dishes were placed in a growth cabinet (Hoffman Manufacturing, Albany, OR, USA) at 26° C in the dark. After 24, 72 or 120 hours on GM, ABA (Grade IV, Sigma Chemical Co., St. Louis, MO, USA) was added to some of the petri dishes at a final concentration of 10^{-4}

molar (M) and the dishes returned to growth cabinets for a further 12 hour incubation in the dark. At the end of this time, treated and control embryos were harvested and placed in sterile tubes and stored at -20° C until extraction of RNA and protein. In experiments using whole grains, the material was surface sterilized (30 minutes in 1% sodium hypochlorite, 6 rinses with sterile distilled water, 10 minutes in sterile 10 millimolar (mM) HCl, followed by an additional 6 rinses with sterile distilled water) and placed in sealed petri dishes on sterile filter paper which had been wetted with sterile water. The sealed petri dishes were placed in a growth cabinet (Environ Air, SRJ Cabinets Sales, Greenacres NSW, Australia) at 25° C in the light (Crompton 18W white). After either 24, 72 or 120 hours on distilled water, ABA was added to some of the petri dishes at a final concentration of 10^{-4} M. These dishes and untreated controls were returned to the growth cabinets for a further 16 hour incubation in the light. After this ABA treatment, treated and control seedlings were harvested, dissected into shoots and roots where possible, and frozen directly in liquid N_2 until protein and RNA were extracted.

The second experiment involved exposing three-day-old wheat seedlings, grown from mature whole grain, to a dehydration stress in an effort to elevate endogenous levels of ABA (Wright, 1969). Whole grains were surface sterilized and sown on sterile filter papers wetted with sterile distilled water and allowed to germinate in conditions

stated above for whole grains. After 3 days on distilled water the seedlings were divided into three groups. One group of seedlings was harvested, dissected into roots and shoots and frozen directly in liquid N_2. A second group of seedlings was placed into a clean tared petri dish, weighed and placed into a desiccator jar to dehydrate slowly over a solution of 25% glycerol. Intact seedlings were left over glycerol from 1 to 4 days and weighed daily to determine the extent of dehydration. At one-day intervals, one group of dehydrated seedlings were harvested, dissected into roots and shoots, weighed and stored in liquid N_2. A third group of seedlings was left on distilled water for the duration of the experiment, and prior to freezing in liquid N_2 were dissected into roots and shoots. ABA, RNA and protein were extracted from the frozen tissue.

RNA Extraction: Total RNA was extracted by one of two methods. RNA extraction from immature embryos and seedlings cultured on GM was by differential precipitation from lithium chloride as published by Williamson et al. (1985). Total RNA from mature embryos and whole grain seedling tissue was extracted as described in Chapter I of this thesis.

Northern analysis: Northern analysis was performed by two methods. The first method (Figures II.1 and II.3) involved electrophoresis of glyoxylated RNA through agarose gels, closely following the method of McMaster and Carmichael (1977) as given by Maniatis et al.(1982). The

second method (Figure II.4 and II.7) involved

electrophoresis of RNA through agarose gels containing

formaldehyde, and was described in detail in Chapter I of

this thesis. Plasmids containing inserts of complementary

deoxyribonucleic acid (cDNA) for Em (p1015) and triticin

(p511) have been described previously (Williamson and

Quatrano, 1988). cDNA fragments used as probes for Northern

analysis were excised from plasmids with appropriate

restriction endonucleases and isolated by separation through

low-melting temperature agarose.

In the first method, samples of total RNA (19 μg)

were denatured by treatment with glyoxal and

dimethylsulfoxide (DMSO). Electrophoresis was through a

1.0% agarose gel, with the gel both poured and run using

0.01 M sodium phosphate buffer (pH 7.0). The glyoxalated

RNA was transferred immediately after electrophoresis to

NYTRAN nylon membrane (Schleicher & Schuell, Keene, NH, USA)

by capillary action with 20X SSPE (20X SSPE: 174 g sodium

chloride, 27.6 g sodium phosphate (di-hydrogen), 7.4 g

ethylene-diaminetetraacetic acid (EDTA) per liter, pH 7.4).

After blotting, the membrane was allowed to air dry before

baking under vacuum, at 80° C for 2 hr. Membranes were

prehybridized at 42° C overnight (12-16 hr) in

prehybridization buffer (50% formamide, 5X Denhardt's (50X

Denhardt's: 1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine

serum albumin (BSA)), 5X SSPE, 0.1% sodium dodecyl sulphate

(SDS) and 100 μg/ml denatured and sheared salmon sperm DNA).

Hybridization was done by removing the prehybridization buffer and replacing it with fresh prehybridization buffer containing radioactive cDNA (2 x 10^6 counts per minute per milliliter (cpm/ml) hybridization solution) and incubating at 42° C overnight (16 to 24 hr). Probe was synthesized using the random hexamer labelling procedure of Feinberg and Vogelstein (1983) using ^{32}P-α-dCTP (3000 Curies per millimole (Ci/mmol), New England Nuclear, Boston, MA, USA) yielding specific activities near 10^9 cpm/μg. Following hybridization, membranes were pre-washed at room temperature in a solution of 50% formamide, 5X SSPE, 0.25% SDS for 15 to 30 minutes. The membranes were washed for 30 minutes each in the following solutions: once with 2X SSPE, 0.1% SDS at 53° C; once with 0.5X SSPE, 0.1% SDS at 53° C; and once with 0.2X SSPE, 0.1% SDS at 63° C. Membranes were wrapped in plastic wrap and placed under Kodak XAR5 X-ray film for exposures of 1 to 4 days using a DuPont Lightning-Plus intensifying screen at -70° C. All exposed X-ray film was developed using Kodak (Eastman Kodak Co., Chicago, IL, USA) developer and fixer. All autoradiographs were quantified by densitometric scanning using a MK III CS microdensitometer (Joyce Lobel & Co. Ltd., Gateshead-on-Tynell, England).

Protein extraction and visualization of total protein by silver-staining or specific antigens by Western Blot analysis: Total protein was isolated by grinding tissue to a powder with liquid N_2 in a mortar and pestle. The ground tissue was extracted in Laemmli sample buffer (Laemmli,

1970) and heated at 80-90° C for 5 minutes. The
concentration of protein in the samples was determined using
the method of Schaffner and Weissmann (1973) and gels were
run on the basis of equal quantities of protein loaded per
lane.

The procedure used for the visualization of total
protein through silver staining shown in Figure II.6 is
given in Chapter I of this thesis.

For those gels to be used for Western blot analysis,
electrophoresis of total proteins was through a 15% (w/v)
SDS-polyacrylamide gel according to the method of Thomas and
Kornberg (1975). The separated proteins were electroblotted
onto nitrocellulose (Schliecher & Schuell). Visualization
of specific polypeptides through the use of antibodies was
done using two different methods, depending on the specific
antibody. Figures II.2, II.5 and II.10 were done using
polyclonal antibodies raised against wheat seed globulins
with visualization of antigens by reaction with horse radish
peroxidase (Bethesda Research Laboratories, Bethesda, MD,
USA) and performed by the method recommended by Vector
Laboratories,Inc. (Burlingame, CA, USA) for their Vectastain
ABC immunoperoxidase system. Figure II.9 was done using
polyclonal antibodies directed against the wheat Em
polypeptide with visualization of antigens by reaction with
alkaline phosphatase and performed by the procedure listed
in Chapter I of this thesis.

ABA assays: The ABA content in the samples was

assayed after the procedure of Walker-Simmons (1987) using a monoclonal antibody to ABA (Mertens et al., 1983). The exact procedure used was given in Chapter I of this thesis.

Results

Total RNA present in wheat embryos during the mid and late stages of development contains mRNA for both Em and triticin (Figure II.1, lanes 1 and 2). When mature embryos are cultured on germination medium (GM) in the absence of added ABA, the level of both messages declines to be undetectable after one day (Figure II.1, lane 3), and no further accumulation of either message is seen up to 120 hours after germination (Figure II.1, lanes 4 and 5). This is parallelled by the disappearance of the corresponding polypeptides. Total protein extracted from dry embryos is found to contain a variety of polypeptides that cross react with an antibody against wheat seed globulins (Figures II.2 lane 1). However, as isolated embryos imbibe and begin to germinate in culture, the number and quantity of these proteins decrease (Figure II.2, lanes 2-5). Mature leaves do not contain any polypeptides that correspond to these seed proteins (Figure II.2, lane 6).

If ABA is added to embryos after an initial 24 hours incubation in ABA-free medium, an accumulation of mRNA for both Em and triticin is seen within 12 hours (Figure II.3, lane 3). The renewed accumulation of both messages, however, results in a lower amount compared to that seen at the highest level during embryo development. At 36 hours, after receiving a 12 hour pulse of ABA, the level of Em mRNA is only 27% that seen in Stage 4 embryos, while the level of

Figure II.1

Northern analysis of total RNA extracted from developing or germinating wheat embryos. Lanes are as follow:

1 = Immature embryos from Stage 3 grains

2 = Immature embryos from Stage 4 grains

3 = Isolated embryos cultured on GM for 24 hr

4 = Isolated embryos cultured on GM for 72 hr

5 = Isolated embryos cultured on GM for 120 hr

The gel was loaded with equal quantities of RNA per lane. The nitrocellulose membrane was probed concurrently with radioactively labelled cDNAs of Em (large arrow) and triticin (small arrow). Migration distances of the 18S and 26S subunits of ribosomal RNA are as shown.
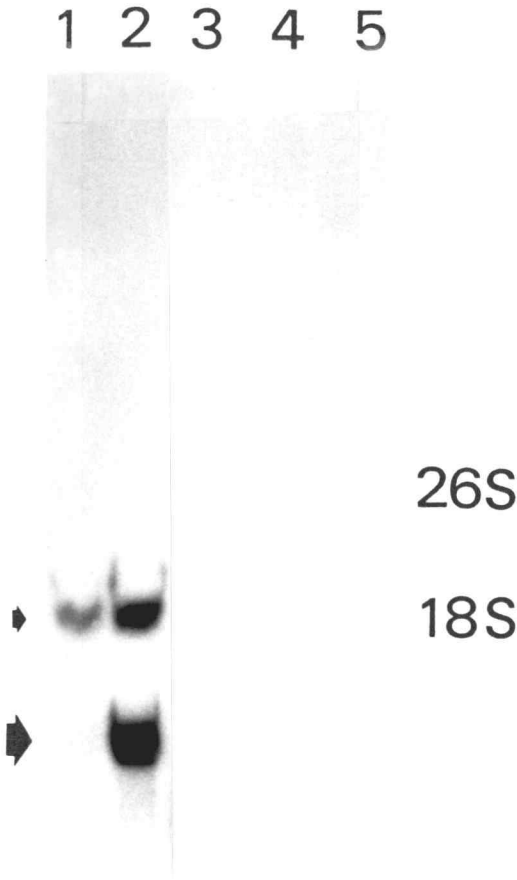
Figure II.1

26S

18S

Figure II.2

Western analysis showing the normal pattern of seed globulin content in wheat embryos and mature leaf tissue. Lanes are as follow:

    1 = Mature embryos from dry Stage 5 grains

    2 = Isolated embryos cultured on GM for 12 hr

    3 = Isolated embryos cultured on GM for 24 hr

    4 = Isolated embryos cultured on GM for 72 hr

    5 = Isolated embryos cultured on GM for 120 hr

    6 = Three-month-old leaf

The gel was loaded with **equal quantities** of protein per lane. The nitrocellulose **membrane** was reacted with polyclonal antibodies **raised against** wheat seed globulins. Approximate molecular **weight markers** given in kD.
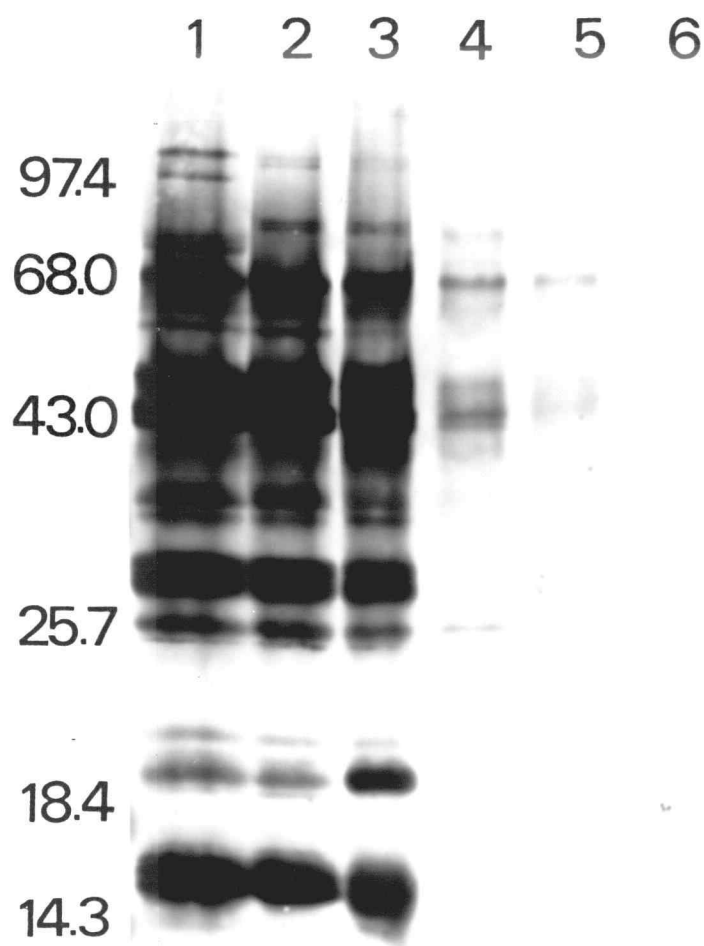
Figure II.2

Figure II.3

Accumulation of embryo maturation mRNAs in cultured isolated embryos. Lanes are as follow:

1 = Immature embryos from Stage 4 grains

2 = Isolated embryos cultured on GM for 24 hr in the absence of ABA

3 = Isolated embryos cultured on GM for 36 hr, with application of ABA (10^{-4} M) at 24 hr

4 = Isolated embryos cultured on GM for 120 hr in the absence of ABA

5 = Isolated embryos cultured on GM for 132 hr, with application of ABA (10^{-4} M) at 120 hr

Graph showing the changes in relative levels of Em (▨) and triticin (▢) mRNA. Values for mRNA abundance are given in arbitrary units (a value of "1" equal to the level of Em expression in Stage 4 embryos, lane 1) derived from scanning the film of a northern analysis (pictured above graph). Equal quantities of total RNA extracted from cultured isolated embryos were separated on a 1% agarose gel and blotted onto nitrocellulose. The membrane was probed simultaneously with radioactively labelled cDNAs of Em (large arrow) and the 7S globulin (small arrow).

Figure II.3
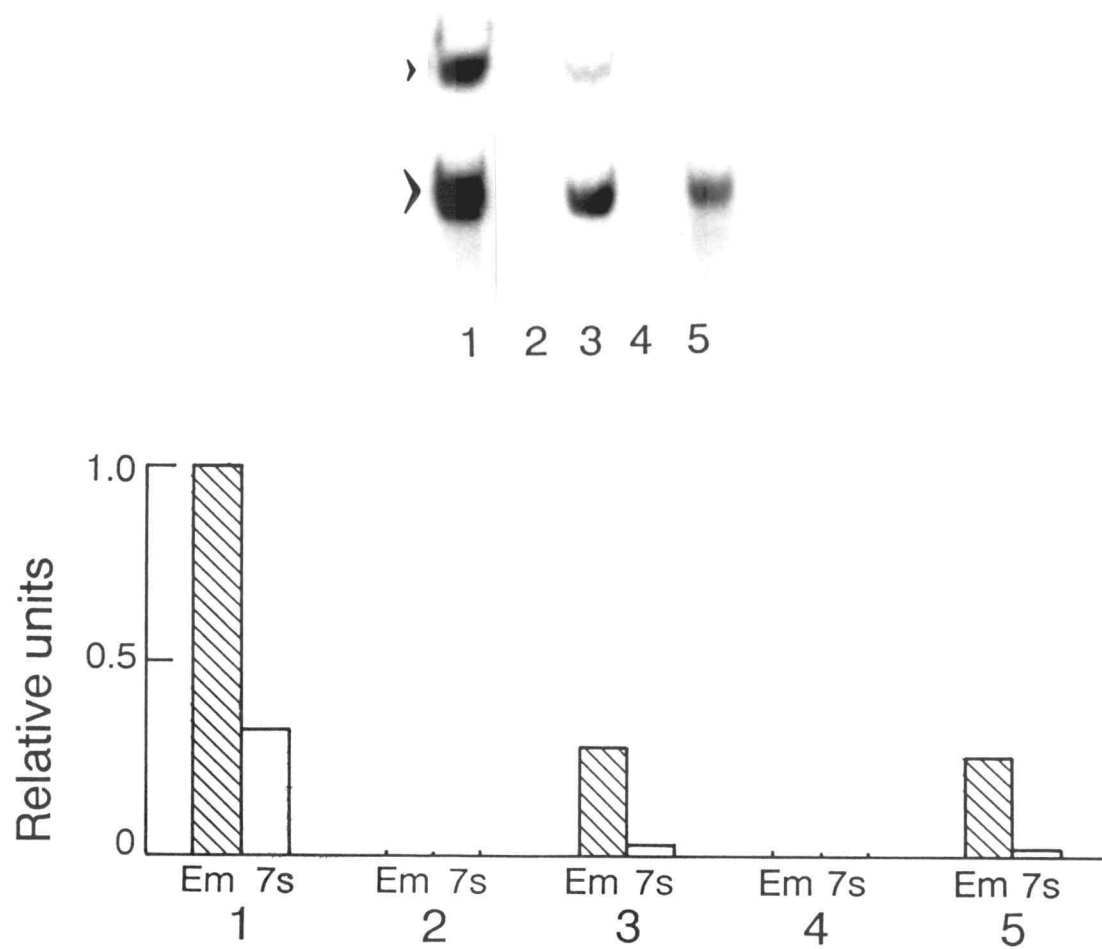
triticin mRNA is approximately 13% the level seen in Stage 4 (Figure II.3). After an initial 120 hours culture in ABA-free medium, the addition of ABA again results in an accumulation of mRNA for Em and triticin (Figure II.3, lane 5). However, at this later time there is an apparent difference in response between the two messages (Figure II.3, compare lanes 3 and 5). While the abundance of Em mRNA at 132 hours reaches nearly the same level as that seen at 36 hours (85%), accumulation of triticin message is only 20% of that found at 36 hours. Seedlings older than five days show a similar but decreasing sensitivity to exogenous application of ABA as measured by mRNA levels of Em and triticin (results not shown). It appears that there is a difference between Em and triticin in the ability to accumulate mRNA in response to exogenous ABA after germination has begun.

There also appears to be a difference between tissue types in the ability to respond to exogenous ABA. If whole grains are imbibed and germinated on water in the absence of ABA, addition of ABA after 24 hours results in an accumulation of Em message (Figure II.4, lane 3). When ABA is added to whole grains after 72 hours on water, both shoots and roots accumulate Em mRNA (Figure II.4, lanes 5 and 7, respectively). Shoots and roots of similar age seedlings which did not receive any ABA treatment show no accumulation of Em mRNA (Figure II.4, lanes 4 and 6, respectively). The roots of the germinated seedlings seem

Figure II.4

Accumulation of Em mRNA in wheat embryos isolated from imbibed and germinating whole grains. Lanes are as follow:

1 = Mature dry embryos

2 = Embryos from whole grains imbibed on water for 40 hr in the absence of ABA

3 = Embryos from whole grains imbibed on water for 40 hr, ABA (10^{-4} M) applied at 24 hr

4 = Shoots of seedlings imbibed and germinated on water for 88 hr in the absence of ABA

5 = Shoots of seedlings imbibed and germinated on water for 88 hr, ABA (10^{-4} M) applied at 72 hr

6 = Roots of seedlings imbibed and germinated on water for 88 hr in the absence of ABA

7 = Roots of seedlings imbibed and germinated on water for 88 hr, ABA (10^{-4} M) applied at 72 hr

Graph showing the changes in relative levels of Em (▧) mRNA. Values for mRNA abundance are given in arbitrary units (a value of "1" equal to the peak of Em expression in mature dry embryos, lane 1) derived from scanning the film of a northern analysis (pictured above graph). Equal quantities of total RNA extracted from dry embryos and whole grain seedlings were separated on a 1% agarose gel and blotted onto nitrocellulose. The membrane was probed with radioactively labelled Em cDNA.

Figure II.4
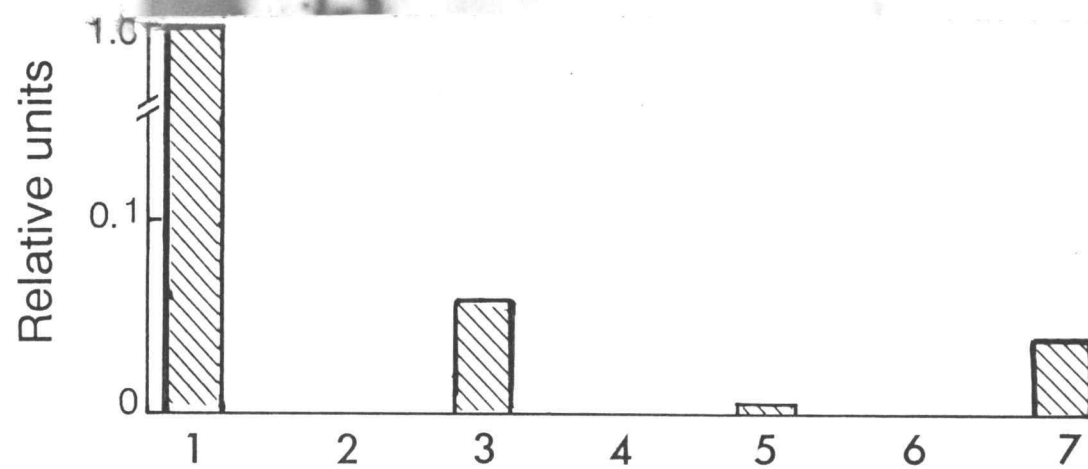
to be responding to the plant growth regulator to a larger extent than the shoots (compare lanes 5 and 7, Figure II.4). The abundance of Em mRNA in shoots is 10% of that seen in roots. A difference in uptake of or contact with ABA may be contributing to the differences in mRNA accumulation observed between the two different tissue types.

A change in the protein composition of cultured embryos given ABA after an initial ABA-free incubation is also observed (Figure II.5). In response to an application of ABA, embryos appear to resume the accumulation of some seed globulins (Figure II.5, lanes 2 and 4, arrows). These storage proteins are not normally found after germination and seedling growth have commenced (compare Figure II.5 to Figure II.2, lanes 4 and 5).

If the endogenous levels of ABA in seedling tissues are increased by subjecting them to water stress, is the response similar to unstressed tissue receiving ABA exogenously? Three-day-old seedlings were subjected to a mild dehydration stress by placing them into a desiccator jar over a solution of 25% glycerol for a period of one to four days (Chandler et al., 1987). Seedlings returned to water at the end of this dehydration stress will resume shoot growth and reinitiate root growth (original roots have died). The level of dehydration experienced by seedling tissues is shown in Figure II.6. Over the first day of dehydration the seedling roots lost 75% of their original fresh weight while shoots on the same seedlings experienced

Figure II.5

Western analysis showing changes in seed globulin content
from cultured isolated wheat embryos. Lanes are as follow:

   1 = Embryos cultured for 72 on GM in the absence of ABA

   2 = Embryos cultured for 84 hr on GM, with application

       of ABA (10^{-4} M) at 72 hr

   3 = Embryos cultured for 120 hr on GM in the absence

       of ABA

   4 = Embryos cultured for 132 hr on GM, with application

       of ABA (10^{-4} M) at 120 hr

The gel was loaded with equal quantities of protein per
lane. The nitrocellulose membrane was reacted with
polyclonal antibodies raised against wheat seed globulins.
Polypeptides that show renewed accumulation on addition of
ABA are denoted by (◀). Approximate molecular weight
markers are given in kD.

Figure II.5
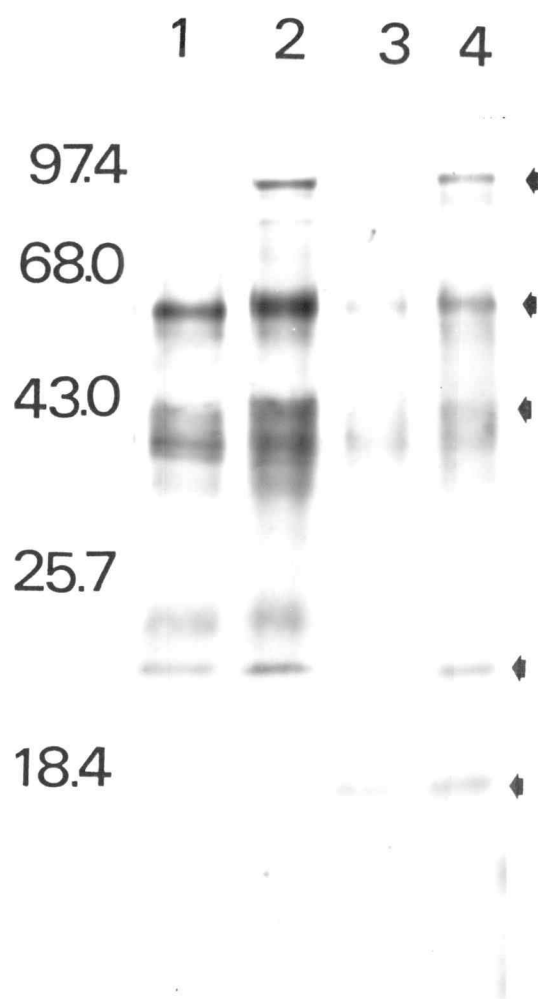
a loss of only 18% original fresh weight. From this observation it is apparent that all tissues within the seedling are dehydrating to differing degrees. Continued dehydration stress resulted in a continued decrease in shoot fresh weight, reaching a final loss of 60% original fresh weight. Roots, having lost the majority of their fresh weight in the first day, remained at approximately the same level of dehydration (retaining only 20% original fresh weight) for the remainder of the experiment.

Normally, the level of ABA found in young wheat seedlings is low (King, 1976). However, when well-watered young seedlings are placed under conditions of water stress, the level of ABA found in their tissues increases quickly and dramatically (Wright, 1969). As expected, the dehydration treatment resulted in an elevation of ABA content within seedling tissues (Figure II.6). Well-watered three-day-old seedlings at the beginning of the experiment have very low ABA levels; 19 pg/mg fresh weight in shoots and 6 pg/mg fresh weight in roots. After one day of water stress the level of ABA increased 50-fold in shoots and 194-fold in roots. Continued dehydration resulted in a slight decrease in ABA content in shoots, but even after 4 days of water stress the ABA content in shoots still showed a 33-fold increase over levels found in well-watered seedling shoots. Seedling roots showed a further increase in ABA levels after an additional day of dehydration stress, but then exhibited a decrease over the remainder of the stress

Figure II.6

Changes in fresh weight and ABA content in roots and shoots
of dehydrated wheat seedlings.

Line graph (left axis) shows the change in ABA content of
roots (•) and shoots (○) of three-day-old wheat seedlings
exposed to a dehydration stress from zero to four days. Bar
graph (right axis) shows the change in fresh weight of roots
(▨) and shoots (▢) from the same seedlings.

Figure II.6
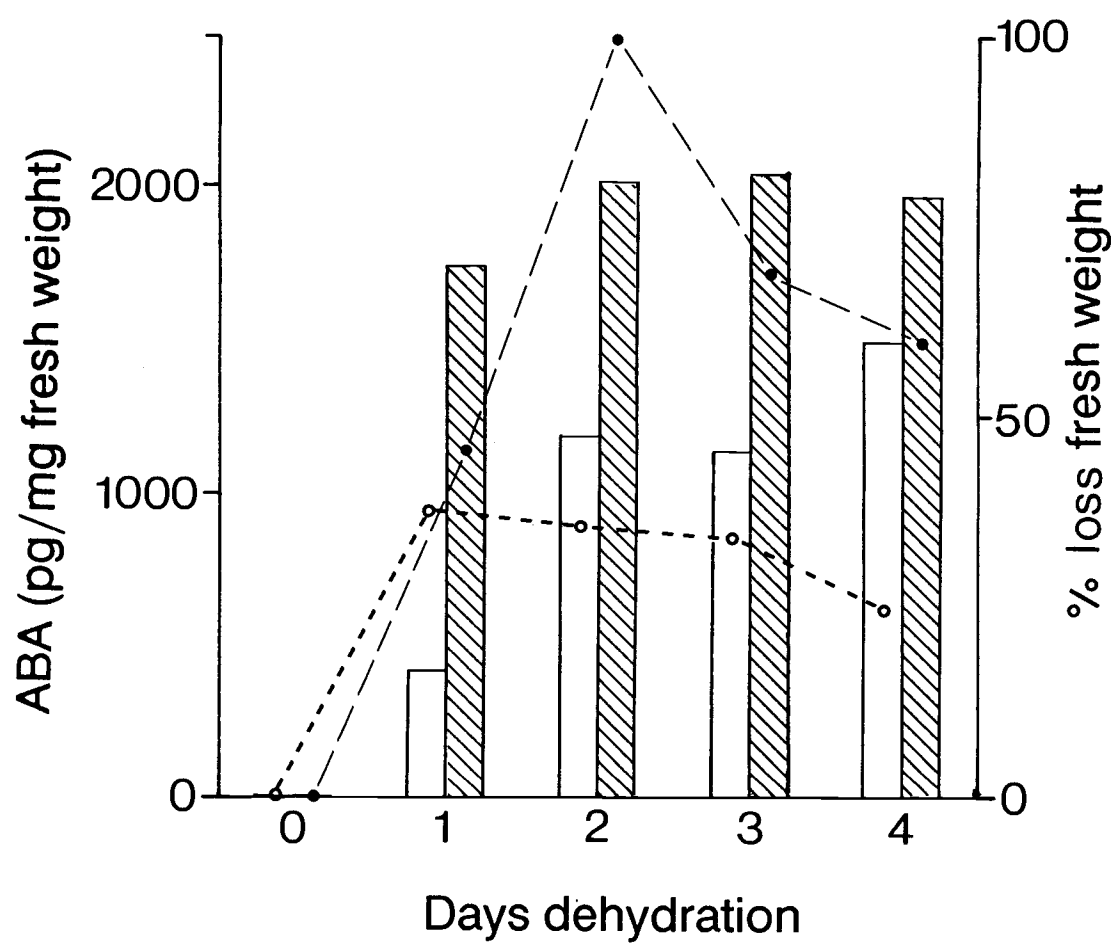
Days dehydration

period. These results demonstrate that increases in the level of ABA varies between tissue type and may reflect differences in the level of dehydration experienced by the different tissue types.

The increase in level of ABA is paralleled by an accumulation of mRNA for Em. Well-hydrated shoots of either three- or six-day-old seedlings have no detectable level of Em mRNA (Figure II.7, lanes 2 and 6 for three- and six-day-old seedlings, respectively). When three-day-old seedlings are subjected to a dehydration stress, an accumulation of Em message is observed in shoots after one day (Figure II.7, lane 3). Further dehydration results in the continued accumulation of the message, but at decreasing levels (Figure II.7, lanes 4 and 5). As shown earlier (Figure II.6), a dramatic initial increase in shoot ABA levels is followed by slightly declining levels of ABA with increasing dehydration stress. Em mRNA levels mirror closely these changing ABA levels. The level of Em mRNA found in dehydrated tissue, while much greater than that found in well-hydrated tissue, is still lower than that found in dry mature embryos (Figure II.7, lane 1). Levels of Em message found in dehydrated shoots ranged from 1% to 20% that found in mature dry embryos.

Many changes in protein composition are observed by silver-staining when total protein extrctsfrom shoots of well-watered or dehydrated seedlings are compared (Figure II.8). The pattern of proteins present in seedling shoots

Figure II.7

Accumulation of Em mRNA in dehydrated wheat seedling shoots.

Lanes are as follow:

  1 = Mature dry embryos

  2 = Shoots of 3-day-old seedlings, on water for 3 d

  3 = Shoots of 4-day-old seedlings, on water for 3 d

     then dehydrated for 1 d

  4 = Shoots of 5-day-old seedlings, on water for 3 d

     then dehydrated for 2 d

  5 = Shoots of 6-day-old seedlings, on water for 3 d

     then dehydrated for 3 d

  6 = Shoots of 6-day-old seedlings, on water for 6 d

Graph showing the changes in abundance of Em mRNA. Values

for Em mRNA abundance are given in relative densitometric

units (a value of "1" equal to the level of accumulation

seen in mature dry embryos, lane 1)) derived from scanning

the film of a northern analysis (pictured above graph).

Equal quantities of total RNA were separated on a 1% agarose

gel and blotted onto nitrocellulose. The membrane was

probed with radioactively labelled Em cDNA.

Figure II.7
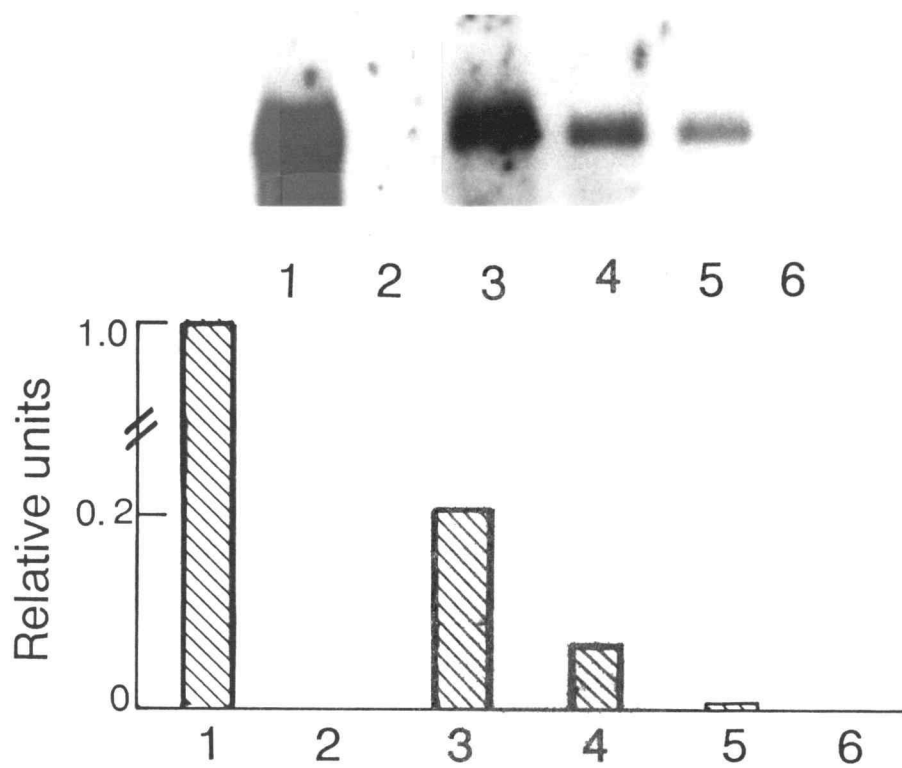
over the first one to two days of dehydration resembles that of the original three-day-old seedling shoot (Figure II.8, lanes 2,3 and 4). As the water stress becomes more severe this pattern changes. The dehydrated shoot tissue contains protien bands that appear identical to proteins found in mature dry embryos (Figure II.8, compare lanes 1, 4 and 5, dots). The prominent band of the large subunit of ribulose-bisphosphate-carboxylase (Figure II.8, arrow) seen in the well-hydrated seedling shoots, appears quite diminished in the dehydrated shoots of the same age (Figure II.8, compare lanes 5 and 6).

Similar protein extracts from dehydrated seedlings and mature leaf tissue were challenged with antibodies directed against wheat embryo maturation gene products (Figure II.9 and II.10). Dry embryos contain the Em polypeptide (Figure II.9, lane 1) but well-watered three- and seven-day old seedlings shoots do not (Figure II.9, lanes 2 and 7). Seedlings subjected to dehydration stress over a period of 4 days do not appear to contain any polypeptides which cross-react with antibodies directed against the Em polypeptide (Figure II.9, lanes 3,4,5 and 6). The same is seen when antibodies directed against wheat seed globulins are used on dehydrated mature leaf tissue (Figure II.10). While dry embryos contain a large number and amount of seed globulins (Figure II.10, lane 1), total protein extracts of mature leaves do not contain any material that cross-reacts with the antibody (Figure II.10, lane 2).

Figure II.8

Changes in proteins from well-watered and dehydrated wheat seedling shoots. Lanes are as follow:

1 = Mature dry embryos

2 = Shoots of 3-day-old seedlings, on water for 3 d

3 = Shoots of 4-day-old seedlings, on water for 3 d then dehydrated for 1 d

4 = Shoots of 5-day-old seedlings, on water for 3 d then dehydrated for 2 d

5 = Shoots of 6-day-old seedlings, on water for 3 d then dehydrated for 3 d

6 = Shoots of 7-day-old seedlings, on water for 3 d then dehydrated for 4 d

7 = Shoots of 7-day-old seedlings, on water for 7 d

Proteins were separated on an SDS 12-25% polyacrylamide gel and visualized by silver-staining. Approximate molecular weight markers are given in kD. Small arrow (▶) denotes the large subunit of ribulose-bisphosphate-carboxylase, and dots (•) denote protein bands in common between dry mature embryos and dehydrated tissue.

Figure II.8
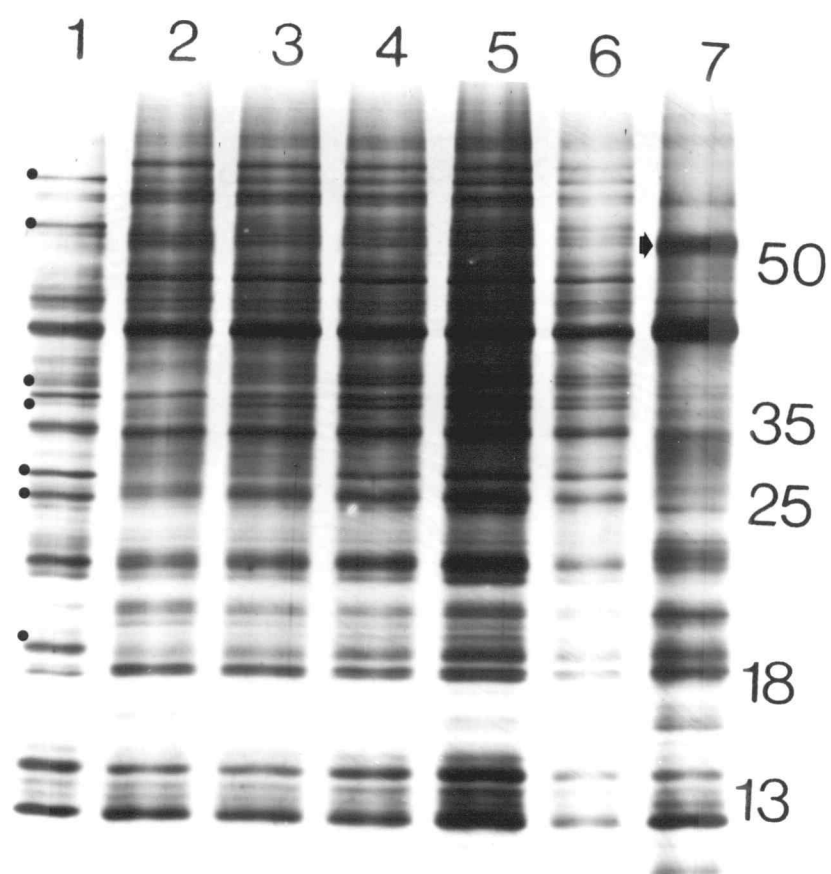
Figure II.9

Western analysis showing changes in Em polypeptide content

from well-watered and dehydrated wheat seedling shoots.

Lanes are as follow:

  1 = Mature dry embryos

  2 = Shoots of 3-day-old seedlings, on water for 3 d

  3 = Shoots of 4-day-old seedlings, on water for 3 d

      then dehydrated for 1 d

  4 = Shoots of 5-day-old seedlings, on water for 3 d

      then dehydrated for 2 d

  5 = Shoots of 6-day-old seedlings, on water for 3 d

      then dehydrated for 3 d

  6 = Shoots of 7-day-old seedlings, on water for 3 d

      then dehydrated for 4 d

  7 = Shoots of 7-day-old seedlings, on water for 7 d

The gel was loaded with equal quantities of protein per

lane. The nitrocellulose membrane was reacted with

polyclonal antibodies raised against the Em polypeptide

(arrow). Approximate molecular weight markers are given in

kD.

Figure II.9
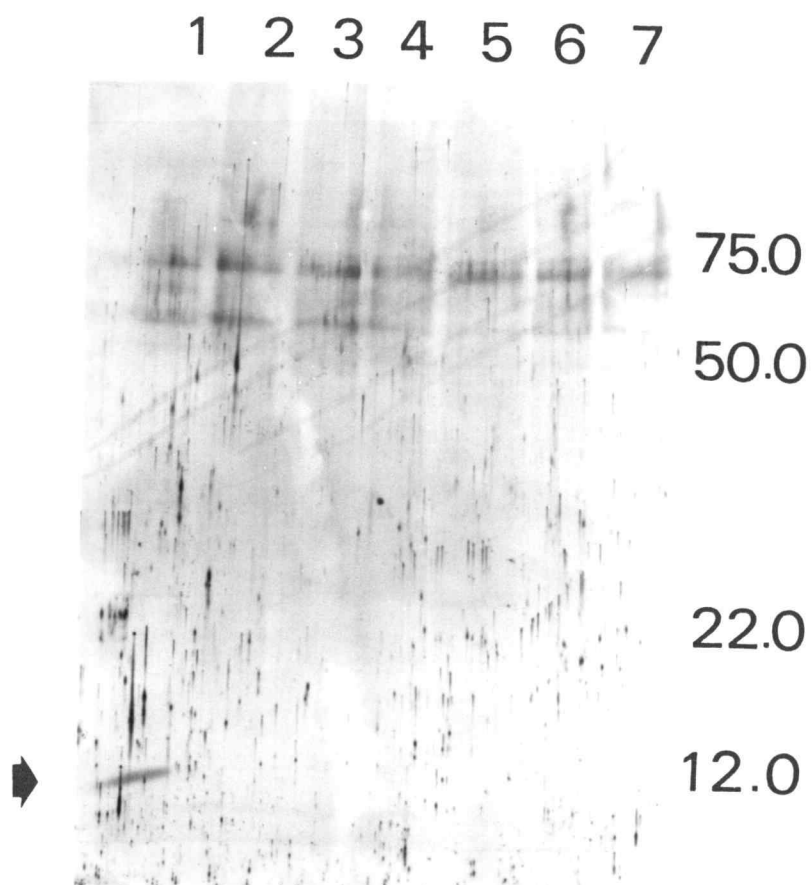
Figure II.10

Western analysis showing changes in seed globulin content
from well-watered and dehydrated mature wheat leaves. Lanes
are as follow:

  1 = Mature dry embryo from Stage 5 grain

  2 = Mature leaf taken from a 3-month-old wheat plant
     and subjected to no dehydration stress

  3 = Mature leaf taken from a 3-month-old wheat plant
     and subjected to a dehydration stress resulting
     in a 10% loss of fresh weight

The gel was loaded with equal quantities of protein per
lane. The nitrocellulose membrane was reacted with
polyclonal antibodies raised against wheat seed globulins.
Approximate molecular weight markers are given in kD.

Figure II.10
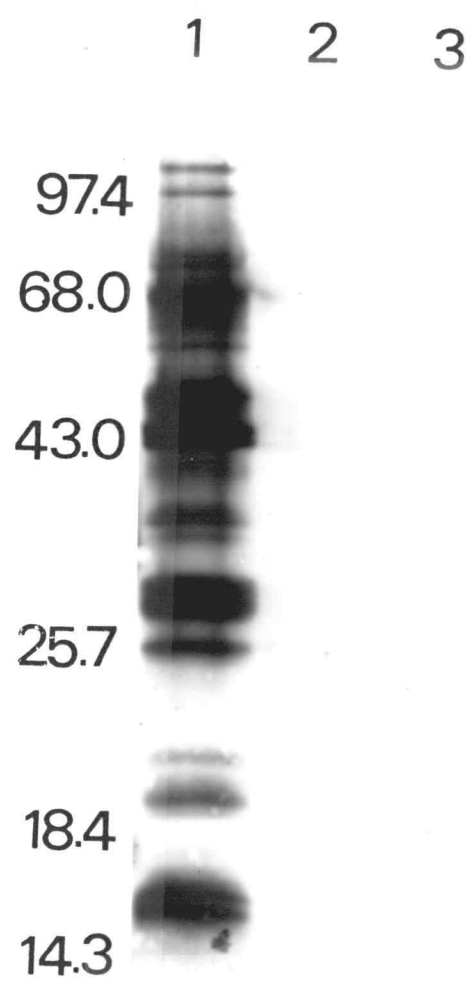
Moreover, when protein extracted from dehydrated leaves is challenged with the same antibody, no cross-reactive polypeptides are found (Figure II.10). Thus, although mRNA for some of the members of the embryo maturation set are accumulated under conditions of water stress (Figure II.7), the polypeptides are not found (Figures II.9 and 10).

Discussion

Results presented here demonstrated the following:

1) Northern analysis of total RNA extracted from developing and germinating embryos indicated that in the absence of applied ABA, mRNAs for members of the embryo maturation set in wheat, Em and triticin, were not detected after 24 hours of imbibition. However, a single application of ABA after 1,3, or 5 days in culture, resulted in an accumulation of Em and triticin mRNAs. The accumulation of Em mRNA in shoots was only 10% of that seen in roots, indicating a difference in sensitivity to, uptake of, or contact with ABA between the two tissues. 2) Polyclonal antibodies to wheat seed globulins indicated that seed globulin proteins present in mature embryos decline steadily with the onset of germination and reach low levels after 72 hours. However, treatment of germinating embryos with ABA resulted in a renewed accumulation of some seed globulins as long as five days into germination. 3) Seedling tissues were able to elevate ABA levels under moderate conditions of water stress, with the shoots of dehydrating seedlings showing a 50-fold increase in ABA content within the first 24 hours of water stress, followed by a decrease in ABA content on continued dehydration. 4) Northern analysis of the shoots of seedlings subjected to varying degrees of water-stress indicated an accumulation of Em mRNA within the first 24 hours of water stress followed by a decrease in mRNA levels on continued dehydration. This pattern of Em mRNA

accumulation closely followed changes in ABA levels in this tissue. 5) Silver-stained one-dimensional SDS-PAGE indicated many changes in the protein profile of dehydrating seedling shoots, but use of polyclonal antibodies directed against wheat seed globulins and the Em polypeptide failed to detect any cross-reactive proteins in this tissue. This contrasted with the accumulation of seed globulins observed in seedlings after a post-germination application of ABA.

The embryo maturation gene set in plants is marked by an apparently strict temporal and spatial regulation (Quatrano, 1987). Expression of mRNA for Em and triticin in wheat was limited to the late stages of embryogenesis, both messages decline and disappear on imbibition (Figure II.1). Proteins corresponding to Em and seed globulins were present in mature dry embryos (Figures I.2 and II.2), but declined on imbibition and germination to low levels after 5 days and were not reaccumulated in mature leaf tissue (Figure II.2). The decline in ABA levels seen in seeds near the completion of maturation (Kermode et al., 1989; Jones and Brenner, 1987; Walker-Simmons, 1987; King, 1976) may play a role in switching off of embryo maturation genes as may the dramatic drop in ABA seen on imbibition in many seeds. Over the initial 24 hours of imbibition and germination, the level of ABA present in lettuce seeds dropped to only 3% of its original value (McWha and Hillman, 1974). If this "signal", i.e. the degradation or removal of ABA, is not perceived, the embryo may continue along the path of maturation

regardless of its actual chronological and/or physiological state of maturity. Certain conditions, if maintained from the onset of seed hydration, will permit and actually promote the continuation of synthesis of messages and proteins produced by the embryo during maturation. This was seen in the continuation of seed storage protein synthesis in mature mustard seeds cultured in the presence of ABA or osmoticum (Fischer, et al. 1987), and also in rapeseed (Finkelstein et al., 1985). Williamson and Quatrano (1988) found that the levels of both Em and triticin message remain at the level found in mature dry Stage 5 embryos for at least 48 hours after imbibition in the continual presence of ABA. From this evidence one can conclude that ABA present at the onset of imbibition does not allow the closure of the embryo maturation set nor the opening of a germination set. This would ensure that the embryo is fully mature before allowing the subsequent expression of germination gene sets.

Presumably however, if germination, either precocious or normal, has occurred and seedling growth commenced, the embryo has been perceived as being "mature" and the expression of the set of genes involved in embryo maturation has been replaced by the expression of genes involved in germination and seedling growth. Nonetheless, from the transcripts seen in both isolated embryos and whole grains which have received a post-germination treatment of ABA, it was clear that the wheat embryo maturation genes for both Em and triticin were still capable of responding to ABA at the

level of mRNA accumulation at least 120 hours after germination (Figures II.3 and II.4). Young wheat seedlings also showed a reaccumulation of some seed globulins on application of ABA (Figure II.5). The transcripts of some embryo maturation genes are able to serve as templates for translation in young seedlings when ABA levels are elevated exogenously. Attempts to detect the Em polypeptide in this tissue were unsuccessful (data not shown) suggesting that these two genes may not be responding in exactly the same fashion.

The ability of wheat embryo maturation genes to respond to ABA at other times in the plant life cycle was further tested by subjecting young seedlings to a dehydration stress. The dehydration of plant tissues has been shown to cause ABA levels to increase up to 50 fold (Henson and Quarrie, 1981). Over the course of the dehydration treatment reported here, the seedling as a whole lost approximately 50% of its fresh weight and this resulted in an elevation of ABA levels in both roots and shoots (Figure II.6). An approximate 50-fold increase in ABA content (on a fresh weight basis) was observed in the shoots of dehydrated plants compared with control shoots (Figure II.6). This elevation of ABA content in dehydrated tissues compared to well-watered tissue was evident on the basis of both dry weight and fresh weight. The accumulation of ABA on dehydration, coincided with an accumulation of Em mRNA in the dehydrated shoots (Figure II.7). It has also been shown

that triticin mRNA accumulates in mature leaves exposed to moderate dehydration stress (Berge et al., 1988).

Dehydration changes the pattern of protein accumulation in water stressed seedling shoots (Figure II.8). In dehydrated tissue there are a number of protein bands which are not found in well-hydrated seedling tissue but appear identical to proteins present in dry mature embryos (Figure II.8, dots). However, from the evidence to date, it is not clear whether any or all of the mRNA for Em or triticin that accumulates in dehydrated vegetative tissues may actually be translated into polypeptides (Figure II.9 and II.10). It is possible that the amount of protein produced by the mRNA for these two genes is so small that it is undetected by the antibodies. It is also possible that the embryo proteins are themselves rapidly degraded in seedlings or that degradation of the protein synthetic apparatus resulting from dehydration does not permit the accumulation of many new products. Attempts were made to pulse label proteins during a number of dehydration experiments. As dehydration continued it became increasingly difficult to the label proteins with ^{35}s-methionine. Therefore the results of these experiments were inconclusive.

The lack of seed globulin accumulation in response to elevated endogenous ABA levels is in contrast to the positive accumulation seen after application of ABA. The polyclonal nature of the antibody may allow it to detect

other storage proteins in addition to globulins. Storage

proteins are not exclusively found in the seed or embryo but

also in leaves (Mason et al., 1988). Alternately, this

difference could be caused by a difference in contact of the

tissue with ABA. Exogenous application may result in a more

widespread exposure of ABA to intracellular "receptors",

while the ABA produced in response to dehydration may be

compartmentalized and not available to certain receptors.

An additional explanation for the lack of detectable

embryo maturation protein products, despite the accumulation

of corresponding mRNAs, may be that the translation of

embryo maturation messages seen during late embryo

development is a tissue specific response. Factors present

in embryonic tissue, while not themselves responsive to ABA

but important for efficient translation of the ABA inducible

gene products, could be lacking in non-embryonic tissues.

Thus the expression of these transcripts would only be at a

basal, undetectable level, if at all. The limited

expression of maturation gene set members may also be due to

a reduction in mRNA stability or translational efficiency in

non-embryonic tissues. Selective translation of messages at

different times in development is seen during wheat

endosperm development. Reeves et al. (1986) showed that

while mRNAs encoding α/β- and γ- type gliadins (the major

group of alcohol-soluble seed storage protein in wheat

endosperm) and ADP-glucose pyrophosphorylase (a regulatory

enzyme of starch biosynthesis) accumulated coordinately, the

accumulation of polypeptides corresponding to these messages differed significantly. The accumulation of gliadin polypeptides was not observed until several days after the detection of its mRNA, while accumulation of the enzyme followed approximately the temporal accumulation of its mRNA. Both post-transcriptional and post-translational processes play an important role in gene expression in cucumber plants. Frevert et al. (1980) showed in that while both mRNA and protein for isocitrate lyase (a glyoxysomal enzyme and a marker for germination) were present in ripening cucumber seeds, no enzyme activity was detected until after germination. An additional line of evidence comes from the finding that mRNA expression of a number of genes in *Brassica napus* L., primarily expressed during seed germination and post-germinative development, is activated during embryogeny (Harada et al., 1988).

Support for the idea that ABA is promoting this effect is largely correlative; when Em mRNA is accumulated, ABA is present in relatively high concentrations (i.e. during late embryogenesis, at times of exogenous application, and during dehydration). Additional evidence comes from studies involving mutants that are deficient in either biosynthesis of or in their response to ABA (Koornneef et al., 1984, 1989). It is clear that dehydration-induced ABA is capable of affecting changes in mRNA and protein synthesis from other work in wheat as well as other plants (Vartanian et al., 1987; Quarrie and Lister,

1988; Close et al., 1989; Mundy and Chua, 1988; Gomez et al., 1988; Heikkila et al., 1984; Cloutier, 1983). Studies done with a viviparous mutant of corn which cannot elevate ABA levels under water stress, showed that the mutant also cannot accumulate mRNA for a dehydration induced mRNA found in a number of other cereals (Chandler et al., 1988). The function of these ABA and/or dehydration induced proteins is unclear.

There is a growing body of evidence showing that some of the proteins expressed under dehydration stress are also expressed during the late stages of embryo development (Chandler et al., 1988; Gomez et al., 1988; Mundy and Chua, 1988). It also appears that some members of the embryo maturation set may be related to proteins expressed during times of dehydration stress (Dure et al., 1989; Close et al., 1989). An ABA-inducible mRNA which normally accumulates late in *Brassica napus* seed development and is also induced in leaves by severe desiccation (Harada et al., 1989) contains regions of homology with four other late embryogenic abundant proteins from three other plant species (Dure et al., 1989). This finding seems reasonable since both situations represent times in which the plant must cope with water stress, strongly suggesting a role for ABA in both situations.

In summary then, it is clear from these results that although desiccation may indeed trigger a change in the developmental program of the embryo, it is not irreversible.

Certain genes that are regulated by ABA and normally expressed only during wheat embryo maturation can be responsive to ABA at other stages in the wheat plant. Accumulation of mRNA for both Em and triticin and some seed globulin proteins was observed in a five-day-old seedling receiving a single 12 hour pulse of ABA. A renewed accumulation of some globulin polypeptides was also observed following application of ABA, but an accumulation of Em polypeptide in the same tissue was not detected. Seedlings subjected to a moderate dehydration stress elevated ABA levels within shoots and roots, and accumulated Em mRNA in shoot tissue following a pattern of accumulation reminiscent to changing ABA levels. The accumulation of Em mRNA n dehydrated shoots was not accompanied by an accumulation of Em polypeptide. In contrast to the accumulation observed on application of ABA to seedlings, seed globulin proteins were not detected in dehydrated leaves. These results suggest a difference in tissue exposure to ABA between the two treatments and/or changes in the relative sensitivities of embryo maturation mRNA translation after desiccation and during vegetative growth.

For these genes then, regulation by ABA appears to be one of the main controlling factors in determining expression at the levels of mRNa at various developmental stages and in different tissues. The explanation for limited expression at the polypeptide level of these transcripts in non-embryonic tissue is less clear.

SUMMARY AND CONCLUSIONS

The mid to late stages in a wheat grain development are characterized by the expression of a set of genes. Two members of this maturation gene set (MGS) in wheat are Em and triticin. Results presented here indicated that the expression of Em mRNA and polyeptide was temporally associated with increasing levels of the plant growth regulator abscisic acid (ABA) during embryogenesis, and that Em mRNA was localized to the embryonic axis within the developing wheat grain. At later stages of the wheat life cycle, when ABA content was low, no accumulation of Em or tricticin mRNA or polypeptide was detected. If ABA levels were elevated either by direct application or by imposing a water stress, non-embronic tissues were able to respond b accumulating mRNA for both these MGS members. However, polypeptides corresponding to these mRNAs were not detected in non-embryonic tissue. This indicated that ABA may act to trigger gene expression at the mRNA level, but other factors may control expression at the polypeptide level.

The study presented here has tried to define the limits of expression for members of the maturation gene set in wheat. The expression of these genes is modulated by ABA in a non-tissue specific manner at the mRNA level but not necessarily at the polypeptide level. The pattern of Em mRNA localization within the embryonic axis of developing wheat embryos suggests that it is not functioning as a storage protein, but the limited expression of the

polypeptide suggests that its function is specific to the embryo. While not directly disputing nor supporting the proposed functions of these proteins, it has attempted to widen the framework within which the proposed functions of these gene products must be carried out.

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