DEHYDRIN SYNTHESES IN RESPONSE TO ABA AND WATER-DEFICIT-RELATED STRESSES: A COMPARATIVE ANALYSIS OF AN ORTHODOX SPECIES (*RICINUS COMMUNIS* L.) AND A RECALCITRANT SPECIES (*CASTANOSPERMUM AUSTRALE*)

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Dehydrin Synthesis in Response to ABA and Water-Deficit-Related Stresses: A Comparative Analysis of an Orthodox Species (*Ricinus communis* L.) and a Recalcitrant Species (*Castanospermum australe*)

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ABSTRACT

During development of *Ricinus communis* L. (castor bean) seeds, substantial changes occurred in synthesis of the subset of LEA (late embryogenesis abundant) proteins referred to as 'dehydrins'. In particular, new dehydrin polypeptides were induced between 40 and 60 days after pollination. In contrast to a rapid loss of *Lea*/dehydrin mRNAs during germination, many of the dehydrin proteins abundant in the dried seed persisted following imbibition or rehydration.

The stress inducibility of dehydrin protein production in immature seeds and seedlings of castor bean was analyzed by subjecting them to abscisic acid (ABA) and various water-deficit-related treatments including desiccation, water stress, high salt, high osmolarity and low temperature. A number of dehydrins increased in seedlings subjected to the various stress treatments; in the endosperm, these appear to be different from the dehydrin-related polypeptides that are induced during late seed development and which persist following germination/growth of mature seeds. In the endosperms of seedlings, ABA, water stress and desiccation induced the same dehydrin polypeptides, while high osmolarity, high salt and low temperature induced a different set. Stress-specific differences in dehydrin synthesis were also found in the cotyledons and radicle of castor bean seedlings; however, dehydrins inducible by exogenous ABA were consistently produced. Immature seeds treated with ABA or subjected to stresses responded by producing dehydrins associated with late development; however, the same proteins were induced following detachment of immature seeds from the parent plant and maintenance on water. When seedlings were exposed simultaneously to GA and either ABA, high salt, or low temperature, dehydrin production was

suppressed.

In contrast to seeds of orthodox species, those of recalcitrant species do not acquire desiccation tolerance during their development. However, dehydrin-related polypeptides (40 KD and 37 KD) were induced in the recalcitrant seeds of *Castanospermum australe* as the seeds matured. When immature seeds were treated with ABA and various water-deficit-related stresses, maturation-related dehydrins were induced. Proteins of 40 KD and 37 KD were induced by ABA treatment; only the 40-KD protein was induced by the water-deficit-related stress treatments. However, when immature seeds of *C. australe* were subjected to a full desiccation treatment, no dehydrins were detected. It is possible that the rapid loss of water killed the seeds. The 40 KD, 37 KD and 31 KD dehydrins present in cotyledons of the mature seeds of *C. australe* were very stable after 5 days of germination. These dehydrins became less abundant following 7 days of treatments (ABA and all water-deficit-related treatments, except desiccation), although most of the dehydrins were still detectable. An exception was the desiccation-treated seedlings, in which no dehydrins were detected. In the roots of *C. australe* seedlings, no dehydrins were found either after 5 days of germination or following 7 days of any of the stress treatments.

Dedication

To My Parents

Acknowledgments

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List of Abbreviations

ABA:	Abscisic acid
ABRE:	ABA-response element
COR:	cold regulated
DAP:	day after pollination
DHN:	dehydrin, dehydration induced
EM:	early methionine labeled
GA:	gibberellin
GLY:	glycine
LEA:	late embryogenesis abundant
LTI:	low temperature induced
LYS:	lysine
RAB:	response to ABA
RFLP:	restriction fragment length polymorphism
SER:	serine

Chapter 1. Introduction

1.1. Proteins Induced by Water-Deficit Related Stresses: LEAs and Dehydrins

There are many reports of different genes or gene products that are positively regulated by the plant hormone abscisic acid (ABA), and in many cases these gene products are accumulated both during late seed development, and under stress conditions imposed on seedlings or plant vegetative tissues, such as dehydration, low temperature, salinity or high osmoticum. Some of these studies have allowed researchers to define relationships between similar gene products from different plant species. The naming of genes and their products has led to some confusion because investigators approached related sets of genes and proteins in different systems and with different interests (Mundy, 1989). The names have been given according to developmental characteristics: **LEA** (late embryogenesis abundant), or regulation of expression: ***rab*** (responsive to ABA) and ***dhn*** (dehydrin: dehydration induced), or amino acid labeling pattern: **Em** (early methionine labeled). In some cases, genes or proteins that are closely related have been given different names: for example, *rab* 17 and *dhn* from corn are almost identical in sequence and in their regulation of expression. In other cases, genes with similar notation are not related in sequence, e.g. *rab* 17 and *rab* 28 (Chandler and Robertson, 1994). Dehydrin-like proteins have now been positively identified in a range of species including barley, wheat, rice, maize, pea, tomato, potato, carrot, cotton, and radish.

The name of 'dehydrins' was given to a subset of a group of LEA proteins which accumulate under osmotic-related environmental stresses, such as water deficit, cold temperature, salinity, increased external osmotic pressure, embryo desiccation, or application of the plant hormone ABA. These

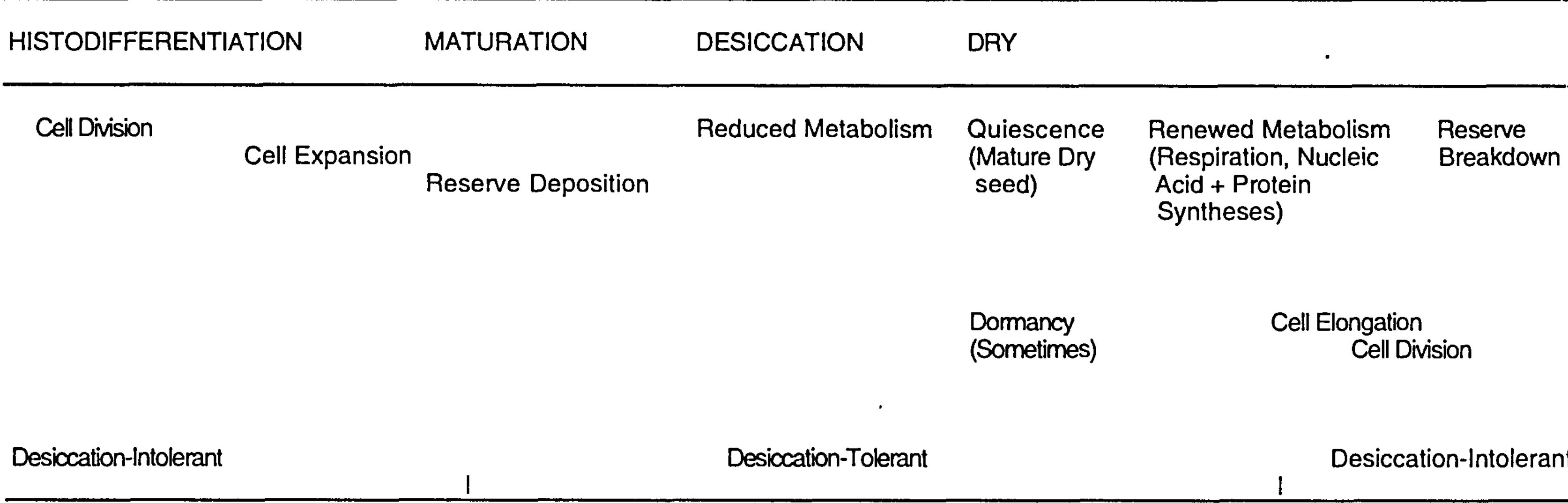
proteins have some common features in their structure and are proposed to play an important role in protecting plant cells under water-deficit-related conditions. It is noteworthy, however, that synthesis of these proteins is not the only response of plants to water deficit (Bray 1991).

1.2. Desiccation as Part of the Seed Development Program

1.2.1. Desiccation Switches the Seed from a Developmental Program to a Germination Program in Orthodox Species

Seed development in orthodox plant species can be divided into three stages. Initially, during histodifferentiation, the single-celled zygote undergoes extensive mitotic division, and the resultant cells differentiate to form the embryo composed of the axis and cotyledons; concurrently the triploid endosperm develops. Later, the maturation or seed expansion stage is characterized by cell expansion and deposition of reserves. Finally, maturation is terminated by some degree of drying, which results in a gradual reduction in metabolism as water is lost from the seed tissues and the embryo passes into a metabolically inactive, or quiescent state (Fig. 1). It is now evident that these events occur under the controlling influence and protection of the maternal environment. Upon imbibition of the mature seed, there is a reactivation of existing metabolic systems, supplemented by synthesis of new components, which leads to renewed cell expansion and cell division as the seedling becomes established. A period of desiccation is the normal terminal event in embryo development; it leads to a state of metabolic quiescence which is naturally interpolated between development and germination. Seed development and germination are distinct physiological stages of the plant life

Figure 1.1. Some events associated with seed development, germination, and growth (From Kermode, 1990).


DEVELOPMENT
GERMINATION
GROWTH
HISTODIFFERENTIATION
MATURATION
DESICCATION
DRY
Cell Division
Cell Expansion
Reserve Deposition
Reduced Metabolism
Quiescence (Mature Dry seed)
Renewed Metabolism (Respiration, Nucleic Acid + Protein Syntheses)
Reserve Breakdown
Dormancy (Sometimes)
Cell Elongation
Cell Division
Desiccation-Intolerant
Desiccation-Tolerant
Desiccation-Intolerant

cycle, in which key metabolic events related to the status of stored reserves contrast markedly. Development is characterized by the rapid accumulation of storage reserves, including proteins, lipids and carbohydrates. On the other hand, germination and subsequent seedling growth is characterized by the mobilization of these stored reserves. There is substantial evidence that desiccation (the final stage of development) plays a role in the switch in cellular activities from an exclusively developmental program to an exclusively germination/growth-oriented program (reviewed by Kermode, 1990; Kermode, 1995).

It has been well documented for cereals that drying is important for the acquisition of germinability of the whole seed (reviewed by Mitchell *et al.*, 1980). As early as 1852, Duchartre noted the beneficial effect of drying upon the subsequent germination of rye, wheat, and barley grains. Subsequent work established that immature grains of barley and wheat will germinate if dried on the straw, while their fresh counterparts (i.e. those of the same developmental stage, but not dried) will not (Harlan and Pope, 1922; Wellington, 1956; King, 1976). These observations are not unique to cereals; immature seeds of several legumes and other dicots also will not germinate on water when removed from the parent plant in the fully hydrated state, but will germinate after drying (Adams and Rinne, 1981; Dasgupta and Bewley, 1982; Kermode and Bewley, 1985; Rosenberg and Rinne, 1986). However, seeds cannot tolerate drying at all stages during their development, but undergo a transition from a desiccation-intolerant to a desiccation-tolerant state at a particular stage in the course of their development. Acquisition of tolerance is usually substantially earlier than the natural drying event itself and seeds of some species may withstand desiccation even before development has reached the midpoint (Fig. 1). Premature desiccation of *Phaseolus vulgaris* axes during the early

developmental stages (up to 22 days after pollination (DAP), during the desiccation-intolerant stage) damages several cellular organelles including protein bodies, mitochondria and the nuclear membrane. This dramatically reduces the metabolic and cellular integrity of the axes upon subsequent rehydration such that the axes deteriorate. In contrast, such severe perturbations do not occur at a desiccation tolerant stage, e.g. at 32 DAP (Dasgupta *et al.*, 1982). The limited damage sustained during drying at this stage is rapidly reversed following rehydration (Kermode, 1990).

During germination, seeds initially remain tolerant of desiccation, but at some stage after axis elongation there is a loss of this tolerance. Germinating soybean seeds are tolerant of drying during early stages, up to 6 h after commencing imbibition, but they become increasingly intolerant after this time. Thus desiccation at 36 h after the start of imbibition leads to a complete loss of seed viability (Senaratna and McKersie, 1983). The membrane appears to be a major site of damage in seeds during the desiccation-intolerant stage of germination (Kermode, 1990). There are two major hypotheses concerning the mechanism involved in protecting cells during desiccation and subsequent imbibition. One mechanism involves sugars which prevent membranes from undergoing a transition from a "liquid crystalline" phase to a "gel" phase during drying or subsequent rehydration, which would otherwise cause a leakage of cellular and membrane components, and result in cell death. The other mechanism implicates a group of proteins (dehydrins) which begin to be expressed during the expansion stage of seed development, and which are proposed to protect cells when seeds begin to desiccate.

1.2.2. Phospholipid Bilayer Leakage and Protection by Sugars

Dehydration of artificial phospholipid bilayers causes fusion. When phospholipid vesicles fuse they leak their contents to the medium. It is likely that when the vesicle is dehydrated it undergoes a phase transition from the liquid crystalline phase to the gel phase. Upon rehydration, the gel phase would attempt to revert back to a liquid crystalline phase. The bilayer may become leaky during both phase transitions (Crowe *et al.*, 1987; Crowe *et al.*, 1989). Disaccharides are thought to prevent membrane fusion during desiccation by interacting with polar head groups of phospholipids and functional groups of membrane proteins. Anhydrobiotic organisms, such as yeast, pollen, and seeds, usually contain high concentrations of disaccharides, particularly trehalose or sucrose. For example, yeast accumulates trehalose, which is required for survival of drying. Trehalose has also been shown to be a particularly effective protectant in nematodes, fungi and bacteria. It is intriguing that most vascular plants seem to lack trehalose, even when subjected to severe changes in external conditions or to complete desiccation. In seeds and pollen, sucrose and stachyose may play the same role (Crowe and Crowe, 1986; Crowe *et al.*, 1992). In soybean seeds, stachyose accumulates at a high level during slow drying of immature seeds (at 34 DAP), but does not increase significantly when seeds are incubated at high relative humidity (Blackman *et al.*, 1992). Many orthodox seeds accumulate high levels of soluble sugars during maturation, and sugar accumulation has been correlated with an increase in desiccation tolerance (Amuti and Pollard, 1977).

Reports about trehalose in vascular plants are scarce, although trehalose was identified as a major carbohydrate in more than 70 species of desiccation-tolerant lower plants (reviewed in Müller *et al.*, 1995). Recently, however,

relatively large amounts of trehalose have been discovered in two desiccation-tolerant angiosperms (resurrection plants). The first is *Myrothammus flabellifolia,* a dicotyledonous plant living in arid, rocky regions in southern Africa. This plant contains about 3% trehalose (on a dry weight basis) in the leaves. The second is the grass *Sporobolus stapfianus,* where trehalose has been found to amount to about 2-5% of total soluble carbohydrates. It appears that trehalose is restricted to primitive phyla of vascular plants and perhaps, as a rare exception, to certain resurrection plants among the angiosperms. Even though higher plants contain very low amounts of trehalose, high activities of trehalase, an enzyme which degrades trehalose, have been found (reviewed in Müller *et al.*, 1995).

Holmström *et al.* (1995) transferred a yeast trehalose synthase gene into tobacco plant. With the synthesis of small amounts of trehalose in tobacco, The ability of the transformed plant to survive drought was greatly increased. The trehalose content was 0.8-3.2 mg per g dry weight in the transformed plants, in contrast to 0.06 mg per g in non-transformed plants. When the seedlings of both transformed and non-transformed (control) plants were subjected to air-drying, the control seedlings showed signs of wilting after 2 h of drying and totally collapsed after 7 h of drying; the transformed seedlings were only marginally affected by this treatment. After rehydration, the trehalose-producing plants recovered turgor and recommenced growth, while the controls died.

Crowe and colleagues (Crowe *et al.*, 1993) have established that these disaccharides preserve dry membranes, liposomes, and labile proteins. Without the sugars, irreversible damage occurs during drying, but this is largely prevented when the sugars are present. For example, phosphofructokinase (PFK) is irreversibly denatured when it is dried without

sugars, but in the presence of trehalose or sucrose, it is stabilized during drying. Similarly, membranes undergo fusion and phase transitions during drying, but if the sugars are added before drying, membranes are completely preserved. This stabilization is effected by hydrogen bonds between -OH groups on the sugars and polar residues of phospholipids and proteins. In the dry state, the sugars apparently replace the water that normally forms hydrogen bonds with these polar residues, an idea that is known as the water-replacement hypothesis. The sugars profoundly alter physical properties of dry membranes or proteins so that they resemble those of fully-hydrated biomolecules. Although the evidence from these experiments using artificial phospholipids is convincing, the role of sugars in protecting cells of the desiccating seed (*in vivo*) remains to be elucidated.

1.2.3. LEA and Dehydrin Proteins Are Produced during Seed Maturation

Seeds of orthodox plant species undergo maturation drying on the mother plant as a normal terminal event in their development, in some cases achieving water contents of less than 5-10% on a fresh weight basis (Kermode, 1995). Seeds are not capable of withstanding desiccation at all stages during their development; the stages may be distinguished by the expression of different groups of genes. An analysis of the changes in mRNA subsets during embryogenesis indicates that the mRNAs detected during seed development and germination can be divided into seven groups. They are: (1) those which are constitutively expressed during embryogenesis and post-germinative growth, including actin, tubulin, and calmodulin mRNAs; (2) those which are early embryogenesis-specific; (3) those which are embryo-specific, i.e., the gene is turned off after embryogenesis is completed; (4) those which are expressed during seed development, such as storage protein and lectin mRNAs; (5) those

which are late embryogenesis-specific mRNAs; (6) those which are expressed during late embryogenesis and germination; and (7) those which are germination/growth-specific such as isocitrate lyase mRNA (Dure, 1985). After midway through seed development, a highly abundant set of hydrophilic proteins called LEAs (late embryogenesis abundant proteins, first described in cotton) are synthesized which exhibit temporal regulation during seed development and are implicated in desiccation tolerance (Dure, 1993b). The genes encoding these proteins arise as highly coordinately regulated sets, which on this basis, comprise two distinct classes in cotton (reviewed in Kermode, 1995). One class, which is made up of six different LEA transcripts, is first detected soon after histodifferentiation is completed and exhibits two transient peaks, before reaching a maximum level 3 days prior to desiccation. The transcript levels of the second class of twelve LEA mRNAs increase sharply during late maturation and peak just prior to, or at desiccation. LEA protein synthesis constitutes a large proportion of the translational activity of the cotton embryo during late maturation (up to 25%), and is regulated at the transcriptional level. In the mature cotton embryos LEA proteins comprise about 2% of the total soluble protein (Dure, 1993b).

Messenger RNAs homologous to the LEA cDNAs of cotton have been found in abundance in mature dry embryos of many diverse plant species including soybean, oilseed rape, pea, loblolly pine, tomato, radish, carrot, castor bean, *Arabidopsis*, *Zizania* and several cereals (Kermode, 1995). LEA proteins may act as desiccation protectants; these proteins can be elicited in early maturation stage cotton embryos by a premature desiccation treatment (Galau *et al.*, 1991).

Unlike the storage proteins, the LEA proteins are not limited to the developing seed. Various members of this group have been shown to

accumulate in other plant tissues under conditions of water deficit, salt, cold, and osmotic stress; application of ABA to plants will also induce the proteins in the absence of environmental stress (Skriver and Mundy, 1990).

1.2.4. ABA May Induce LEA and Dehydrin Proteins during Seed Development

The role of the hormone abscisic acid (ABA) in plant growth and development is multifunctional; it is involved in stomatal function, seed development and germination, and in the plant's response to drought, salinity and cold stress. While the mode of action of ABA on these physiological and developmental processes is unclear, ABA is suggested to act by changing membrane function and altering gene expression (Skriver and Mundy, 1990). ABA levels change during seed development in cotton, oilseed rape, wheat, barley and castor bean (Finkelstein *et al.*, 1985; Quarrie *et al.*, 1988; Kermode, 1990). For example, during the development of castor bean seeds, ABA starts to accumulate at 25 DAP (early expansion stage of seed development) in the embryo and endosperm and reaches a peak around 40 DAP (late expansion stage in seed development). It then declines rapidly thereafter as the seed undergoes maturation drying and is barely detectable in the mature seed (60 DAP) (Kermode, 1990). In developing embryos, a programmed accumulation of ABA occurs prior to seed desiccation (Bewley and Black, 1985). Some results support the contention that an increase of ABA may elicit the production of LEA proteins (Willams and Tsang, 1991; Pla *et al.*, 1991; Dure, 1985). In maize, LEA 3 proteins accumulate during late embryogenesis (as embryos begin to dehydrate); the proteins are also induced in immature embryos by exogenous ABA (Thomann *et al.*, 1992). These proteins decline gradually in abundance during germination, but high levels accumulate when seedlings are grown

under conditions of water deficit. Similarly, Williams and Tsang (1994) found changes in transcripts encoded by the *Rab 17* gene in *Zea mays*. The mRNA of *Rab* 17 was first detected during mid embryogenesis and accumulated to high levels during late embryogenesis. In an ABA-deficit mutant, *Rab* 17 transcripts accumulated only to very low levels. Treatment with 1 μM ABA during early and mid embryogenesis, but not during late embryogenesis, enhanced the accumulation of RAB 17 mRNA in both the wild type and the ABA-deficit mutant.

The mechanism of ABA modulation of embryo-specific and LEA proteins is unknown, and it is still unclear whether the hormone directly or indirectly regulates these proteins in different plants. To discover the regulatory programs that control developmentally induced roles of ABA, Thomann *et al.* (1992) compared the accumulation of LEA proteins in wild type maize embryos and in mutants that are deficient either in ABA synthesis (*vp5* mutants) or ABA response in seeds (*vp1* mutants). After culture of the immature embryos (18 DAP) in 10 μM ABA for 5 days, the wildtype and *vp5* mutants accumulated LEA proteins which were not found in *vp1* mutants.

1.2.5. Development and Germination of Recalcitrant Seeds

The terms 'orthodox' and 'recalcitrant' have been used to describe the storage behavior of seeds. Orthodox seeds are shed from the parent plant at low moisture contents, having undergone maturation drying prior to this event, and can generally be further dried to moisture contents in the range of 1~5% without damage. In this dehydrated state, the seed can resist the vicissitudes of the environment, and unless dormant, will resume full metabolic activity, growth and development when conditions conducive to

germination are provided. Because of these properties, such seeds can be stored for long periods. Recalcitrant seeds, on the other hand, do not undergo maturation drying, and are shed at relatively high moisture contents. Such seeds are highly susceptible to desiccation injury, and thus are not storable under conditions suitable for orthodox seeds. Furthermore, most recalcitrant seeds are sensitive to chilling injury at lowered temperatures (Farrant *et al.*, 1988).

The changes that occur on dehydration of recalcitrant seeds of the mangrove, *Avicennia marina*, are similar to changes brought about by desiccation of orthodox seeds during the intolerant stage following germination. In fact, recalcitrant seeds may be desiccation intolerant because germination is initiated upon abscission, and attempting to store these seeds is akin to air-dry storage of germinated, orthodox seeds (Kermode, 1990). There is no clear-cut event delineating the end of seed development and the start of germination; thus it is difficult to separate these two phases. At all stages recalcitrant seeds appear to remain metabolically active.

Water deficit and high endogenous ABA levels are thought to be elicitors of dehydrin gene expression. The resultant dehydrins are hypothesized to function in the acquisition of desiccation tolerance. Dehydrins are not detectable during the late stages of development of recalcitrant *A. marina* seeds. It was suggested that the lack of desiccation tolerance of *A. marina* seeds might be related to the absence of desiccation-related dehydrins (Farrant *et al.*, 1992). However, these workers examined only heat-stable proteins and use of the dehydrin antibody would be necessary to confirm their results. Work with *Zizania palustris*, a minimally recalcitrant seed type, indicates that dehydrins can be detected by the maize dehydrin antiserum in both embryos and seedlings (Bradford and Chandler, 1992). The *Z. palustris*

embryos and seedlings are also capable of ABA accumulation during limited dehydration. The intolerance of *Z. palustris* seeds to dehydration at low temperature does not seem to be due to an absence of dehydrins or an inability to accumulate ABA (Bradford and Chandler, 1992). Finch-Savage *et al.* (1994) have detected dehydrins in mature seeds of many desiccation sensitive (recalcitrant) trees, and dehydrins can also be induced by exogenous ABA and limited drying treatments during seed development. Although recalcitrant seeds are shed at high water content and are desiccation-sensitive, the degree of water loss tolerated varies with the species. The mechanisms of desiccation tolerance are complex; the limited desiccation tolerance might partially depend on insufficient accumulation of dehydrins. Dehydrin gene expression in recalcitrant seeds needs to be investigated in a wider range of different recalcitrant seed types and this is one objective of the present thesis.

1.3. The Regulation of Dehydrin Gene Expression

1.3.1. Dehydrins are Encoded by Multiple Genes

Nucleic acid hybridization using DNA from wheat-barley addition lines and several different barley cultivars, and immunological data (Close and Chandler, 1990) suggest that the barley genome encodes at least 10 dehydrins. So far, three of seven Betzes barley chromosomes have been shown to carry dehydrin genes. At least two dehydrin genes (*dhn* 3 and *dhn* 4) have been mapped to the same point on the restriction fragment length polymorphism (RFLP) map of barley chromosome 6 (6I) (Heun *et al.*, 1991); one (*dhn* 6) has been placed accurately on the RFLP map of barley chromosome 4 (4I) (Close *et al.*, 1993b) and at least two (*dhn* 1 and *dhn* 2) have been mapped to an

undetermined position on barley chromosome 7 (5I) (Close and Chandler, 1990). The dehydrin locus on chromosome 7 may be a major cold-tolerance locus in barley (Blake *et al.*, 1993). In maize, only one dehydrin locus (UMC 170) has been identified so far, and it is on the long arm of chromosome 6 (Close *et al.*, 1989). However, the exact number of dehydrin genes at this locus and in the maize genome are not yet known. Hybridization studies have indicated that there may be at least two *dhn* genes in maize (Vilardell *et al.*, 1990). The different dehydrin genes may respond differentially to different stresses and these responses may also be tissue-specific.

1.3.2. Regulation of Dehydrins by ABA and Water-Deficit-Related Stresses

Dehydrins are a subset of LEA proteins whose expression is not confined to developing seeds but is also induced in seedlings or plant vegetative tissues exposed to mild water-deficit-related stresses. During a period of mild drought stress, a plant undergoes a number of physiological and metabolic changes together with an increase in the biosynthesis of the plant hormone ABA. Since ABA levels increase as plants are exposed to water stress, it has been proposed that the changes in gene expression which occur in a water-stressed plant (including dehydrin expression) are mediated by the elevated levels of endogenous ABA (Mundy and Chua, 1988; Close *et al.*, 1989; Claes *et al.*, 1990; Curry *et al.*, 1991; Plant *et al.*, 1991; Cohen *et al.*, 1991; Robertson and Chandler, 1992; Chandler *et al.*, 1993). Besides water stress, Plant *et al.* (1991) found that other water-deficit-related stresses (including PEG, salt, cold) also elevate ABA levels. Further evidence for the role of ABA in dehydrin gene expression in vegetative tissues has come from studies on ABA-deficient mutants of maize (Pla *et al.*, 1989) and tomato (Cohen and Bray, 1990). When exposed to

dehydration stress, these mutants fail to elevate ABA levels, and show a corresponding inability to express dehydrins.

Yamaguchi-Shinozaki and Shinozaki (1994) analyzed the expression of two genes in *Arabidopsis thaliana* (*rd*29A and *rd*29B) under ABA and some stress treatments. The treatments applied were: dehydration (60% relative humidity), ABA (100 μM), NaCl (250 mM) and cold (4°C). The two genes, *rd*29A and *rd*29B, which are closely located on the *Arabidopsis* genome, were differentially induced by the above treatments. The transcript of the *rd*29A gene is induced within 20 min after dehydration began and was strongly expressed after 2 h; it was induced within 5 h by cold temperature and was detectable for at least 24 h. Under high salt conditions, *rd*29A mRNA was detected after 1 h and reached its maximum at 2 h. In contrast, the *rd*29B mRNA took longer to accumulate to detectable levels after all three stress treatments. Deletion analysis of the 5' upstream regions of the two genes showed that *rd*29A has at least two *cis*-acting elements: one was responsive to salt and showed ABA-dependent regulation and the other was induced only by changes in osmotic potential (drying and cold treatments). In contrast, the analysis showed that *rd*29B contains at least one *cis*-acting element which is involved in ABA-responsive, slow induction. Thus, different stresses may trigger different signal transduction pathways (affecting one gene) and the same stress often has different effects on different genes.

1.3.2.1. ABA-Regulated Genes and Cold Tolerance

An early study that indicated a role for ABA in cold acclimation investigated two potato species, *Solanum tuberosum*, whose leaves failed to cold acclimate, and *S. commersonii*, whose leaves hardened from a lethal

temperature of -5°C to -12°C and survived over a 15-day period at 2°C (Chen *et al.*, 1983). During hardening of leaves, the ABA content of *S. commersonii* showed a transient rise (by approximately 2.5-fold). Application of ABA to *S. commersonii* plants growing either at 25°C or at 2°C resulted in cold acclimation even at the higher growth temperature.

Recently, the expression of cold- and ABA-regulated genes has been associated with cold acclimation in both wild-type and mutant *A. thaliana* plants. Two types of mutants isolated by Koornneef and colleagues (Koornneef *et al.*, 1982; Koornneef *et al.*, 1984) have been used to study the role of ABA in cold acclimation and desiccation tolerance: (a) *aba* (ABA deficient) mutants are impaired in the epoxidation reaction converting zeaxanthin to antheraxanthin and most likely in the subsequent step leading to violaxanthin (Duckham *et al.*, 1991; Rock and Zeevaart, 1991) and (b) *abi* (ABA insensitive), denoting mutants at any of three different loci (*abi*1, *abi*2, *abi*3); the *abi*3 locus is active only in seeds. Phenotypically, no alteration of ABA response is detected in the vegetative tissues of *abi3* mutant plants. The *abi*3 gene is transiently expressed after seed germination (in young seedlings), but this expression in strictly confined to the organs of embryonic origin (reviewed in Giraudat *et al.*, 1994). The *abi*1 and *abi*2 mutants are defective in numerous ABA-responses during vegetative growth. The *abi*1 gene regulates stomatal aperture in leaves and mitotic activity in root meristems (Leung *et al.*, 1994; Meyer *et al.*, 1994). The*aba* mutants are impaired in cold acclimation; neither the *abi*1 nor *abi*2 mutants are altered this process. The *abi*1 and *abi*2 mutants differentially affect the ABA-dependent morphological and molecular responses of *Arabidopsis* plants to progressive drought stress.

Wild-type *A. thaliana* cold hardens at 4°C so that its freezing resistance increases from approximately -3°C to approximately -8°C (Gilmour *et al.*, 1988;

Lang *et al.*, 1989). Treatment of *A. thaliana* plants with ABA results in cold hardening occurring at 20°C. An alternative assay for freezing tolerance, leaf electrolyte leakage, showed a similar range over which cold tolerance increases in response to ABA. The *aba*-1 mutant showed no cold acclimation nor inducible cold acclimation related to the wild-type (Chandler and Robertson, 1994). Therefore it can be concluded that the low levels of ABA in the mutant plants result in an impaired ability or failure to develop cold tolerance, which can be corrected by applied ABA. It is not presently clear to what extent the poor cold adaptation is a direct result of the ABA deficiency; pleiotropic effects of the ABA deficiency might also be involved (Gilmour and Thomashow, 1991). The *abi* mutants (*abi*1, *abi*2) show cold acclimation at 4°C similar to the wild-type (Leung *et al.*, 1994; Meyer *et al.*, 1994). Thus ABA responses may be necessary for the development of cold tolerance.

Using a low-temperature-induced (*lti*) cDNA clone as a probe, Nordin *et al* (1993) isolated a genomic fragment that carries two closely located *lti* genes of *A. thaliana*. The nucleotide sequences of the two genes, *lti* 78 and *lti* 65, predict novel hydrophilic polypeptides with molecular weights of 78 KD and 65 KD respectively. Around half of the amino acids are identical between the two polypeptides. Both *lti* 78 and *lti* 65 are induced by low temperature, exogenous abscisic acid and drought, but the responsiveness of the genes to these stimuli is markedly different. Expression of *lti* 78 is mainly responsive to low temperature, while that of *lti* 65 is most responsive to drought and ABA. The induction of *lti* 78 follows separate signal pathways during low-temperature, ABA and drought treatment, whereas the drought induction of *lti* 65 is ABA-dependent and the low-temperature induction appears to be coupled to the ABA biosynthetic pathway. This differential expression of the two related genes may indicate that they have somewhat different roles in the stress

response. Similar to the *lti78/lti* 65 gene pair, four of the cold regulated genes that have been isolated from *A. thaliana* are members of gene pairs: *kin*1/*kin*2, *cor*15a/*cor*15b. In each case, the members of the gene pair have a high degree of nucleic acid sequence identity and are physically linked in tandem array. The individual members of each gene pair display differences in regulation (Lin and Thomashow, 1992, Wilhelm and Thomashow, 1993). Many low-temperature-induced genes have been studied (Mäntylä *et al.*, 1995; Takahashi *et al.*, 1994; Berkel *et al.*, 1994; Welin *et al.*, 1994), and expression of these *lti* genes can be ABA dependent or ABA independent. One environmental cue, such as cold may not be sufficient for developing full freezing tolerance (Mäntylä *et al.*, 1995); a shorter treatment time may also be a problem since only low amounts of dehydrins are induced (Muthalif and Rowland, 1994). Treatments by ABA, dehydration and low-temperature increase the freezing tolerance of plants, suggesting overlapping responses to these environmental cues (Mäntylä *et al.*, 1995). A general response in stressed plant cells, then, is the accumulation of dehydrin proteins. Although different dehydrin polypeptides may be differently induced in response to different cues, all may have a unique function, i.e., to protect plant cells under environmental stress.

1.3.2.2. ABA-Regulated Genes and Desiccation Tolerance

The same set of *A. thaliana* mutations is of value for elucidating the roles played by ABA during seed development, particularly in the induction of desiccation tolerance. For these studies, double mutants were constructed (involving the *aba* and *abi3* loci), since the combination of mutations affecting both ABA levels and ABA sensitivity generates a more extreme ABA null phenotype than either mutation alone. The *aba/abi3* seeds that develop on *aba/abi3* plants lack desiccation tolerance (<10% of the seeds are desiccation

tolerant). However, application of ABA confers desiccation tolerance on up to 60% of the seeds. SDS-PAGE indicate that the *aba/abi3* double mutant fails to accumulate the 12S and 2S storage proteins, in contrast to *aba* or *abi3* single mutants (Koornneef *et al.*, 1989). More importantly, the synthesis of a set of heat-stable polypeptides, expected to include LEA-type proteins, is absent specifically from the *aba/abi3* double mutant, but is restored by treatment with an ABA analog, as is desiccation tolerance (Meurs *et al.*, 1992).

1.3.2.3. ABA-Regulated Genes and Salt Stress

In response to exogenous ABA and salt stress, plant cells start to synthesize dehydrins which are suggested to protect plant tissues from damage caused by water loss. Gulick and Dvorak (1992) analyzed the transcription of 11 genes whose expression is enhanced in roots of *Lophopyrum elongatum* after treatment with 250 mM NaCl. All 11 genes are induced within 2 h after exposure to NaCl solution and reach peak expression after 6 h. Timing of the decline of gene expression distinguishes two groups, one in which mRNA concentrations return to basal levels by 24 h and the other in which the decline occurs between 3 and 7 days. Only one of the 11 genes was found to be homologous to LEA genes responsive to ABA.

Similarly, Bostock and Quatrano (1992) analyzed the Em mRNAs of ABA- and NaCl-treated cells from rice suspension cultures. Their results demonstrate that NaCl and ABA are strongly synergistic in controlling Em mRNA abundance; NaCl operates not only through changes in ABA levels but also through an independent pathway that changes the sensitivity of rice cells to ABA.

1.3.3. Mechanism of ABA-Regulated Gene Expression

Recent interest in understanding the molecular basis of gene regulation by ABA has focused on transcriptional activation and a general model in which a *trans*-acting protein factor interacts with an element in the promoter region of a gene. There are three essential steps in this analysis (1) identification of an ABA-responsive *cis*-acting element within the promoter of an ABA-regulated gene, (2) characterization of the *trans*-acting factor that interacts with the defined element, and (3) functional analysis of the factor and the element to test whether ABA-regulated gene expression is conferred by their interaction *in vivo*. A *cis*-acting ABA-response element (ABRE) is one of the sequence motifs conserved between several ABA-regulated genes. The ABRE is a minimum 11-bp sequence, and is sufficient to confer ABA-responsiveness to a reporter gene when tested in transient expression assay systems (Guiltinan *et al.*, 1990; Skriver *et al.*, 1991). Recently, Pla *et al.* (1993) identified an ABRE from the promoter region (-194 to -60) of a maize gene *rab* 28. The ABRE contains a conserved 8-bp sequence CCACGTGG (called the G-box) which has been reported in other plant genes as the element conferring responsiveness to ABA (Quatrano *et al.*, 1992). A transient expression system shows that a 134-bp fragment of the *rab* 28 gene (-194 to -60; containing the G-box) fused to a truncated cauliflower mosaic virus (35S) promoter is sufficient to confer ABA-responsiveness upon the GUS reporter gene (Pla *et al.*, 1993). *Trans*-acting factors are nuclear proteins that bind to *cis*-acting regulatory elements in DNA. Several nuclear proteins specifically bind to the ABRE. One such protein, EmBP-1, cloned from wheat (Guiltinan *et al.*, 1990), was found to interact specifically with an eight base sequence within the ABRE containing a G-box core. The functional analysis shows that the factor acts as a

transcriptional activator regulated by ABA and water stress. *In vitro* footprinting and gel retardation experiments have also demonstrated nuclear protein(s) binding to motifs of the rice *rab* 16A gene promoter (Mundy *et al.*, 1990) and the maize *rab* 28 gene (Pla *et al.*, 1993). The nuclear proteins which bind to the ABRE of the maize *rab* 28 gene are stable in the presence of detergent and high salt (Pla *et al.*, 1993).

As the number of characterized regulatory sequences in plant genes has increased it has become clear that G-box like motifs are associated with many genes that are regulated by diverse factors (Chandler and Robertson, 1994). In tobacco, small differences in *cis*-acting sequences containing a G-box core result in different temporal and spatial promoter activity patterns (Salinas *et al.*, 1992).

More information on the structure of dehydrin genes comes from the research on a barley *dhn* 7 gene. Robertson and co-workers analyzed the *dhn* 7 sequence (2138 bp) and its deduced amino acid sequence. The sequence of 2138 bases includes 935 bp of 5'-flanking sequence, 72 bp of 5' untranslated region, two exons of 198 and 216 bp separated by a 99 bp intron, 190 bp of 3' untranslated region and 428 bp of 3'-flanking sequence (Robertson, personal communication). A putative TATA box (TATAAA) is located at position -47, and a putative poly (A) addition signal (AATAAA) is located at position +751. In order to test the ABA-responsiveness of the promoter, chimeric gene constructs were made between the 5'-flanking sequence of the *dhn* 7 gene (-935 to +44) and a reporter gene (GUS) (Robertson, personal communication). To test possible involvement of this sequence in ABA-regulated gene expression, the dehydrin promoter was deleted from the 5'-end towards the TATA box, and the ABA responsiveness of the 5'-deletion series constructs was analyzed. The results indicate that the region between -285 and -211 is responsible for

ABA-regulated expression of the *dhn* 7 gene.

A sequence characteristic of *cis*-acting regulatory elements responsible for conferring ABA-responsiveness in a number of ABA-regulated genes is found in the 5'-flanking region of *dhn* 7 between position -225 and -200. The sequence is composed of a G-box-like motif, TACGTGGcgcccagacgaGGCCGCG, followed by a GC-rich sequence.

1.3.4. ABA-GA Interaction

Many members of the dehydrin gene family are induced, albeit to different degrees, as part of a dehydration stress response which also includes elevated levels of endogenous ABA (Close *et al.*, 1989). The absence of dehydrins in non-stressed plants is likely due to insufficient levels of inducing signals, such as ABA. In addition, antagonizing factors, such as GA may also play an important role in regulating the expression of dehydrin genes. The balance of promotive (ABA) and inhibitory (GA) factors may well govern whether expression *in vivo* will occur.

In many cases, ABA antagonizes GA-induced gene expression. The most extensively studied interaction between ABA and GA is in respect to alpha-amylase expression in the cereal aleurone layer. Aleurone cells and protoplasts synthesize alpha-amylase in response to GA and this synthesis is accompanied by an increase in the steady-state levels of alpha-amylase mRNA. Run-on transcriptions with nuclei isolated from barley and oat aleurone protoplasts have demonstrated that the GA-induced increase in alpha-amylase mRNA is due primarily to increased transcription of alpha-amylase genes which can be overcome by ABA (reviewed in Hooley, 1994). Promoter analysis of the wheat low pI alpha-amylase gene revealed that elements involved in

directing GA- and ABA-regulated expression lie within 289 bp upstream of the start of transcription (Huttly, *et al.*, 1989). Similar analysis of a barley high pI *Amy* gene promoter showed that the main elements important for GA and ABA regulation lie between 174 and 41 bp upstream from the start of transcription (Jacobsen and Close, 1991).

It remains to be seen whether ABA and GA interact with the same *cis*-acting element in the promoters of dehydrin genes. A number of experiments were performed to elucidate the interaction between ABA and GA on dehydrin gene expression (Robertson, personal communication). When ABA is added to barley aleurone protoplasts, high levels of dehydrin mRNAs accumulate within 24h. When protoplasts are incubated with both ABA and GA, the accumulation of dehydrin mRNAs is reduced. The simplest interpretation of these observations is that GA antagonizes the effect of ABA on transcription of the dehydrin gene. But when this hypothesis was tested by examination of the effects of GA on the expression of the *dhn* 7 (-935 to +44)-GUS construct in aleurone protoplasts, no reduction in the level of ABA-induced GUS activity was observed. Robertson and co-workers suggest that the antagonistic effect of GA on ABA-induced dehydrin gene expression could potentially occur via a down-regulating element further upstream than the -935 in the promoter. A longer promoter fragment (+44 to -2600) of the *dhn* 7 gene was tested for ABA and GA interaction. This longer fragment of the *dhn* 7 promoter was responsive to ABA, leading to slightly higher GUS activity levels than the +44 to -935 promoter fragment, but the increase in transcription was similarly not reduced by GA.

The contrasting results from the above experiments show that GA may affect a *cis*-acting element which is located in the 3'-downstream region of the *dhn* 7 gene and replaced by the GUS reporter gene and nopaline synthase 3'

end when the chimeric construct is made. Alternatively, the *cis*-acting element may be even further upstream than -2600. The interaction between GA and ABA on dehydrin gene expression is a very interesting topic. To understand more about GA-regulated dehydrin gene expression, we clearly need to know more about the location of *cis*-acting elements in dehydrin genes, and the effects of GA on these *cis*-acting elements. We also need to elucidate the level of influence of GA on ABA and the effect of GA on other stress responses in plants.

1.4. Structure and Functions of Dehydrins

1.4.1. The Common Motif of Dehydrin Amino Acid Sequences

The primary amino acid sequences of over 30 dehydrins have been deduced in various laboratories, principally from nucleotide sequences of cDNA and genomic clones. Dehydrins are characterized by a consensus 15-amino acid sequence domain EKKGIMDKIKEKLPG that is always present at or near the carboxyl terminus (with the only exception being cotton D-11), and repeated upstream of the terminus once or more (Close *et al.*, 1993b). Among this domain, lysine-rich boxes (KIKEKLPG) are the most highly conserved regions (Robertson and Chandler, 1992). Many dehydrins contain a stretch of serine residues adjacent to the consensus domain. These serine residues are postulated to be possible phosphorylation sites (Vilardell *et al.*, 1990).

A schematic illustration of the positions and repetition of the consensus regions discussed above is shown in Figure 1.2. An alignment of the conserved carboxyl-terminal amino acid sequences of numerous dehydrins from monocots and dicots is shown in Table 1. The table also notes that dehydrin gene induction occurs in response to any type of environmental stress or

developmental change that has a dehydration component, or in response to ABA.

The primary structure shows dehydrins are lysine-rich, glycine-rich, and free of the amino acids tryptophan and cysteine. Dehydrin proteins remain stable at 100°C (Close *et al.*, 1989). The high temperature solubility of dehydrins and their low hydropathy scores have led to the general view that dehydrins are hydrophilic. Structure prediction algorithms predicted from primary amino acid sequences showed that a portion of the consensus polypeptide forms an amphiphilic alpha-helix (Dure, 1989).

Besides the most fundamental lysine-rich amino acid consensus sequence EKKGIMDKKIKEKLPG, other features of dehydrins are frequently observed, such as: (a) a run of serine residues; (b) repeating GT residues; (c) a DEYGNP consensus sequence upstream of the N-terminal-most lysine-rich motif; (d) glycine-rich tandem repeats to the amino side of the DEYGNP consensus. But , since these features are not always present, they cannot be the basis of fundamental properties shared by all dehydrins. The strict conservation of amino acids in the lysine-rich motif implies conservation of some higher order structure, presumably an amphiphilic alpha-helix, which may be the key to dehydrin function (Close *et al.*, 1993b).

1.4.2. A Repeating 11-mer Amino Acid Motif: An Example of Dehydrin 3-D Structure

Dehydrins contain a funtional amphiphilic alpha-helix. The relationship between primary amino acid sequences and their specific alpha-helix 3-D structure have been studied in detail in a LEA protein (D-7) which contains a randomly repeated 11-mer amino acid motif. Members of this

Figure 1.2. Common features of plant dehydrins (LEA D-11 family). The boxed areas indicate protein-coding regions, and the shading indicates regions of similarity for barley dehydrins DHN1, DHN4, DHN5, maize dehydrin DHN1 (RAB17) and cotton dehydrin D147b. The darker the shading, the greater is the similarity. The Lys-rich consensus sequence (K) occurs at the end of each dehydrin, and in most cases at one additional position adjacent to a block of Ser (S). There are examples, such as barley DHN5, where the Ser block is not present or there are more than two occurrences of the Lys-rich consensus block. Lesser conserved tandem repeating units are numbered. (From Close *et al.*, 1993b).

Barley DHN1
14.2 kD

Barley DHN4
22.6 kD

Barley DHN5
58.6 kD

Maize DHN1
17.0 kD

Cotton D147b
14.7 kD

Table I. *Conserved Amino Acid Sequences Near the Carboxy Terminus of Monocot and Dicot Dehydrins (LEA D-11 family)*

Sequence	Plant	Gene	Acc #	Induction[a]
EKKGVMDKIKEKLPG	*H. vulgare*	*dhn1*	P12951	SD
EKKGVMDKIKEKLPG	*H. vulgare*	*dhn2*	P12952	SD
EKKGIMDKIKEKLPG	*H. vulgare*	*dhn3*	P12948	SD
EKKGIMDKIKEKLPG	*H. vulgare*	*dhn4*	P12949	SD
EKKSLMDKIKDKLPG	*H. vulgare*	*dhn5*	M95810	LIB
EKKGVMDKIKEKLPG	*H. vulgare*	*dhn7*	X71362	LIB
EKKSLMDKIKDKLPG	*T. aestivum*	*wcs120*	M93342	COLD
EKKGIMDKIKEKLPG	*T. aestivum*	*rab15b*	X62476	SD
EKKGIMDKIKEKLPG	*T. aestivum*	*rab15*	X59133	SD
EKKGIMDKIKEKLPG	*Z. mays*	*dhn1*	P12950	SD
EKKGIMDKIKEKLPG	*Z. mays*	*rab17*	X15994	ED
EKKGIMDKIKEKLPG	*O. sativa*	*rab16A*	Y00842	ABA
EKKGFMDKIKEKLPG	*O. sativa*	*rab16B*	P22911	LIB
EKKGFMDKIKEKLPG	*O. sativa*	*rab16C*	P22912	LIB
EKKGIVDKIKEKLPG	*O. sativa*	*rab16D*	P22913	LIB
EKKGIVEKIKEKLPG	*O. sativa*	*rab25*	X57327	ABA
EKKGMMDKIKDKIPG	*L. esculentum*	*le4*		ABA
EKKGMMDKIKDKIPG	*L. esculentum*	*tas14*	X51094	SALT
EKKGFLDKIKEKLPG	*L. chilense*	*plc3015*	M97211	ABA
EKKGVMEKIKEKLPG	*C. plantagineum*	*dsp14*	P22238	LD
EKKGIMDKIKEKLPG	*C. plantagineum*	*dsp16*	P22239	LD
EKKGFLEKIKDKIPG	*S. oleracea*	*cap85*	M96259	COLD
EKKGILEKIKEKLPG	*R. sativus*	*lea2*	X56280	ED
EKKGILEKIKEKLPG	*A. thaliana*	*cor47*	X59814	COLD
EKKGMMEKIKEKLPG	*A. thaliana*	*xero1*	P25863	LIB
EKKGMMDKIKEKLPG	*A. thaliana*	*rab18*	X68042	COLD
EKKGVMDKIKEKLPG	*G. hirsutum*	*d147b*	M81655	COTY
EKKGIMDKIKEKLPG	*P. sativum*	*dhn1*	P28639	COTY
EKKGIMDKIKEKLPG	*P. sativum*	*dhn2*	P28640	COTY
EKKGIMEKIKEKLPG	*P. sativum*	*dhn3*	P28641	COTY
EKKGILEKIKEKLPG	*P. sativum*	*dhn-cog*	Z14145	COTY
EKKGIMEKIKEKIPG	*M. sativa*	*cas18*	L07516	COLD
EKKGIMEKIKEKLPG	*L. gibba*	*npr1*	X64327	DARK

[a]First inducing conditions observed: SD, seedling desiccation; LIB, screened genomic library with existing clone; COLD, low temperature; ED, embryo desiccation; ABA, abscisic acid; SALT, salinity; LD, leaf desiccation; COTY, cotyledon development; DARK, darkness. (From Close *et al.*, 1993b).

family have been found in barley, wheat, *Brassica napus*, carrot and cotton (Dure, 1993c). This motif is characterized by apolar residues in positions 1, 2, 5 and 9; positively charged residues in positions 6 and 8; and negatively charged or amide residues in positions 3, 7 and 11 of the 11-mer repeat. The number of repeats among these proteins ranges from 5 in the smallest protein (cotton) to 13 in the largest protein (*Brassica*). In all but cotton, the tandem arrangement of the 11-mer is interrupted once by a stretch of 11 amino acids that does not show the 11-mer motif. In the barley and wheat proteins, other interruptions also occur. These interruptions may represent "hinge" regions in the predicted 3-D structure formed by the 11-mer repeat motif. These repeating elements most likely exist as alpha-helices. Such helices would be amphiphilic in that positions 1, 2, 5 and 9 would provide a hydrophobic stripe along the helix surface and the polar residues of positions 3, 6, 7, 8 and 11 would present a wider hydrophilic stripe. Figure 1.3 gives the consensus sequence for the 11-mer unit. An intramolecular salt bridge is possible between #8 Lys and #11 Glu and an intramolecular charge-dipole interaction between #3 and #6 Lys. Although evidence for such structures has yet to be accumulated, molecular modeling of the D-7 family consensus sequences in the 11 residues helical unit has been initiated and produced the structures illustrated in Figure 1.4a and b (Dure, 1993c). Figure 1.4a is a view from the N-terminus down the helix axis of a 33-mer peptide composed of three 11-mer consensus repeats. The molecule is tilted slightly to the left to give an oblique view of the apolar surface. This surface is composed entirely of the methyl groups of alanine and threonine residues in positions 1, 2, 5 and 9 of the repeating units.

The lysine residues of position 8 are seen to protrude from the top of the molecule and the lysine residues of position 6 from bottom (Fig. 4a). The surface of the left side of the molecule is seen to be composed of the glutamine

and glutamic acid residues of the repeating units.

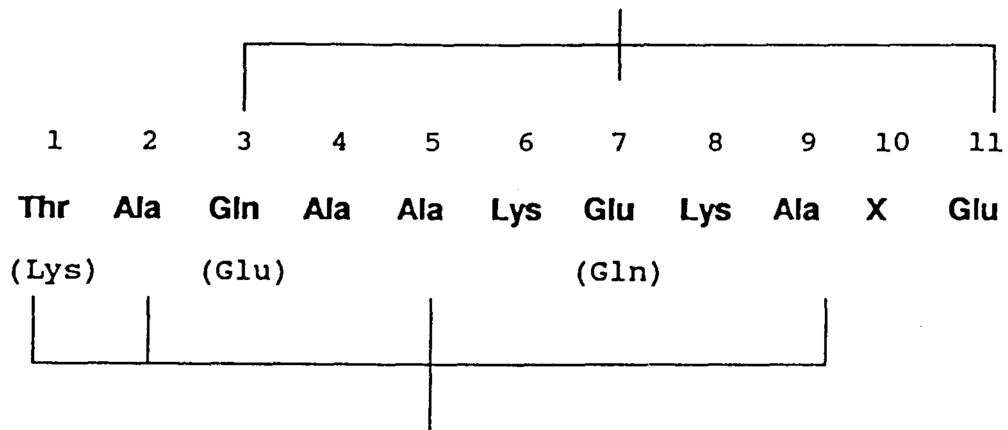
In Figure 1.4b, two of these 33-mer peptides have been placed in parallel, in-register configuration with their hydrophobic surfaces juxtaposed. The view is from the N-terminus of the helix dimer. The potential for tight packing of the alanine and threonine residues of positions 1, 2, 5 and 9 is apparent.

1.4.3. Function of Proteins Composed of the 11-mer Repeating Motif

Although there are no obvious indications of the role played by the repeating motif (or of dehydrins in general) in protecting the plant during water stress or allowing the seed/plant to survive severe desiccation, some of the problems faced by these tissues in these situations can be imagined. One such problem would be that of dealing with the extremely high ionic environment in the dehydrating cell.

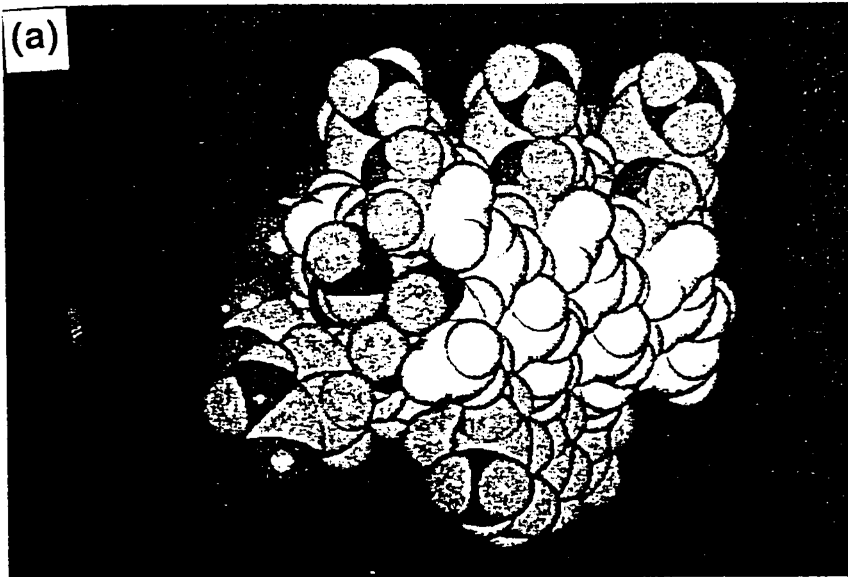
A dimer shown in the configuration of Figure 1.4b shows the lysine residues of positions 6 and 8 and the negative or amide residues of positions 3, 7 and 11 comprising the solvent-exposed surface (Dure, 1993c). As mentioned previously, lysine 8, glycine 3 and glutamine 7 and 11 can be involved in intramolecular electrostatic interactions. In high salt environments these internal interactions could be replaced by the formation of amino acid salts. In view of the large number of charged residues in the 11-mer repeats, the function of these molecules may be simply that of an ion carrier whose existence forestalls the precipitation/ crystallization of ions inside the cells of tissues that are destined to dry up to approximately 5% water content on a fresh weight basis. The percentage of charged residues contained in these proteins ranges from 26 to 39%. This percentage in the 11-mer repeat domains of these proteins ranges from 33 to 43% (Dure, 1993c). When it is recognized that 4 of the 11 positions of the repeating domain are occupied by apolar residues to

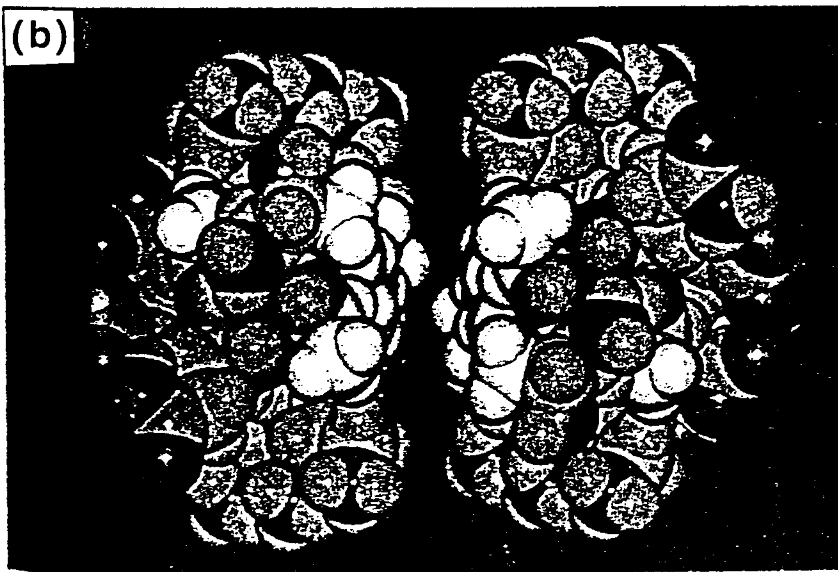
Figure 1.3. Consensus sequence of 11-mer repeating element showing amino acids comprising hydrophilic and hydrophobic surfaces of putative alpha-helix (From Dure, 1993c).



Backside of helix
1 2 3 4 5 6 7 8 9 10 11
Thr Ala Gln Ala Ala Lys Glu Lys Ala X Glu
(Lys) (Glu) (Gln)
Apolar surface of helix

Figure 1.4 (a) A slightly oblique view of LEA D-7 from the N-terminus down the long axis of an helix composed of three 11-mer consensus repeats (Figure 1.3). (b) Two of the molecules of (a) are juxtaposed along their apolar faces. The view is from the N-terminus, the molecules are parallel and in-phase (From Dure, 1993c).


(a)


(b)

form the hydrophobic stripe, the extremely high charge density of the solvent face of these domains becomes even more apparent.

Of particular interest is the pocket formed by the threonine 1 and lysine 8 of one strand and lysine 6 of the second strand when two monomers bind along their hydrophobic faces to dimerize parallel and in phase (Dure, 1993c). Such a pocket readily accommodates divalent anions such as phosphate by the formation of two salt bridges and a dipole-dipole interaction between the phosphate and threonine hydroxyls. Glutamic acid 7 and 11 are available to bind the phosphate counterions. In such a scheme one molecule of K_2HPO_4 could be bound for each 11-mer repeating unit (Dure, 1993c). Such an ion carrier role would require that the protein molecules be in abundance if the capacity for ion sequestration is to be meaningful in the cell. In mature cotton embryo cells, D7 has been found to represent about 4% of the non-organellar cytosolic protein (approximately 0.34 mM). This would allow for the sequestration of over 1.5 mM phosphate salts by the repeating 11-mer domain of D7 molecules alone (Dure, 1993c).

By immunochemical methods, the levels and cell type distribution of D7 and D113 have been determined in mature cotton embryos (Roberts *et al.*, 1993). D7 and D113 proteins accumulate to $\sim 10^9$ and $\sim 1.3 \times 10^9$ molecules per average cell respectively, both proteins appear to be evenly present in the cytosol of all cell types present in the embryo, including cotyledon and axis epidermal cells. The very high concentration of the two proteins and the uniformity of their distribution in tissues, is suggestive of their unique ion trap function.

1.5. Summary

Dehydrins are a group of proteins which share a number of common characteristics. For example, most contain a consensus amino acid sequence that usually repeats several times. The conserved primary structure motif predicts an amphiphilic alpha-helix in the 3-D structure of the protein, which may function as an ion carrier when cellular ion concentrations increase as a result of dehydration. Dehydrins are synthesized during seed development and are present in the mature dry seeds of many orthodox plants, and their mRNAs are induced in vegetative tissues when the plants are subjected to environmental stresses - namely water-deficit-related stresses (e. g. drought, high salt, and cold). However, little is known about how dehydrin proteins are regulated by water-deficit related stresses or by endogenous ABA (*in situ*) and whether this hormone serves as a primary trigger to elicit their production.

Recalcitrant seeds can not be stored under normal (dry and cold) conditions in contrast to orthodox seeds. They have to be stored under high moisture conditions. Their water content is high at maturity, and they are desiccation sensitive, but the underlying bases for this are not understood. Some researchers have reported that recalcitrant seeds do not tolerate desiccation because of an absence of dehydrins in the seeds. Others report that dehydrins are found in recalcitrant species. An insufficient accumulation of dehydrins in recalcitrant seeds might be the major problem resulting in desiccation sensitivity in these species, and one of the objectives of my research is to clarify this. Part of my analyses examines the expression pattern of dehydrins regulated by exogenous ABA and water-deficit-related stresses in developing seeds and seedlings of recalcitrant species.

1.6. Research Objectives

This study will determine the accumulation patterns of dehydrin gene products during castor bean seed development (an orthodox species). The effects of some water-deficit related stresses and the plant hormones ABA and GA on dehydrin gene expression will also be analyzed in different organs/tissues of castor bean seeds and seedlings. Furthermore, as part of my comparative analysis of orthodox vs. recalcitrant seeds, dehydrin gene expression will be examined in *Castanospermun australe* and *Artocarpus heterophyllus* (recalcitrant species) which differ in their degree of desiccation intolerance. Most of my analyses are at the protein level using Western blot analysis. The fate of the dehydrins during normal development and germination and following premature desiccation and subsequent rehydration will also be studied. This research will attempt to answer the following questions.

Question 1. What is the pattern of accumulation of the dehydrins during seed development and during germination and seedling growth? Are dehydrins stable or do they turn over rapidly during rehydration following premature drying or normal maturation drying?

Question 2. Do different stresses, such as water stress, desiccation, cold (4°C), high-salt (0.2M NaCl), high-osmolarity (0.3M mannitol), and exogenous ABA induce the same type of dehydrins or do different stresses cause a differential induction of dehydrins? Is dehydrin production organ/tissue-specific and developmentally regulated?

Question 3. Does a high concentration of ABA (i.e. 10^{-3} M) reduce dehydrin production by suppressing some cellular functions? How does GA affect ABA- and stress-induced dehydrin production?

Question 4. Are dehydrins present in the two recalcitrant species? If dehydrins are present, are there differences in dehydrin production between the orthodox seeds, *Ricinus communis* L. (castor bean), and the recalcitrant seeds (*Castanospermum australe* and *Artocarpus heterophyllus*)?

Chapter 2.

An Analysis of Late Embryogenesis Abundant (LEA) messenger RNAs and Dehydrin Proteins in Castor Bean Seeds: Fate during Development, Germination, and Premature Desiccation

2.1. Introduction

In most dicot and monocot plant species, seed development can be divided into three stages. Initially, during histodifferentiation, the single-celled zygote undergoes extensive mitotic division, and the resultant cells differentiate to form the embryo composed of the axis and cotyledons, and the triploid endosperm. Later, the seed expansion stage is characterized by cell expansion and deposition of reserves. Finally, maturation is terminated by some degree of drying, which results in a gradual reduction in metabolism as water is lost from the seed tissues and the embryo passes into a metabolically inactive, or quiescent state (reviewed in Kermode, 1990, 1995). Key metabolic events associated with these distinct stages of seed development are reflected by changes in the mRNA subsets prevalent within the developing seed. In particular, storage protein synthesis which is prevalent during the expansion stage, declines dramatically during desiccation and does not occur during subsequent germination. Residual mRNAs for storage proteins present in the dry seed are degraded upon subsequent imbibition (Dure, 1985; Bewley and Marcus, 1990; Kermode, 1990). In contrast, there are other mRNA classes which are up-regulated during late maturation and desiccation. Included among these are mRNAs encoding a highly abundant set of hydrophilic proteins exhibiting temporal regulation, i.e., the late embryogenesis abundant [LEA] polypeptides, first described in cotton. The genes encoding these proteins are expressed as highly coordinately regulated sets, which on this basis, comprise two distinct classes in cotton (Galau *et al.*, 1987; Galau and Hughes, 1987; Hughes and Galau, 1989). One class, which is made up of six different *Lea* transcripts, is first detected soon after histodifferentiation is completed and exhibits two transient peaks, before reaching a maximum level 3 days prior to

desiccation. The transcript level of the second class of 12 *Lea* mRNAs increases sharply during late maturation and peaks just prior to, or at desiccation. LEA protein synthesis constitutes a large proportion of the translational activity of the cotton embryo during late maturation (up to 25%), regulated at the level of transcription, i.e., by the abundance of *Lea* mRNAs (Hughes and Galau, 1987). In the mature cotton embryos they comprise about 2% of the total soluble protein (Dure, 1993a) or about 30% of the non storage protein moiety (Hughes and Galau, 1987).

Although seeds of orthodox plant species are not capable of withstanding desiccation at all stages during their development, their acquisition of tolerance is usually substantially earlier than the onset of the natural drying event itself. The pattern of accumulation of some of the *Lea* mRNA classes during seed development (i.e., primarily commencing around mid-development and highest at incipient desiccation), widespread occurrence, highly conserved sequence motifs as well as their general physical characteristics (e.g., extreme hydrophilicity, resistance to denaturation) (Pages *et al.*, 1993; Gomez *et al.*, 1988; Mundy and Chua, 1988; Close *et al.*, 1989) have led to the suggestion that LEA polypeptides play a decisive role in the acquisition of desiccation tolerance (reviewed in Dure, 1993a, 1993b, 1993c; Close et al, 1993b). Transcription of their genes can be elicited precociously in early maturation-stage cotton embryos following a premature desiccation treatment (Galau *et al.*, 1991). However, the basis of desiccation tolerance in seeds is likely not restricted to the production of specific polypeptides (reviewed in Bewley and Oliver, 1992).

As noted earlier, only some classes of *Lea* mRNAs are associated with the late developmental stages (Hughes and Galau, 1989). The name "dehydrins" (Close *et al.*, 1993a) was given to the subset of a group of LEA proteins which accumulate at the mid- to late-stages of seed development

(before or during desiccation) (including LEA group 2 proteins such as LEA D11 in cotton and some denoted RAB [Responsive to ABA]) (reviewed in Dure *et al.*, 1989; Skriver and Mundy, 1990; Galau and Close, 1992).

Seeds of the orthodox species, castor bean (*Ricinus communis* L.) acquire a tolerance of slow desiccation around mid-development (Kermode and Bewley, 1985a). In this study, the abundance of several *Lea* mRNAs in the endosperm of castor bean seeds was examined during development, following rehydration after premature desiccation, and following imbibition of the mature dry seed. *Lea* mRNAs representative of the two classes of cotton genes exhibiting different expression characteristics during development were studied. Some previous studies have focused primarily on changes in *Lea* mRNAs or LEA proteins alone and have not attempted to examine both. Use of a dehydrin antibody (Close *et al.*, 1993a) allowed me to follow changes in dehydrin-like polypeptides and to correlate these changes with mRNA abundance. This chapter is a result of some collaborative research (Han *et al.*, 1996). The Northern blot analyses were carried out by G. Galau and W. Hughes at the University of Georgia; I am responsible for the Western blot analyses.

2.2. Materials and Methods

2.2.1. Plant Materials

Ricinus communis L. plants cv. Hale or Zanzibar were grown from seeds as outlined in Greenwood and Bewley (1982). *Ricinus communis* L. plants cv. Hale plants were grown in controlled-environment conditions (23 °C day/18 °C night, 16h photoperiod, RH 80%) and fully opened female flowers were individually tagged and pollinated and the tagging date recorded as 0 days after

pollination (DAP). Capsules were harvested at 5-d intervals. Endosperms were obtained from seeds at different times during development (from 25-60 DAP) (Greenwood and Bewley 1982), and also following imbibition of mature (60 DAP) seeds, and upon rehydration of prematurely dried (30 DAP) seeds.

Ricinus communis L. plants cv. Zanzibar were grown in the field from seed from May to November at Simon Fraser University on Burnaby Mountain, B.C. Seeds were collected at 30 d after pollination (DAP) and at maturity (60 DAP). The staging of seed was according to Greenwood and Bewley (1982).

Northern blot analyses utilized endosperms isolated from seeds of the Hale cultivar. Due to greater availability of developing seed, protein analyses utilized seeds of the cultivar. No differences in the Western blot profiles of heat-stable proteins were observed between the Hale and Zanzibar cultivars.

2.2.2. Drying treatments

Developing (30 DAP) seeds were dried slowly to 25% water content as described in Kermode and Bewley (1985a). This involved placing seeds for one day sequentially in desiccators containing stirred saturated salt solutions maintaining RH values of 92, 86(2d), 65 and 44% and then for one day on the laboratory bench (approx. RH 25%).

2.2.3. Seed germination

After removal of the coat, mature or prematurely dried seeds were placed in Petri dishes containing filter paper moistened with sterile water and maintained in the dark at 25 °C.

2.2.4. Isolation of bulk RNA and poly(A)$^+$ RNA

Bulk RNA was isolated using the method according to Kermode et al. (1989). Poly (A)$^+$ RNA was isolated from bulk RNA by affinity chromatography using type III oligo (dT) cellulose (Collaborative Research, Bedford, MA., U.S.A.). Chromatography was carried out at 23 °C using a buffer containing 25 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.5% SDS as the binding buffer; sterile water was used to elute the poly(A)$^+$-rich mRNA which was purified further by a second cycle of chromatography.

2.2.5. Northern blot analysis of RNA

Poly (A)$^+$ RNA (30 ng) was analyzed by formaldehyde-gel electrophoresis (Galau et al. 1986). RNA transfers were onto GeneScreen membranes (DuPont-New England Nuclear, DuPont Canada, Inc., Mississauga, Ontario, Canada). The membranes were then washed, air dried, irradiated with UV light, and heated for 1 h at 80 °C under vacuum (Galau et al. 1986). They were hybridized at Tm - 15 °C (Galau et al. 1983, 1986) with ^{32}P-labeled cloned cDNA probes from cotton (Galau et al. 1983, 1986; Hughes and Galau 1989). The cDNA probes used included 1 MAT gene (encoding a legumin storage protein), 7 LEA genes and 3 GERM genes (encoding proteins that are induced post-germinatively). During cotton seed development, the maturation or seed expansion stage starts at 26 DAP and ends at 45 DAP, and is followed by a postvascular separation stage (postabscission) starting at 46 DAP and ending at 55 DAP when cotton seeds reach maturity. The MAT mRNA encoding a legumin-type storage protein is present only during the maturation stage and

declines at ~ 40 DAP. The 7 LEA genes fall into two classes. LEA 2, 6, 10 and 20 are in class I, the class II LEAs (i.e. dehydrin) include *lea* 3, 4 and 12. See the introduction and results section for the patterns of expression of the 2 *lea* classes. The GERM mRNAs are present at high levels following germination (1-4 days after the start of imbibition).

2.2.6. Extraction of heat-stable proteins and western blot analysis

Heat-stable proteins were extracted from endosperms isolated from developing seeds, from prematurely dried and rehydrated seeds and from mature-imbibed seeds as outlined in Close et al. (1993a). Briefly, total soluble proteins were extracted by grinding seed endosperms in 30 mM TES buffer pH 8.0, containing 20 mM NaCl and 1 mM PMSF using a ground glass homogenizer. This was followed by centrifugation at top speed (13,000 rpm) in a microcentrifuge for 15 min at 4 °C. The supernatants were boiled at 100 °C for 10 min, kept on ice and then centrifuged as before. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Mississauga, Ontario, Canada) (Bradford 1976) using bovine serum albumin as a standard. Aliquots containing equal amounts of heat stable proteins were precipitated with four volumes of acetone and then resuspended in SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.7, 2% SDS, 10% glycerol, 0.003% bromphenol blue and 5% ß-mercaptoethanol). The mixture was heated for 10 min at 95 °C, cooled and then fractionated by SDS-PAGE on 12% gels using a Hoefer Model SE 280 apparatus (Hoefer Scientific Instruments, San Fransisco, CA, U.S.A.) according to the method of Laemmli (1970). After electroblotting onto nitrocellulose (using a Bio-Rad Trans-Blot Semi-dry Transfer Cell), dehydrin proteins were detected with rabbit anti-dehydrin (serum diluted 1/600

before use), followed by goat anti-rabbit IgG (Promega, Madison, WI, U.S.A.) to which was conjugated alkaline phosphatase. The anti-dehydrin serum was produced from a synthetic polypeptide containing a highly conserved sequence (KIKEKLPG) (kindly provided by T.J. Close) (Close et al. 1993a, 1993b). The pre-immune serum was utilized as a control.

2.3. Results

2.3.1. The Effect of Premature Desiccation and Rehydration on Developmental and Post-Germinative Messenger RNAs

As a prelude to studies of the expression of different *Lea* genes, the fate of mRNAs encoding developmental and post-germinative proteins was examined during development and following natural and imposed desiccation of castor bean seeds. Figure 2.1 shows Northern blot analyses of developmental and germination/growth-related messenger RNAs in the castor bean endosperm. The LEG B MAT 1 clone is for a developmental protein (a cotton legumin storage protein), the message for which was prevalent during the major phase of storage protein synthesis in castor bean endosperm (30-40 DAP) (Kermode and Bewley, 1985b). This mRNA was not present in the mature dry seed (at 60 DAP) nor upon imbibition (Fig. 2.1, 5-96 h). Premature drying of the seed at 30 DAP resulted in a substantial loss of this developmental mRNA, which did not increase during germination or growth. Germ D91, D30, and D38 clones are for mRNAs that arise post-germinatively. These were predominant in the castor bean endosperm at 48 h (and thereafter) following imbibition of mature seeds (Fig. 2.1). They were present in low abundance during development. After premature drying and rehydration at 30 DAP, these postgerminative mRNAs increased at the same time after the start of

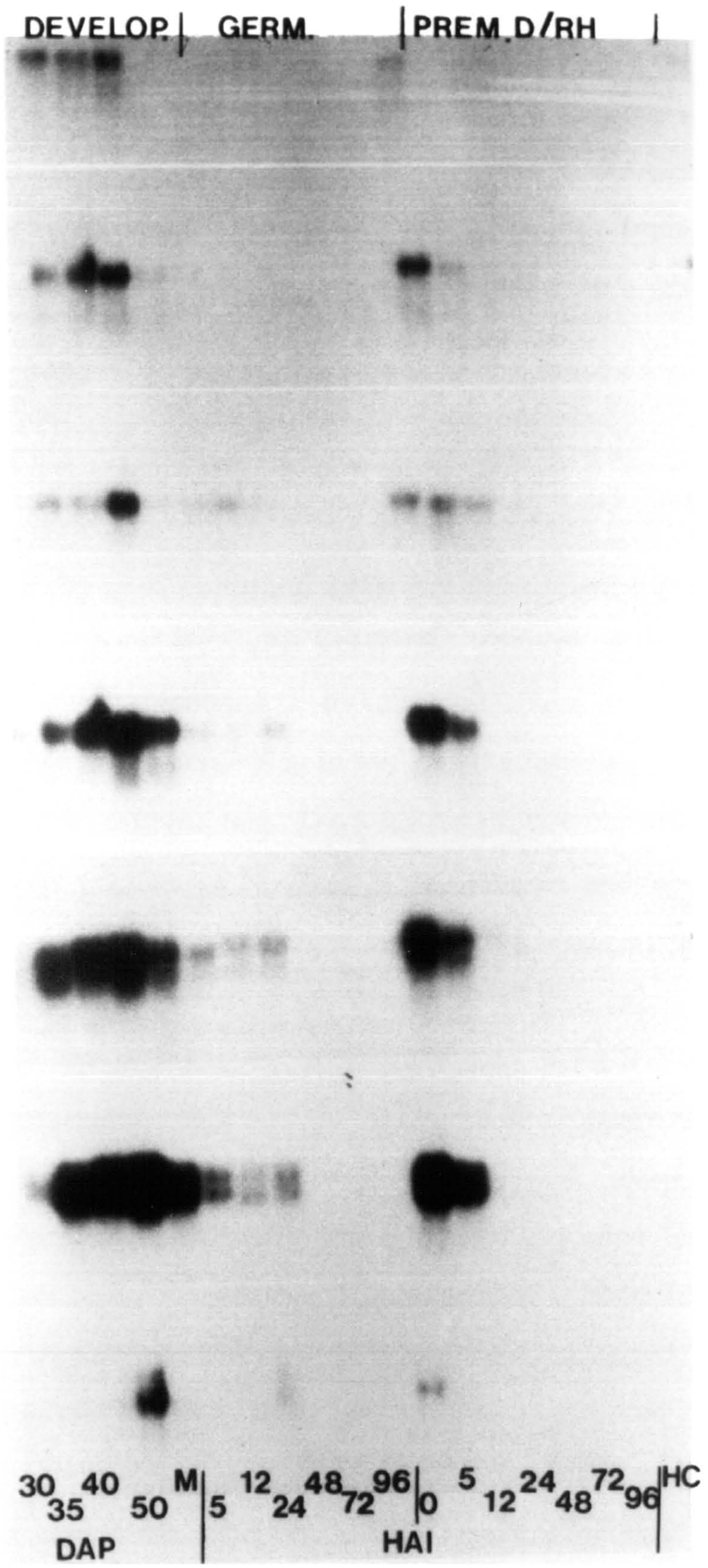
imbibition as in the mature seed. Thus, desiccation effects an off- or down-regulation of the synthesis of developmental mRNAs, and an on-regulation of mRNA synthesis associated with germination and growth.

2.3.2. Changes in *Lea* Messenger RNAs During Development and Germination and in Response to Premature Desiccation

LEA transcript abundance in castor been endosperm during development (30-60 DAP) was analyzed (Fig. 2-2). Class I *Lea* mRNAs (2, 6, 10 and 20) were detected around 30 DAP and increased thereafter, showing a maximum at 50 DAP, around the onset of desiccation. With the expection of *Lea* 2 mRNA, they become much less abundant in the endosperm as the seed matured and dried to 60 DAP. *Lea* 2 mRNA was the most abundant of the Class I group and showed a pattern of accumulation more characteristic of the Class IIA *Lea* representatives in the castor bean endosperm. Similar to transcripts belonging to the Class IIA group, *Lea* 2 mRNA was also abundant in the dry seed (at 60 DAP). The mRNAs representative of Class IIA in cotton (*Leas* 3, 4, and 12) were generally more abundant in the endosperm than those belonging to Class I. Similar to the Class I mRNAs, the Class IIA messages increased during development from 30 DAP to 50-55 DAP. However, in contrast to the Class I mRNAs, most of the Class IIA messages were still abundant in the mature dry seed at 60 DAP (an exception was the *Lea* 12 mRNA which behaved more like a class I message) (Fig. 2.2).

Similar to storage protein mRNAs, *Lea* mRNAs present in high amounts within the endosperm of the mature dry seed (*Lea* mRNAs 2, 3, and 4) declined dramatically following imbibition of the mature seed (Figure 2.2). *Lea* 2 and 4 transcripts showed a short lag following imbibition before

Figure 2.1. Northern blot analyses of developmental and germination/growth-related messenger RNAs in the castor bean endosperm. Poly $A^{+}RNA$ was isolated from endosperm of developing seeds at 30-60 DAP, (M: mature or 60 DAP seed), mature seeds following imbibition from 5-96 h (germinated), and prematurely dried 30 DAP seeds rehydrated for 0-96 h. DAP: days after pollination; HAI, hours after imbibition. The LEGB, MAT1 clone is for a developmental protein (a legumin storage protein); Germ D91, D30 and D38 clones are for post-germinative mRNAs (From Kermode, 1995).


DEVELOP.
GERM.
PREM.D/RH
LEA 10
20
6
3
4
2
12
30 35 40 50 M
5 12 24 48 72 96
0 5 12 24 48 72 96
HC
DAP
HAI

becoming undetectable after 24 h of imbibition.

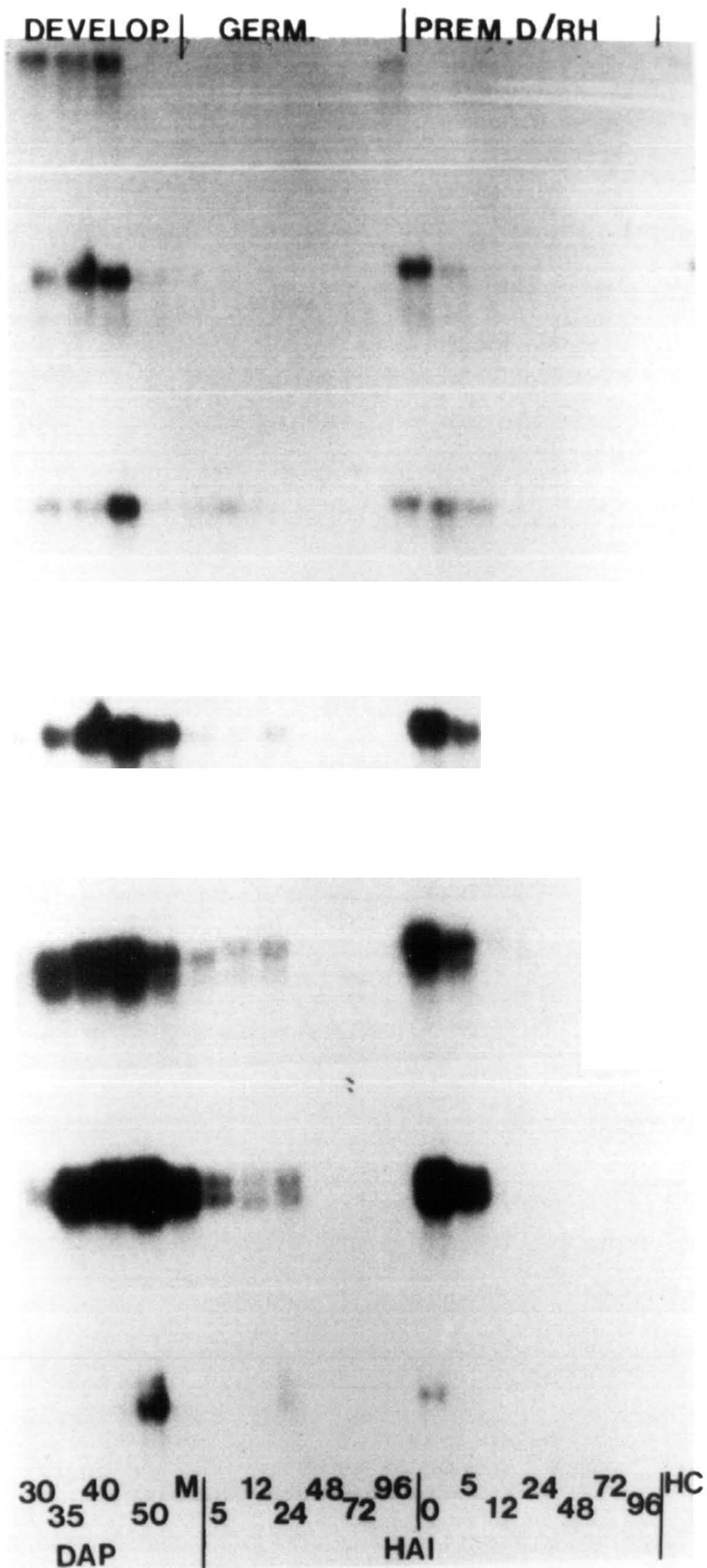
We also examined *Lea* mRNAs in endosperm of 30 DAP seeds subjected to premature drying and their fate following rehydration after premature drying. In most cases, the abundance of *Lea* mRNAs following premature drying did not change and was approximately the same in the dried 30 DAP seed as in the developing 30 DAP fresh seed (Fig. 2.2). However, following rehydration of the prematurely dried seed all Lea mRNAs declined dramatically, becoming undetectable after 5 h. The hydrated control seeds (30 DAP fresh seeds detached and hydrated for 5 d, equivalent to the drying treatment, plus 96 h) showed no detectable *Lea* mRNAs (Fig. 2, HC). These control seeds do not exhibit a 'switch' in gene expression to a germinative mode (Kermode *et al.*, 1989). Therefore a decline in these messages may be a response to deterioration during the long period of hydration of the detached seed (9 d).

2.3.3. Changes in Dehydrin Proteins During Development and Germination and in Response to Premature Desiccation

We extended our studies on *Lea* gene expression at the mRNA level to examine the fate of LEA proteins at different stages. I analyzed the dehydrins of castor bean endosperm with the use of a specific dehydrin antibody from rabbit produced from a synthetic polypeptide containing the most conservative sequence (KIKEKLPG) (Close *et al.*, 1993a) (kindly provided by T. J. Close). Changes in heat stable and dehydrin proteins occur within the castor bean endosperm during seed development (25 to 60 DAP). During the early-to-mid-stages of seed development (i.e., 25 to 40 DAP), prominent bands on the Western blot have Mr ~ 25, 32, 33, 46 and 60 KD (Fig. 2.3B). Of these, only the

Figure 2.2. Northern blot analyses of *Lea* mRNAs in castor bean endosperm during development, following imbibition of mature seed and following premature desiccation/rehydration.

Lea clones 2,6,10 and 20 are representatives of cotton Class I *Leas*; clones 3,4 and 12 are representatives of Class IIA. HC: hydrated control seed; 30 DAP seed hydrated for 5 d (equivalent to the drying treatment), plus 96 h.


DEVELOP.
GERM.
PREM.D/RH
LEA 10
20
6
3
4
2
12
30 35 40 50 M
5 12 24 48 72 96
0 5 12 24 48 72 96
HC
DAP
HAI

25-KD and 33-KD products are likely dehydrin-related polypeptides; the others are detected on control Western blots using the pre-immune serum (Fig. 2.4). After 40 DAP (i.e., in mature dry seed at 60 DAP) the nature of the dehydrin-related polypeptides accumulated in the endosperm changed dramatically (Fig. 2.3B). In particular, new polypeptides were detected with Mr ~ 28-30 KD and 41 KD (Fig. 2.3B).

In Figure 2.5, changes in heat-stable and dehydrin-related polypeptides following imbibition of the mature (60 DAP) seed are shown. The Western blot (Fig. 2.5B) shows a persistence of most of the dehydrin-related polypeptides (prominent in the mature dry seed) during germination and growth (up to 96 h after imbibition). Thereafter, at 48-96 h after imbibition, proteins in the 33 and 46 KD range declined, whereas proteins in the 28~30 KD and 41 KD range persisted even at 96 h after imbibition. This is somewhat surprising given the dramatic decline in dehydrin mRNAs (e.g., *Lea* 3 mRNA) following imbibition of the mature seed. The profiles of heat-stable proteins in endosperm of mature-imbibed seeds were similar to those of developing seeds up to 24 h (Compare Figs. 2.3A and 2.5A).

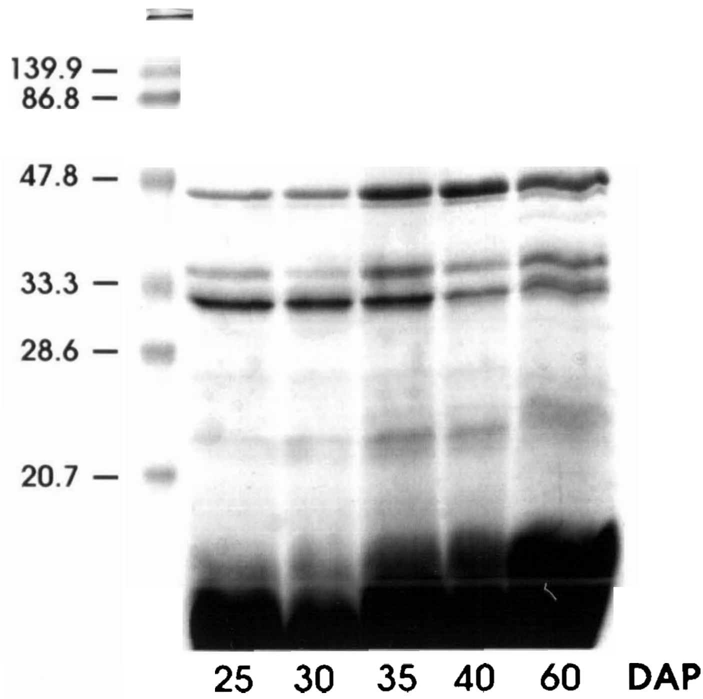
Figure 2.6 shows the changes in heat-stable and dehydrin-related polypeptides in the prematurely dried 30 DAP seed and their fate following rehydration after desiccation. Within the dried 30 DAP seed, some of the dehydrin-related polypeptides characteristic of the mature dry seed (and not found in developing fresh 30 DAP seeds) were induced (Mr ~ 28-30 KD, Fig. 2.5B). In contrast to the mature-imbibed seed, in which these polypeptides (Mr ~ 28-30) persisted throughout the 96-h study period, polypeptides of this set declined in the prematurely dried seed after 24 h following the start of rehydration. There were considerable differences in the profiles of heat stable

Figure 2.3. Changes in heat-stable proteins (A) and dehydrin-related proteins (B) in castor bean endosperm during development.

A. Coomassie blue-stained SDS-polyacrylamide gel of heat-stable proteins extracted from developing endosperm of seeds harvested at 20-60 DAP. (A) Samples were loaded with equal protein (36 µg in 18 µl sample buffer containing 5% v/v ß-mercaptoethanol). B. Western blot of heat-stable proteins (64 µg in 32 µl sample buffer) using dehydrin antibody to detect dehydrin-related polypeptides.

DEVELOPMENT

A. Stained Gel



B. Western Blot

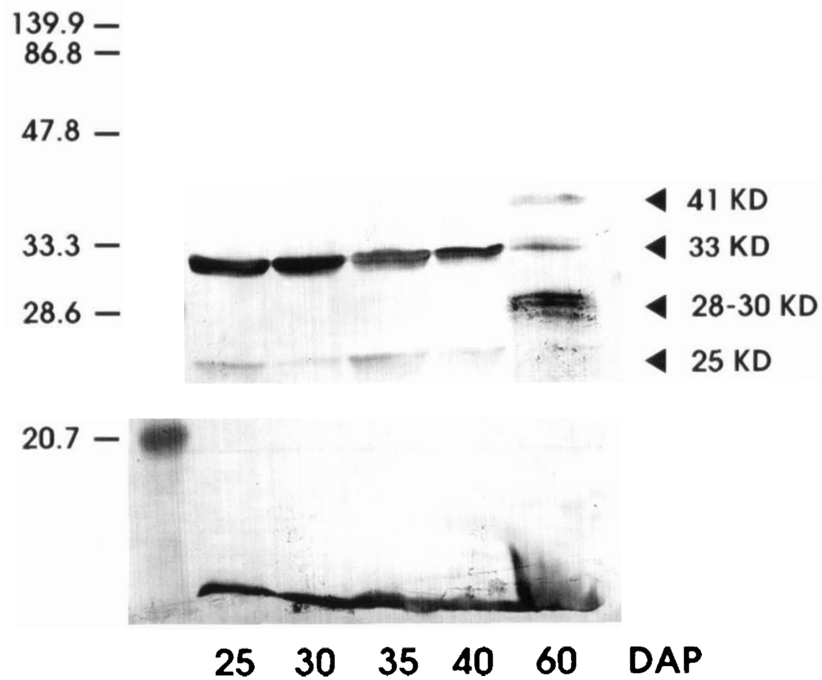


Figure 2.4. Proteins detected in extracts from castor bean endosperm on control Western blots using a pre-immune serum.

Samples were loaded with equal protein (64 μg in 32 μl sample buffer). Lane 1: 30 DAP fresh seed; Lane 2: mature seed (60 DAP); Lane 3: prematurely dried seed (30 DAP); Lane 4: Prematurely dried seed subsequently rehydrated for 24 h.

CONTROL WESTERN
(PRE - IMMUNE)

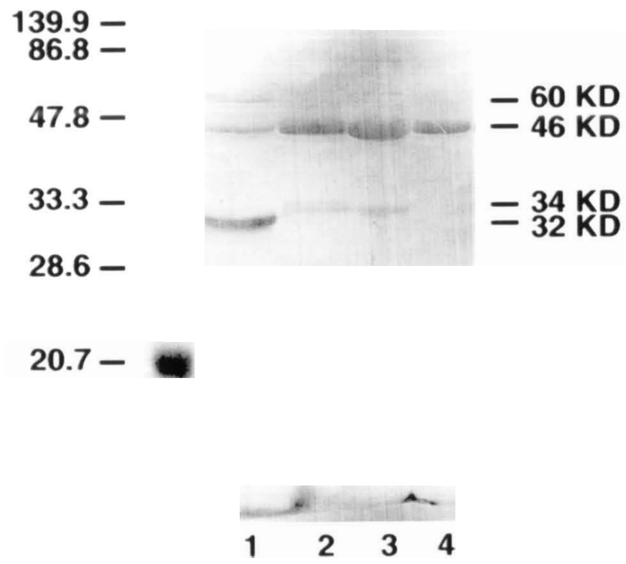
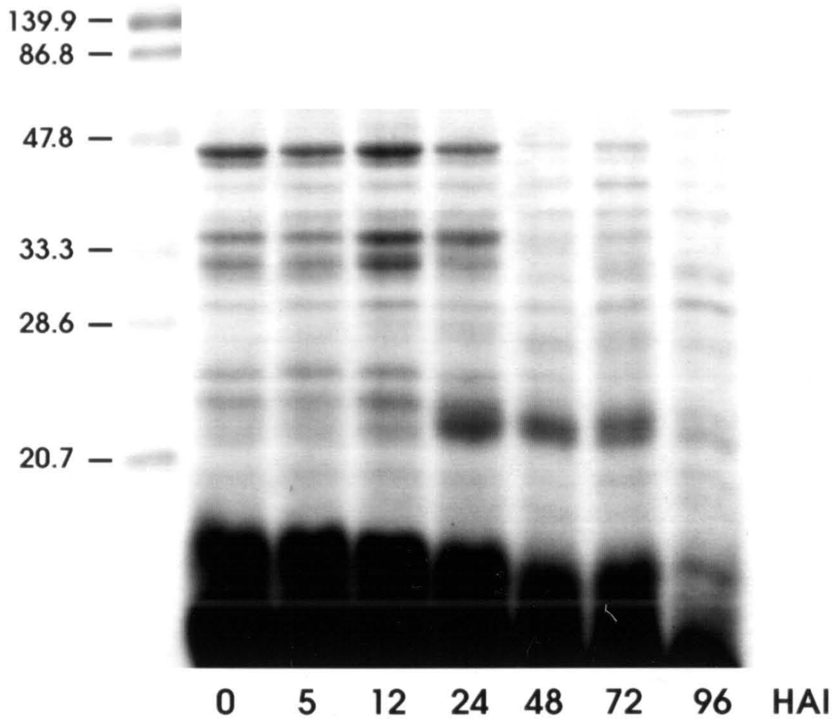


Figure 2.5. Changes in heat-stable proteins (A) and dehydrin-related proteins (B) in castor bean endosperm during germination and growth of mature seed.

A. Coomassie blue-stained SDS-polyacrylamide gel of heat-stable proteins extracted from endosperm of mature seeds imbibed for 0-96 h. B. Western blot of heat-stable proteins using dehydrin antibody to detect dehydrin-related polypeptides. Samples were loaded with equal protein as in Fig. 2.3.

MATURE - IMBIBED

A. Stained Gel



B. Western Blot

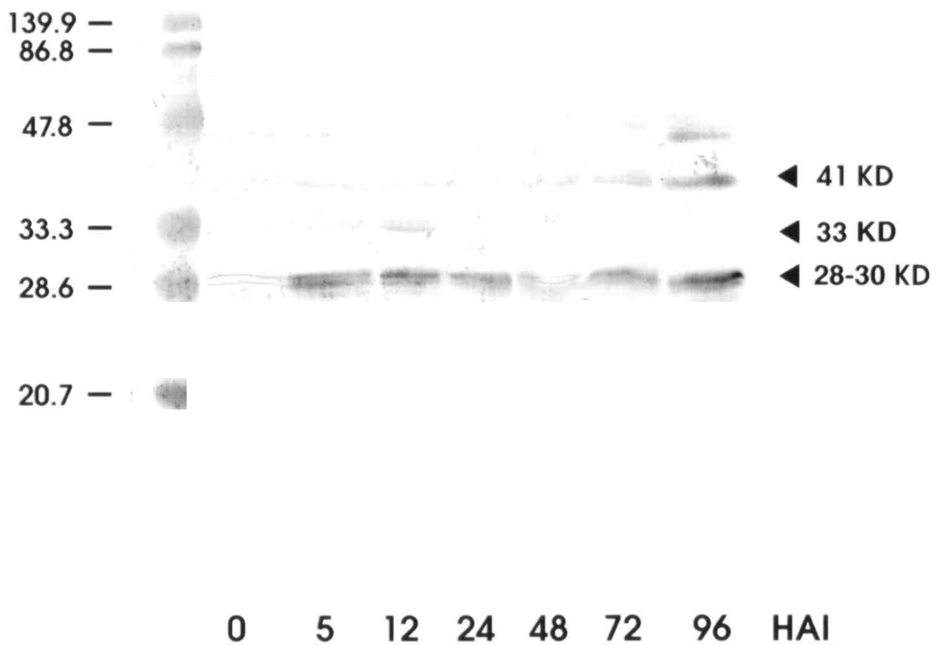
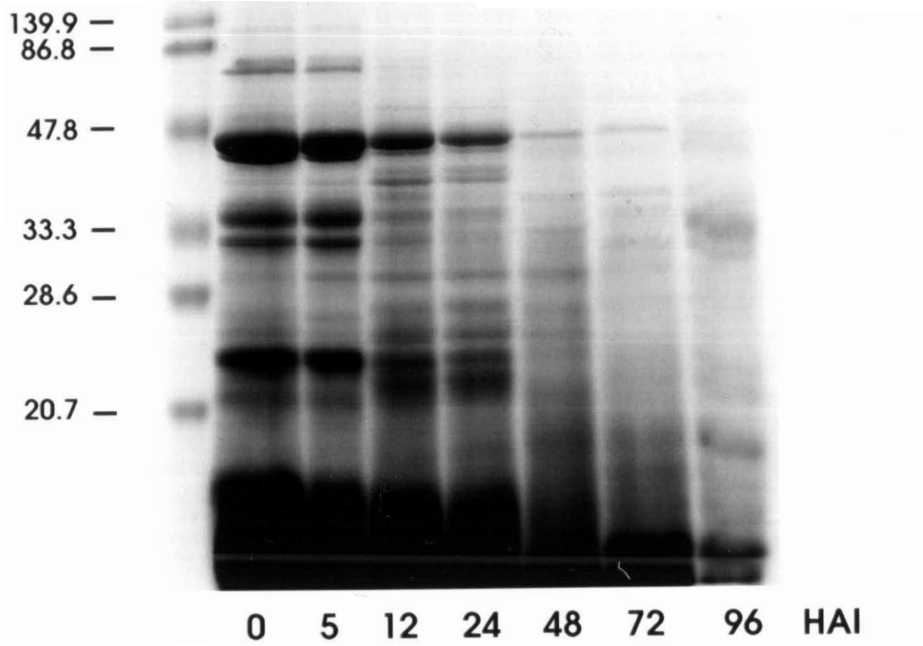


Figure 2.6. Changes in heat-stable proteins (A) and dehydrin-related proteins (B) in castor bean endosperm following premature desiccation/rehydration.

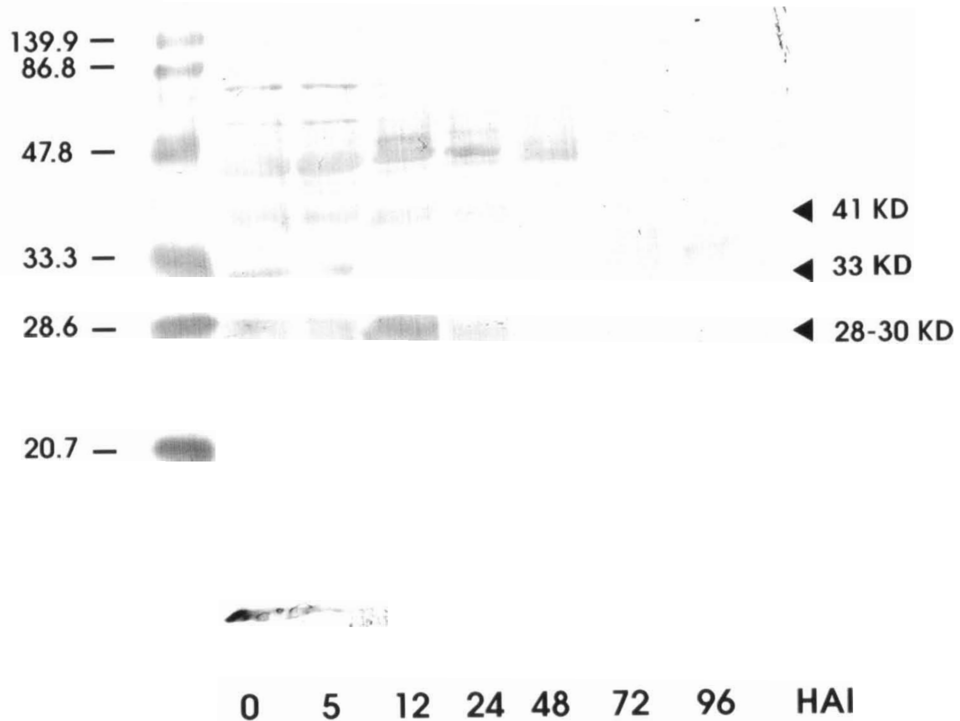
A. Coomassie blue-stained SDS-polyacrylamide gel of heat-stable proteins extracted from endosperm of prematurely dried 30 DAP seeds rehydrated for 0-96 h. B. Western blot of heat-stable proteins using dehydrin antibody to detect dehydrin-related polypeptides. Samples were loaded with equal protein as in Fig. 2.3.

PREMATURELY DRIED - REHYDRATED

A. Stained Gel



B. Western Blot



proteins following rehydration of the prematurely dried seed (Fig. 2.6A) *versus* the mature-imbibed seed (Fig. 2.5A).

2.4. Discussion

Drying, whether occurring as part of the maturation process or imposed prematurely has a marked effect upon the metabolism of developing seeds. Developmentally-related events such as storage protein synthesis cease as a consequence of drying and the expression of genes for storage proteins becomes permanently suppressed. The synthesis of mRNAs and their translation products normally associated with the events that occur after germination is induced upon rehydration following drying. It is clear from this study and from previous studies (Kermode et al. 1989; Kermode 1990) that all of the changes in the mRNA population, from those characteristic of development to those specific to germination or growth, do not occur during drying per se. The appearance of mRNAs for germination occurs after hydration of the dry seed (after a lag of a few to several hours), and the loss of, or substantial decline in, developmental mRNAs is largely completed at this time. Thus, while most developmental (storage-protein) mRNAs decline during drying itself (which may be due to a direct effect of water loss on the transcription of these genes) (Jiang et al. 1995), hydration appears to be the crucial event leading to the loss of any residual developmental mRNAs that remain stable throughout desiccation (Kermode et al. 1989). These mRNAs are presumably degraded upon rehydration by the normal turnover processes and are not replaced because their genes have been off- or down-regulated. With respect to these changes in gene expression, there is little quantitative or temporal difference in the response to normal and premature desiccation.

Such is not the case in relation to the synthesis of LEA (dehydrin) proteins, however, as we demonstrate here.

The dehydrin genes represent a class exhibiting a highly flexible expression repertoire in that they are responsive not only to developmental cues but also to environmental ones. Transcription of these genes is also induced in virtually all seedling tissues subjected to water stress (i.e., nonlethal desiccation); other water deficit-related stresses, such as high salt, high osmolarity, and low temperature also elicit their production, as does abscisic acid. Furthermore, different dehydrin genes may be induced by different stresses and these responses may also be tissue-specific. Three barley dehydrin mRNAs respond differently to salt, cold, mannitol and ABA (Espelund et al. 1992). These dehydrin mRNAs also show different expression patterns during seed development (Espelund et al. 1992). In this study we examined the abundance of several *Lea* mRNAs in the endosperms of castor bean seeds during development, following rehydration after premature desiccation, and following imbibition of the mature dry seed. *Lea* mRNAs representative of the two classes of cotton genes exhibiting different expression characteristics were studied, namely *Lea*s belonging to Class I (the 'gradual' class) and Class II A (the 'abrupt' class). Not surprisingly, our analyses of the various *Lea* mRNAs in the castor bean endosperm revealed that they did not behave in a manner identical to that found in the embryonic cotyledons of cotton. Nonetheless, in the castor bean seed there were different patterns of accumulation and decline of the two classes of *Lea* mRNAs within the endosperm, a triploid storage organ separate from the embryo. In castor bean seeds, representatives of the two classes of *Lea* mRNAs began to accumulate around the same time during endosperm development (30-35 DAP). However, during the later stages of development there were distinct

differences in *Lea* gene expression. In particular, most of the Class I *Lea* mRNAs declined substantially during maturation drying, in the same manner as the storage protein messages. Class II mRNAs remained abundant in the mature dry seed. In contrast, all *Lea* mRNAs were preserved in endosperms of the prematurely dried seed; they did not decline during the 5-d drying treatment. Hence the response of *Lea* mRNAs to premature drying is different from that during maturation drying, and also different from the response of the major storage protein mRNA, which declines precipitously during both types of drying (Fig. 2.1, Fig. 2.2). This demonstrates that the persistence of the *Lea* mRNAs exhibits a differential temporal response in relation to desiccation during development and these mRNAs are more stable during premature drying. Following rehydration of the prematurely dried seed, preserved *Lea* mRNAs declined very rapidly, as in the mature seed following imbibition, showing that the genes for these transcripts are down-regulated by drying. Thus, in *Ricinus* endosperms, full expression of germination and growth-related genes can occur even after only about half of the developmental period has been completed; the only requirement for the switch from development to germination is a desiccation period (Fig. 2.1, Fig. 2.2).

Heat-stable proteins are present during development of the castor bean endosperm, but there is an increase in specific dehydrin-type proteins (~ 28-30 KD) as detected by western blotting during the final stages, as maturation drying occurs at 50-60 DAP. The dehydrin protein is encoded by the *Lea* 3 gene, although in this study we have not proven there is a direct relationship between the product of this gene and the various dehydrins synthesized. There is an increase in the ~ 28-30 KD proteins in prematurely dried endosperms at 30 DAP during the 5 d of drying, albeit to a reduced extent compared to naturally dried (60 DAP) endosperms. Interestingly, dehydrins

persist in the endosperm of germinating and germinated mature castor bean seeds, even at the time when the major protein reserves are being mobilized (Kermode et al. 1985). Moreover, there is no mRNA for dehydrin present in the endosperms following imbibition and thus there is little turnover of the proteins for at least 4 d after the start of imbibition. In contrast, following premature desiccation at 30 DAP the proteins exhibit a marked decline and are undetectable within 48 h after the start of imbibition. The patterns of decline of *Lea* mRNAs upon rehydration following premature desiccation are similar to those occurring in mature endosperms following imbibition, although there is more in the prematurely dried endosperms during the first few hours following imbibition than in rehydrated mature dried ones. However, by 24-48 h after imbibition no *Lea*-type mRNAs are present following either mode of drying.

Thus, endosperms exhibit differences in their response to prematurely-imposed and maturation drying in relation to the expression of some genes for developmental and post-germinative metabolism, especially those for LEA proteins. In contrast, the response to the different modes of drying is very similar for storage protein genes and their products (Kermode and Bewley 1985b; Kermode et al. 1985, 1989; Jiang et al. 1995). *Lea* mRNAs, including that for a putative dehydrin, are more stable during premature drying, although the capacity of *Lea* genes to be transcribed upon subsequent rehydration is down-regulated by both modes of drying. In contrast to *Lea* mRNAs, dehydrin proteins are very stable following maturation drying and persist for several days following the start of imbibition; such is not the case following premature drying, following which the dehydrins are degraded after a lag of about 48 hours of rehydration. The reason for this is not apparent, although endosperms at 30 DAP contain less storage protein than those which have

completed germination (Kermode and Bewley 1985b; Kermode et al. 1985) and thus non-storage proteins may become mobilized, or more susceptible to proteinases, earlier in the rehydrated 30 DAP endosperms to provide nutrients for the growing embryo.

Chapter 3.

Dehydrin Proteins Are Differentially Produced in Castor Bean Seeds and Seedlings in Response to ABA and Water-Deficit-Related Stresses

3.1. Introduction

Plants undergo a series of physiological, biochemical and molecular changes in response to adverse environmental conditions or stresses such as drought, low temperature or high salt. Following exposure of the plant to a water-related stress for a few days (or, for several hours, depending on the plant or tissue), a group of proteins is induced that is presumed to play a protective role. This group of proteins, termed dehydrins, (a subset of the proteins termed LEAs; *L*ate *E*mbryogenesis *A*bundant or RABs; *R*esponsive to *A*BA) have some common features in their structure. In particular, they are characterized by a conserved 15-amino acid, lysine-rich sequence near the carboxyl terminus. The consensus polypeptide forms an amphiphilic alpha-helix which may serve as an ion trap in dehydrating cells, sequestering ions that become concentrated (Close *et al.*, 1993b; Dure, 1993a, 1993b). The elevated levels of endogenous ABA, which occur as a result of stress imposition, are thought to induce dehydrin gene expression (reviewed in Bray, 1991; Chandler and Robertson, 1994; Kermode, 1995). For example, there is excellent correlation (e.g., in barley and maize) between the amounts of mRNA and ABA in shoots, roots, and aleurone layers from either well-watered, dehydrated, or dehydrated/rehydrated seedlings (Gomez *et al.*, 1988; Chandler *et al.*, 1988). The most convincing evidence for the role of ABA in dehydrin gene expression comes from studies of ABA-deficient mutants of maize (Pla *et al.*, 1989, 1991), tomato (Bray, 1991) and *Arabidopsis thaliana* (Mäntylä *et al.*, 1995). When exposed to dehydration stress, these mutants fail to elevate ABA levels, and show a corresponding inability to produce dehydrins. Thus, the expression of Lea/dehydrin genes appears to be intimately connected with ABA; however, the extent to which expression is regulated (directly or indirectly) by ABA

alone, or whether other factors are important requires further investigation. Antagonizing factors, such as GA, may also play an important role in regulating the expression of dehydrin genes. For example, in barley aleurone protoplasts, GA suppresses the ABA-induced increase in transcription of a dehydrin gene (Robertson, personal communication). It remains to be determined whether similar interactions occur between GA and ABA in other organ/tissue types and if GA is capable of suppressing dehydrin synthesis elicited by water-deficit-related stresses.

As mentioned in the introduction (Chapter 1), dehydrin genes exhibit a flexible expression repertoire, being responsive to both developmental and environmental cues (reviewed in Thomas *et al.*, 1991). In addition to being expressed under abnormal (stress) conditions, they exhibit temporal regulation during seed development where expression is most intense during mid- to late-development. Thus, the protective role of dehydrins in the survival of water loss is purported to be dual: during maturation drying of the developing seed and following germination/growth of the mature seed (i.e., in seedlings or plant vegetative tissues undergoing mild water stress). Precocious appearance of the proteins and their mRNAs can be induced in cultured immature embryos by exogenous ABA treatment. Specifically, it has been hypothesized that, during normal development, high levels of ABA induce the accumulation of these polypeptides and hence prepare the embryo for desiccation or possible cellular disruption upon subsequent rehydration. However, the recent finding of Lea/dehydrin transcripts and proteins in the so-called recalcitrant seeds, which are desiccation-intolerant, suggests that an intolerance to dehydration is not due to an absence of dehydrin-like proteins (Bradford and Chandler, 1992; Finch-Savage *et al.*, 1994; Han *et al.*, 1995). Further, an inability to accumulate ABA does not account for the dehydration-

intolerance of *Zizania* (wild rice) seeds, which are non-viable when subsequently rehydrated at low temperature (e.g., 10 °C) (Bradford and Chandler, 1992).

Dehydrins are encoded by multiple genes (Close and Chandler, 1990) and their expression is likely regulated by a complex mechanism, in some cases resulting in developmental regulation, tissue/organ specificity and/or stress-specific expression.

In this chapter, I examine the stress inducibility of dehydrin protein production in immature seeds and mature seedlings of castor bean by subjecting them to ABA and various water-deficit-related treatments including desiccation, water stress, high salt, high osmolarity and low temperature. Inducibility of dehydrins in shoots of castor bean plants in response to dehydration was also examined. A further objective was to examine the effects of GA on dehydrin induction in seedlings exposed to ABA and the water-deficit-related treatments.

3.2. Materials and Methods

3.2.1. Plant material

Ricinus communis L. plants cv. Zanzibar were grown in the field from seed from May to November at Simon Fraser University on Burnaby Mountain, B.C. Seeds were collected at 30 d after pollination (DAP) and at maturity (60 DAP). The staging of seed was according to Greenwood and Bewley (1982). Endosperms were obtained from 30 DAP seeds subjected to the various treatments outlined below. Endosperms, cotyledons and radicles were obtained from five-d-old seedlings (obtained by germinating mature seed) subjected to

the same treatments.

3.2.2. Seed germination

Mature seeds were surface-sterilized in a solution of 1% hypochlorite, 0.01% SDS for 10 min, and then rinsed three times in sterilized water. The seed coats were then removed under asceptic conditions in a laminar flow hood. Seeds were placed in Petri dishes containing sterile filter paper moistened with 10 ml of sterile water and allowed to germinate at 26°C in the dark. After 5 d, seedlings were used for the various treatments.

3.2.3. ABA treatments

Five-d-old seedlings were incubated for 7 d in different concentrations of ABA (10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M). For these treatments, the seedlings were placed in Petri dishes containing sterile filter paper and 10 ml of the different ABA solutions (sterilized by passing through a 0.22 μm filter). The 7-d treatment was carried out under ambient laboratory conditions.

3.2.4. Water stress treatment

Developing seeds and five-d-old seedlings were subjected to water stress by equilibrating them in a desiccator over 25% glycerol for 7 d. The osmotic potential of the glycerol solution was determined to be -12.6 MPa, which produces a RH value in the chamber of approximately 87%. The water content was measured in the following formula: fresh weight after treatment / fresh weight before treatment x 100%. Average and standard deviation of water

content have been calculated.

3.2.5. Desiccation treatment

Developing seeds and seedlings were dried slowly to known percentage water contents by placing them in a desiccator over stirred saturated salt solutions. The drying regime used for 30 DAP seeds and for five-d-old seedlings was as described in Kermode and Bewley (1985). This involved placing seeds or seedlings for one day sequentially in desiccators containing stirred saturated salt solutions maintaining RH values of 92, 86 (2 d), 74, 65, 44 and 22%. The water content was measured in the following formula: fresh weight after treatment / fresh weight before treatment x 100%. Average and standard deviation of water content have been calculated.

3.2.6. High salt and high osmolarity treatments

Developing seeds and five-d-old seedlings were placed in Petri dishes containing sterile filter paper moistened with either 10 ml of 0.2 M NaCl (high salt treatment) or 10 ml of 0.3 M mannitol (high osmolarity treatment). Both solutions were previously sterilized by passing through a 0.22 µm filter. The treatment was carried out for 7 d under ambient laboratory conditions.

3.2.7. Cold treatment

Developing seeds and five-d-old seedlings were placed in Petri dishes containing sterile filter paper moistened with 10 ml of sterile water, and then maintained at 4°C for 7 d.

3.2.8. GA_3 treatment

To determine the effect of exogenous GA_3 on the response to cold treatment, seedlings were placed in Petri dishes containing sterile filter paper moistened with 10 ml 10^{-4} M GA_3 (previously sterilized by passing through a 0.22 μm filter) and then maintained at 4°C for 7 d.

To examine the effect ,of GA_3 on the responses to the ABA and water-deficit-related treatments, seedlings were placed in Petri dishes containing sterile filter paper moistened with 10 ml of the following solutions: ABA (10^{-5} M) + GA_3 (10^{-4} M); NaCl (0.2 M) + GA_3 (10^{-4} M); Mannitol (0.3 M)+ GA_3 (10^{-4} M). All solutions were sterilized by passing through a 0.22 μm filter. The treatments were carried out for 7 d under ambient laboratory conditions.

3.2.9. Dehydration treatment of shoots of castor bean plants

Shoots were excised from young plants (25-30 cm in height) and then dried at the lab bench until the weight was 88% of the original weight (~ 45 min). Immediately the shoots were sealed in plastic bags, and left for 6 h under ambient laboratory conditions. Control shoots were sealed in plastic bags immediately after excision from plants.

3.2.10. Extraction of heat-soluble proteins and Western blot analysis

Heat-soluble proteins were extracted from endosperms isolated from immature seeds and seedlings, and from cotyledons and radicles isolated from seedlings as outlined in Close *et al.* (1993a). Briefly, total soluble proteins were extracted by grinding tissues in 30 mM TES buffer pH 8.0, containing 20 mM

NaCl and 1 mM PMSF using a ground glass homogenizer. This was followed by centrifugation at top speed in a microcentrifuge for 15 min at 4°C. The supernatants were boiled at 100°C for 10 min, kept on ice and then centrifuged as before. Protein concentrations in the supernatants were determined by the Bio-Rad protein assay (Bradford, 1976) using bovine serum albumin as a standard. Aliquots containing equal amounts of heat-soluble proteins were precipitated with four volumes of acetone and then resuspended in SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.7, 2% SDS, 10% glycerol, 0.0003% bromophenol blue and 5% ß-mercaptoethanol). The mixture was heated for 10 min at 95°C, cooled and then fractionated by SDS-PAGE on 12% gels using a Hoefer Model SE 280 apparatus, according to the method of Laemmli (1970). After electroblotting onto nitrocellulose (using a BioRad Trans-Blot Semi-Dry Transfer Cell), dehydrin proteins were detected with rabbit anti-dehydrin serum (diluted 1/6000 before use), followed by goat anti-rabbit IgG (Promega, Madison, WI) to which was conjugated alkaline phosphatase. The anti-dehydrin serum was produced from a synthetic polypeptide containing a highly conserved sequence (KIKEKLPG) (kindly provided by T.J. Close) (Close *et al.*, 1993a, 1993b). The pre-immune serum was utilized as a control.

3.3. Results

3.3.1. The effects of desiccation and water stress treatments on the water content of castor bean seedlings

During the desiccation treatment, castor bean seedlings progressively lost water over the 7 d period. The water content of castor bean seedlings during the desiccation or water stress treatment, are shown in Figure 3.1. Seedlings

underwent the greatest loss of FW (approx. 25%) over the first 4 d of the desiccation treatment; the water-stress treatment resulted in very gradual water loss over 7 d in which 25% of the original FW was lost, while the desiccated seedlings lost 35% FW (Fig. 3.1). For both immature seeds and seedlings, the desiccation treatment resulted in a much faster rate of water loss than the water stress treatment and the extent of water loss at the end of the 7-d period was greater.

3.3.2. Dehydrins induced in developing seeds, seedlings and shoots of castor bean by ABA and different water-deficit-related treatments

I analyzed the stress inducibility of dehydrin protein synthesis in immature seeds of castor bean by subjecting them to ABA and various water-deficit-related treatments including desiccation, water stress, high salt, high osmolarity and low temperature. Further, I examined whether the dehydrins induced in immature seeds were similar to those induced following the transition to germination and growth (i.e. in mature seedlings subjected to stress or ABA treatment). Figure 3.2 (A) shows dehydrin proteins produced in developing fresh seeds at different stages of development. At 30 DAP, prominent bands on the Western blot had Mr ~ 25, 32, 46 and 60 KD. Of these, only the 25-KD products are dehydrin-related polypeptides; the others were detected on control Western blots using the pre-immune serum (Chapter 2). This profile of dehydrin production persists within the developing endosperm until at least 40 DAP. In the mature dry seed at 60 DAP, the nature of the dehydrin-related polypeptides accumulated in the endosperm changed dramatically. In particular, new polypeptides were detected with Mr ~ 28-30 KD, 33 KD and 41 KD (Fig. 3.2A). Figure 3.2 (B) shows the effect of

Figure 3.1 The water content of castor bean seedlings after desiccation and water stress treatments.

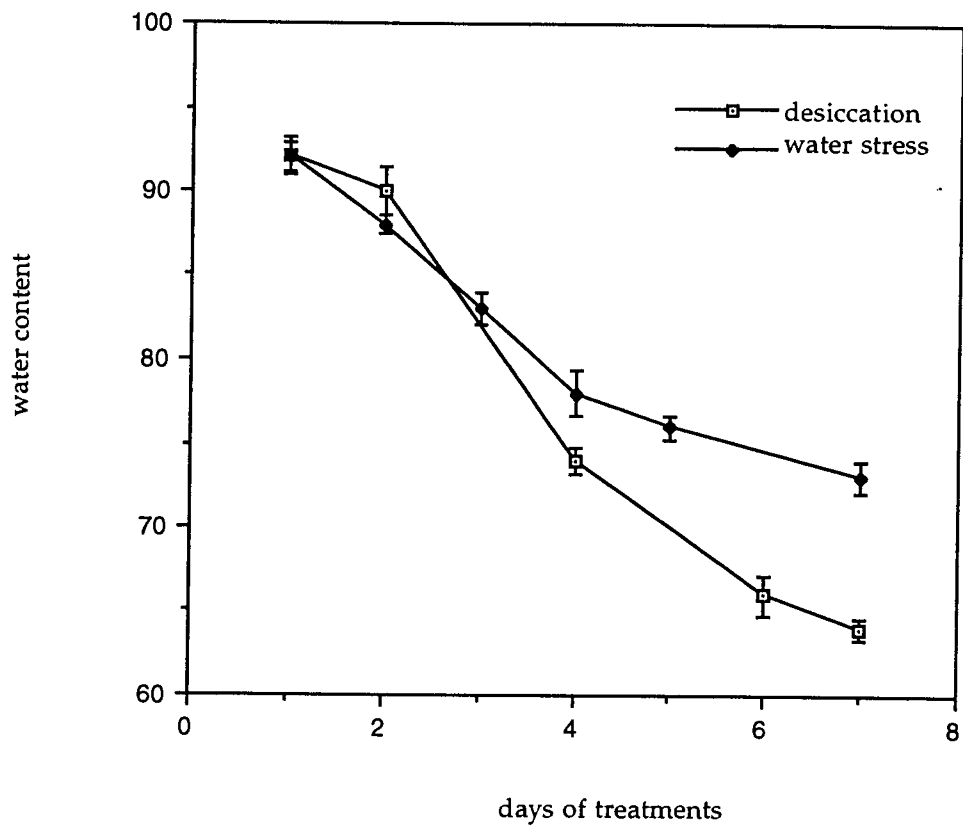
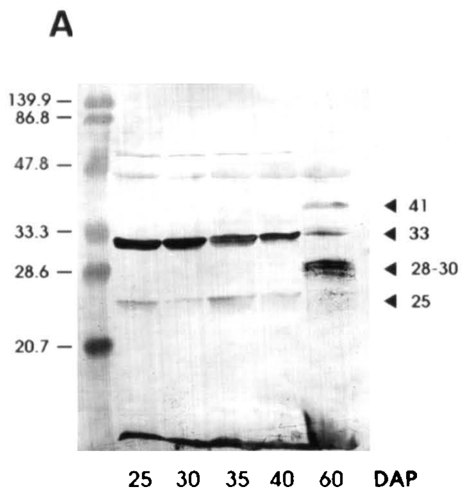

100
90
80
70
60
0
2
4
6
8
desiccation
water stress
water content
days of treatments

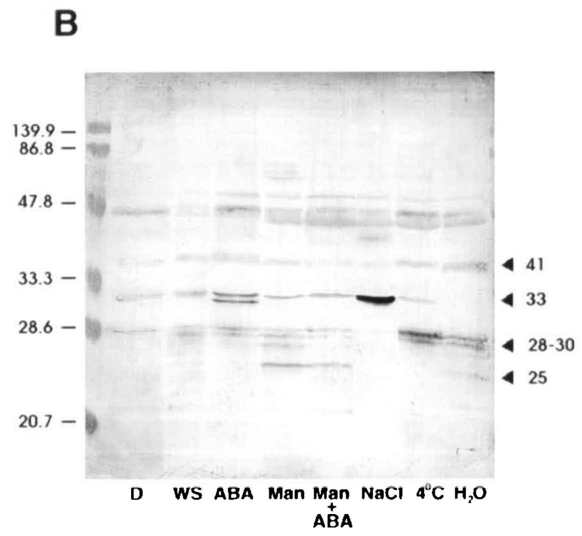
Figure 3.2. Changes in dehydrin-related proteins in the endosperm of immature (30 DAP) and mature (60 DAP) castor bean seeds (A) and in immature seeds (30 DAP) subjected to ABA and water-deficit-related stresses (B).

A and B are Western blots of heat-stable proteins (64 μg in 32 μl sample buffer) using a dehydrin antibody to detect dehydrin-related polypeptides. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated. D, desiccation; WS, water stress; ABA, abscisic acid (10^{-5} M); Man, mannitol.

A



B



exogenous ABA and the various stress treatments on dehydrin production in developing 30 DAP castor bean seeds. Most of the dehydrins produced in response to ABA or the various stress treatments in the endosperm (that do not appear on control blots, using the pre-immune serum; see table 3.1) were similar to those induced during normal late development, and are prominent in the mature dry (60 DAP) seed (Fig. 3.2A). However, the induction of this late developmental pattern may not be due to the stress imposed, but rather the period of detachment from the parent plant required to effect the treatment. For example, a similar profile of dehydrins was induced in 30 DAP seeds detached and hydrated on water for the same period required to effect the stress treatments (Fig. 3.2B; H_2O). However, there were some minor differences between different treatments, and some proteins were not induced by detachment alone. A doublet around 33 KD was detected in ABA treated seeds, a 21-KD band was induced in seeds subjected to Man+ABA and an intense 33-KD band was present following salt treatment. These developing seeds responsed differentially to these treatments.

The dehydrin-related polypeptides that are predominant within mature dry seeds persist following germination/growth of mature seeds for at least 48-72h after the start of imbibition (see Fig. 2.5; Chapter 2). To determine whether these polypeptides increase in seedlings subjected to stress or to exogenous ABA, five-d-old castor bean seedlings were treated with different concentrations of ABA. As shown in Figure 3.3, dehydrin-related polypeptides induced in the endosperm by ABA (when applied at concentrations in the range of 10^{-3} to 10^{-5} M) had Mr ~ 48 KD, different in size from those produced during late seed development. Surprisingly, these dehydrins were not produced in response to 10^{-6} M ABA, but did increase when seedlings were subjected to 10^{-3} M ABA, even though this latter concentration is probably

Table 3.1 Proteins detected by control Western blots using pre-immune serum*

treatments (7 days)	5-d-old seedling			immature seed (35 DAP)	mature seed (60 DAP)
	endosperm	cotyledon	radicle	endosperm	endosperm
ABA		51		48	
D		51, 43	25	48	
WS		51, 43	25	48	
man	48	51		48	
NaCl		51	51	48	
4°C		51		48	
H_2O					
GA**					34, 46
ABA+GA		51	32		
man+GA					
NaCl+GA					
4°C+GA					
no***				32, 64, 60	34, 46

ABA: abscisic acid, D: desiccation, WS: water stress, man: mannitol, GA: Gibberellin.

* The numbers in the table indicate the molecular weights (KD) of protein.

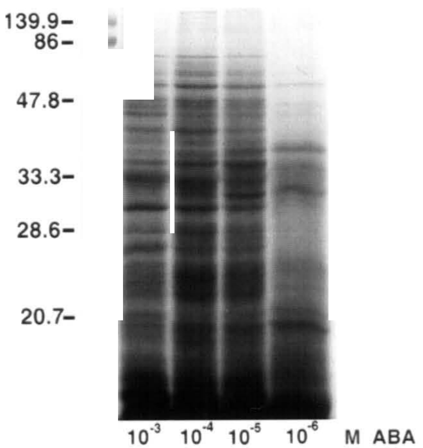
** Imbibed in GA solution (10^{-4} M) for 12 h.

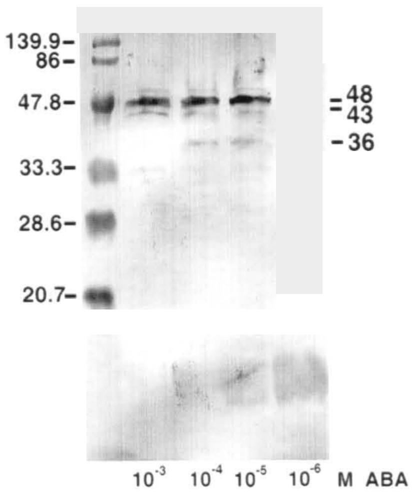
*** no treatment applied.

Figure 3.3. Changes in heat-stable proteins (upper panels) and dehydrin-related proteins (lower panels) in the endosperm, cotyledons and radicle of castor bean seedlings following treatment with different ABA concentrations.

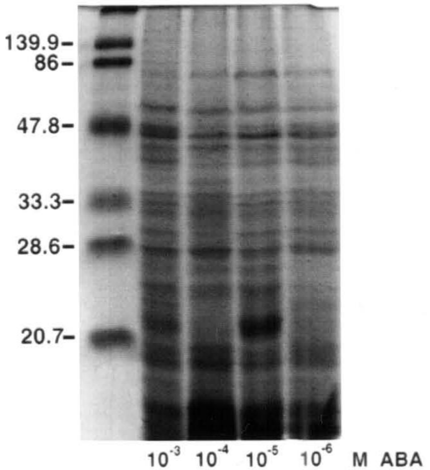
Upper panels are Coomassie blue-stained SDS-polyacrylamide gels of heat stable proteins extracted from the endosperm, cotyledons and radicle of ABA-treated seedlings. Samples were loaded with equal amount of protein (36 µg in 18 µl sample buffer containing 5% v/v ß-mercaptoethanol). Lower panels are Western blots of heat-stable proteins (64 µg in 32 µl sample buffer) using a dehydrin antibody to detect dehydrin-related polypeptides. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated.

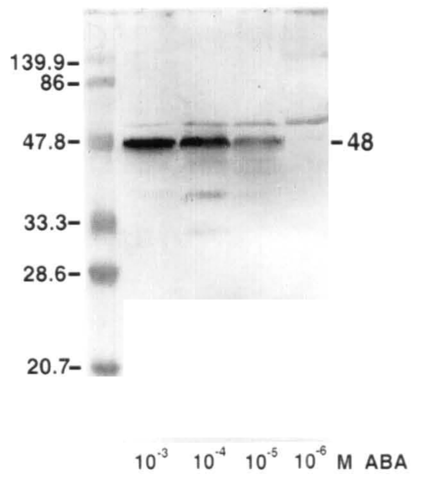
ENDOSPERM



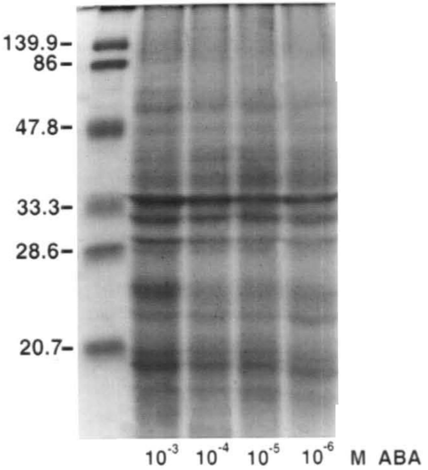


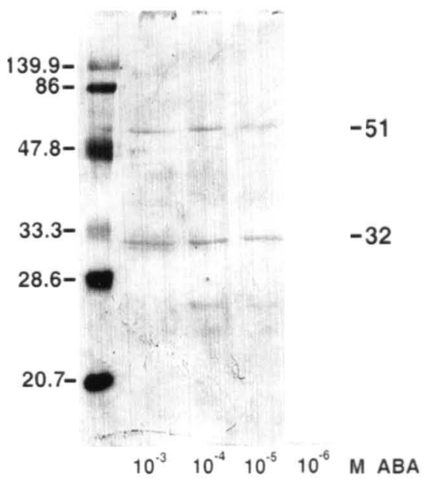
COTYLEDONS





RADICLE





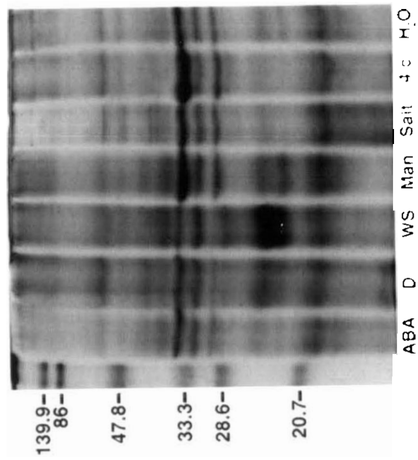
higher than endogenous ABA concentrations in stressed seedlings and might be expected to suppress cellular metabolism and dehydrin synthesis. This effective range of exogenous ABA in respect of eliciting dehydrin production was apparent in all three organs/tissues of the treated seedlings (endosperm, cotyledons and radicle; Fig. 3.3). As in the endosperm, Western blot analysis detected polypeptides of similar molecular weights (~ 48 KD) in the cotyledons of seedlings subjected to ABA treatment; in contrast, those produced in radicles had Mr ~ 51 and 32 KD (Fig. 3.3). Considerable changes in the profiles of extant proteins were observed in cotyledons and endosperm subjected to concentrations of exogenous ABA higher than 10^{-6} M (Fig. 3.3, upper panels).

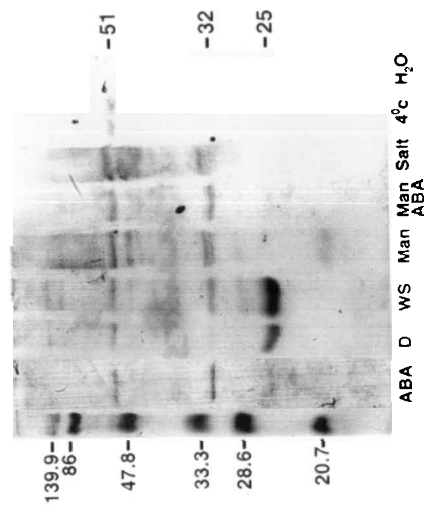
Different dehydrin genes may be induced by different stresses and these responses may also be tissue-specific. In barley seedlings, three dehydrin mRNAs respond differentially to salt, cold, mannitol and ABA (Espelund *et al.*, 1992). These dehydrin mRNAs also show different expression patterns during seed development. To determine differences between the responses of immature seeds versus mature seedlings, I examined the stress-inducibility of dehydrin production in mature seedlings. I was also interested in whether the various stress treatments would induce a set of dehydrin polypeptides similar to those induces by exogenous ABA treatment. Two distinct patterns of dehydrin production were elicited in the endosperm of castor bean seedlings subjected to the different stresses, in which there were both quantitative as well as qualitative differences (Fig. 3.4, Endosperm). Western blot analysis showed the induction of one set of dehydrins (Mr ~ 48 KD) in response to ABA, desiccation and water stress; another set, in which proteins with Mr ~ 43 KD were particularly prominent, was elicited by the high osmoticum (mannitol), high salt, and cold temperature treatments. Production of some of the Mr ~ 43-KD proteins also occurred following water stress and desiccation,

Figure 3.4. Changes in heat-stable proteins (upper panels) and dehydrin-related proteins (lower panels) in the endosperm, cotyledons and radicle of castor bean seedlings following ABA treatment and different water-deficit-related treatments.

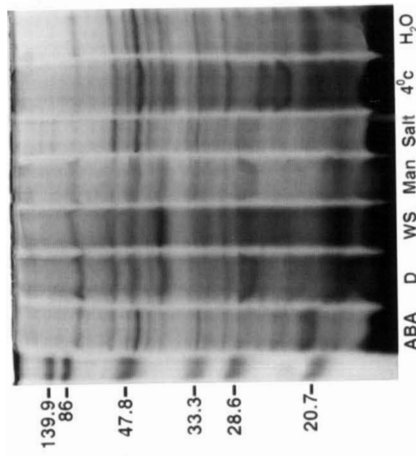
Upper panels are Coomassie blue-stained SDS-polyacrylamide gels of heat stable proteins extracted from the endosperm, cotyledons and radicle of treated seedlings. Lower panels are Western blots of heat-stable proteins using a dehydrin antibody to detect dehydrin-related polypeptides. Samples were loaded with equal amounts of protein as in Fig. 3.3. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated. (An exception is the 25-KD protein in radicles that is barely detectable on control blots of water stressed/desiccated seedlings). D, desiccation; WS, water stress; ABA, abscisic acid (10^{-5} M); Man, mannitol.

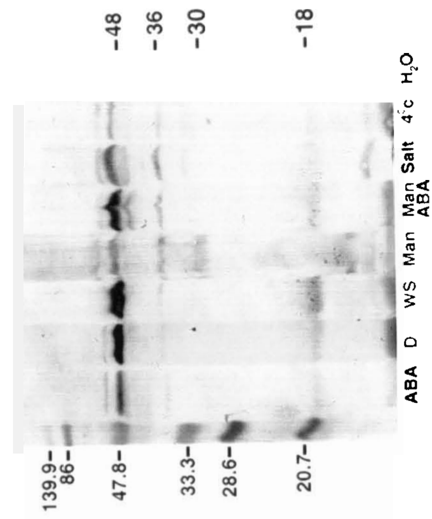
RADICLE



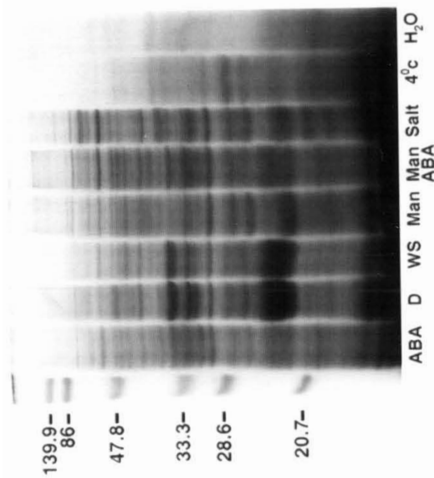


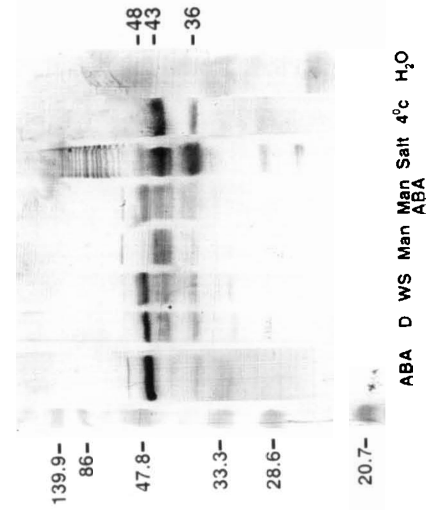
COTYLEDONS





ENDOSPERM





albeit in lower amount; in ABA-treated seedlings these proteins were barely detectable. Similarly, other dehydrin-related proteins (Mr ~ 36 KD) were common to most of the water-related stresses examined, although they were produced in much higher amounts in response to high salt and low temperature. Consistent with this, the combination of ABA and high osmoticum (ABA and Man, Fig. 3.4) resulted in the production of the 36-, 43- and 48-KD proteins in the endosperm. It is possible that ABA, desiccation and water stress share one signal transduction pathway which is ABA-dependent. On the other hand, high salt, high mannitol (high osmoticum), and cold may induce other dehydrin gene(s) via an additional signal transduction pathway that is ABA-independent.

Somewhat different results were found in relation to dehydrin production in the cotyledons and radicles of the stressed castor bean seedlings as compared to the ABA-treated seedlings. Here, most of the differences in dehydrin production appeared to be quantitative, rather than qualitative. In the cotyledons, all of the water-deficit-related stresses elicited the production of the Mr ~ 48-KD polypeptides (similar to exogenous ABA treatment), and to a lesser extent, proteins of Mr ~ 36-KD. Additional polypeptides of Mr ~ 30 KD (induced in mannitol-treated seedlings) and Mr ~ 18-KD (particularly evident in the seedlings subjected to desiccation, water stress and the combination of ABA and mannitol; Fig. 3.4) were also induced in response to ABA at 10^{-4} M (Fig. 3.3, Cotyledons). Proteins of Mr ~ 43 KD appeared to be elicited exclusively in the cotyledons of seedlings treated with high salt or the combination of ABA and mannitol. Like exogenous ABA treatment, the various water-related stress treatments induced the production of 51- and 32-KD proteins in the radicle (Fig. 3.4, Radicle). Proteins of Mr ~ 25-KD were particularly prominent in the radicles of seedlings subjected to desiccation and water stress, but these were

also detected (faintly) on control Western blots using pre-immune serum (Table 3.1).

In all three organs/tissues of castor bean seedlings, there were substantial changes in the profiles of extant proteins as a result of the different treatments (Fig. 3.4; Endosperm, Cotyledons, Radicle, upper panels). Most notable was the increased synthesis of proteins of Mr ~ 25 KD in the radicles of seedlings subjected to water stress.

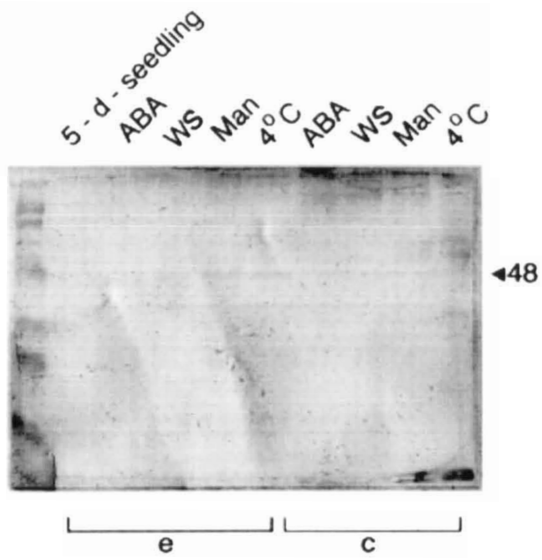
The finding that some dehydrin production is stress/organ-specific may indicate that dehydrin genes respond differently to certain inducers or the inducers stimulate dehydrin synthesis by different pathways. Dehydrin synthesis is organ/tissue-specific, and the signal transduction pathways may also be organ/tissue-specific.

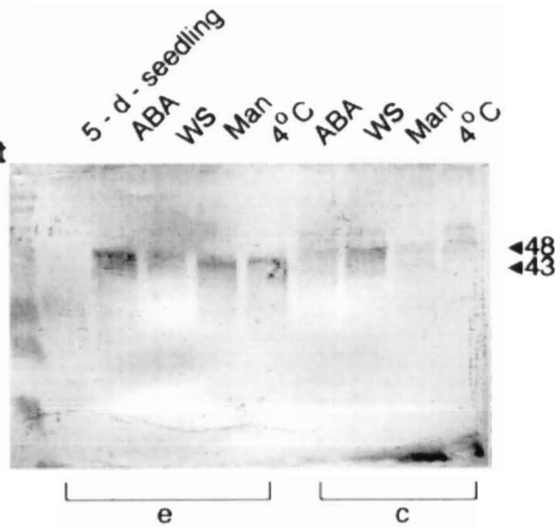
3.3.3. Dehydrins become detectable following the imposition of the water-deficit-related stresses between 2 and 4 days

Dehydrins were barely detectable in the endosperm and cotyledons of castor bean seedlings at 2 days following treatment with ABA (10^{-5} M) or the imposition of the water-deficit-related stresses (Fig. 3.5A). The dehydrins accumulated to much higher amounts after 4 days of treatment (Fig. 3.5B), but showed little change in abundance thereafter and in endosperm some of the dehydrins declined (Fig. 3.5C). In endosperm, a 36-KD band became more prominent after 7 days in seedlings subjected to the water stress and high mannitol treatments. (Fig. 3.5C).

Figure 3.5. Western blot analysis showing the timing of induction of dehydrin-related proteins in the endosperm and cotyledons of castor bean seedlings following ABA and some water-deficit-related treatments.

Samples were collected at 2 days (A), 4 days (B), and 7 days (C) following the start of the treatments. Samples were loaded with equal amount of protein as in Fig. 3.3. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated. ABA, abscisic acid (10^{-5} M); WS, water stress; Man, mannitol; 5-d-seedling, no treated 5 days old seedlings; e, endosperm; c, cotyledon.


(A) 2-d treatment
5 - d - seedling
ABA
WS
Man
4 °C
ABA
WS
Man
4 °C
◂48
e
c


(B) 4-d treatment
5 - d - seedling
ABA
WS
Man
4 °C
ABA
WS
Man
4 °C
◂48
◂43
e
c

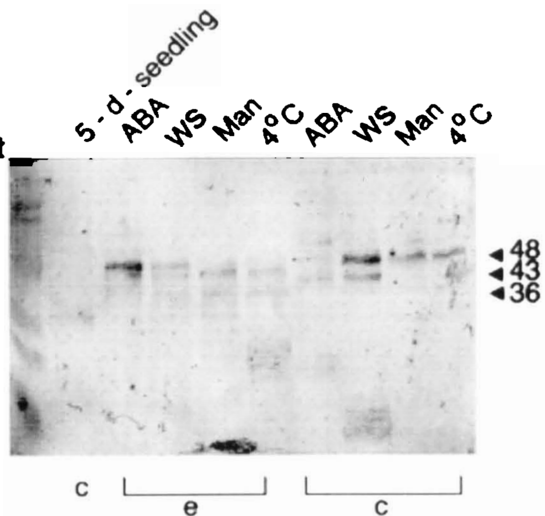
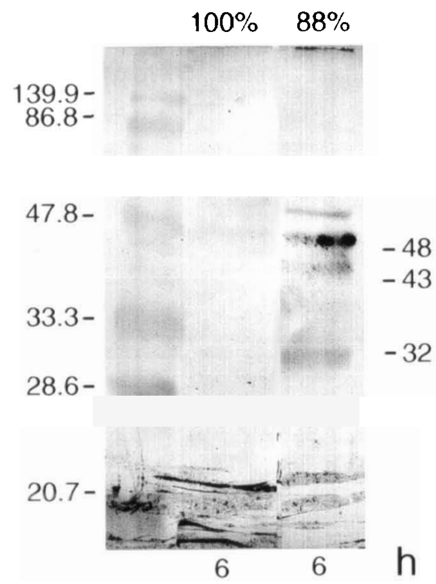

(C) 7-d treatment
5 - d - seedling
ABA
WS
Man
4 °C
ABA
WS
Man
4 °C
◂48
◂43
◂36
c
e
c

Figure 3.6 Western blot analysis of dehydrins induced in the shoots of castor bean plants after a short dehydration treatment.

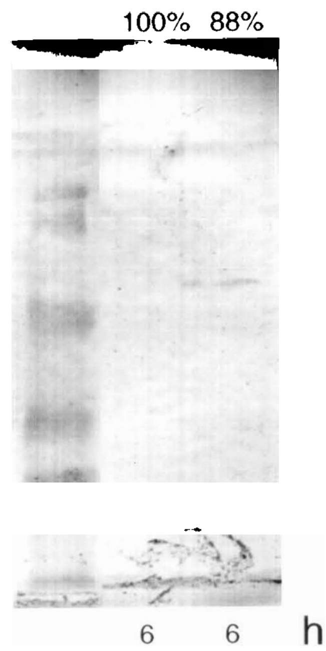
Samples were loaded with equal amount of protein as in Fig. 3.2. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated. 100% and 88%: the percentage of original fresh weight (fresh weight after treatment / fresh weight before treatment x 100%); 6 h: duration of maintenance of shoots in sealed plastic bags.

Shoots

immune



pre-immune



3.3.4. Dehydrins are induced in vegetative tissues (shoots) by drying treatment

Three major dehydrin polypeptides (48 KD, 43 KD and 32 KD) were detected in castor bean shoots after their maintenance for 6 h in sealed plastic bags following a 12% loss of fresh weight (Fig. 3.6). Similar dehydrin proteins were detected in different organs of seedlings (Fig. 3.4) when the seedlings were treated by water stress (Compare Figs. 3.6 and 3.4).

3.3.5. GA_3 and the fate of dehydrin proteins following germination of mature seeds

Figure 3.7 shows the changes in heat-stable and dehydrin-related polypeptides of mature seeds following imbibition in 10^{-4} M GA_3 solution. Many of the dehydrin-related polypeptides (28-30 KD, 41 KD and 45 KD) (Fig. 3.7B) declined more rapidly when mature seeds were imbibed in GA_3 solution (after 72 h of imbibition) than when they were imbibed in water (Fig. 2.5). The more rapid decline of dehydrin proteins in the presence of GA_3 may be due to the hormone speeding up germination or the result of a larger amount of proteinases stimulated by GA_3.

3.3.6. Effects of GA_3 on dehydrin production in castor bean seedlings elicited by ABA and water-deficit-related stresses

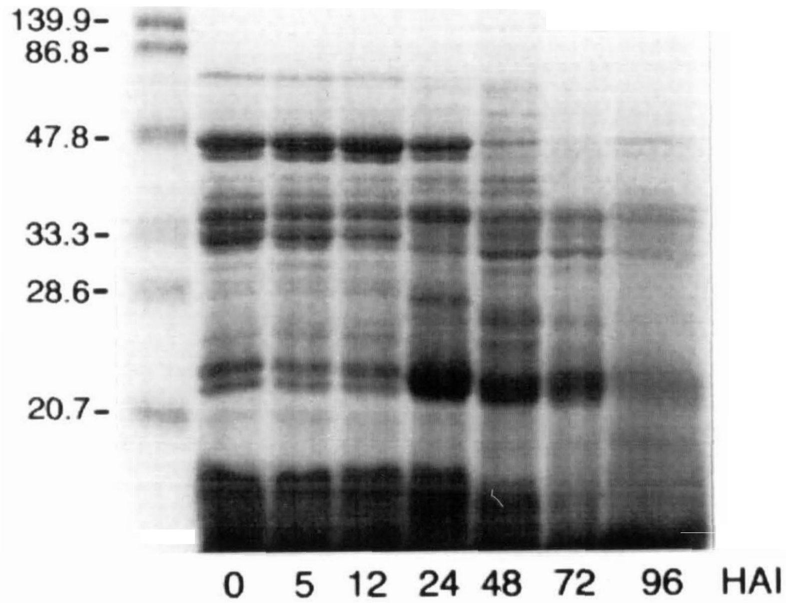
In order to further address the possible role of ABA in mediating plant responses to water-deficit-related stresses, I examined the effects of an antagonist of ABA action, i.e. gibberellic acid (GA_3). Figure 3.8 shows the effects of GA_3 on dehydrin induction when castor bean seedlings were exposed

Figure 3.7. Changes in heat-stable proteins (A) and dehydrins (B) in castor bean endosperm during germination and growth of mature seeds imbibed in GA_3 (10^{-4} M).

A. Coomassie blue-stained SDS-polyacrylamide gel of heat-stable proteins extracted from endosperm of mature seeds imbibed from 0-96 h. B. Western blot of heat-stable proteins using dehydrin antibody to detect dehydrin-related polypeptides. Samples were loaded with equal protein as in Fig 3.3. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated.

Mature - Imbibed in GA

A. Stained Gel



B. Western Blot

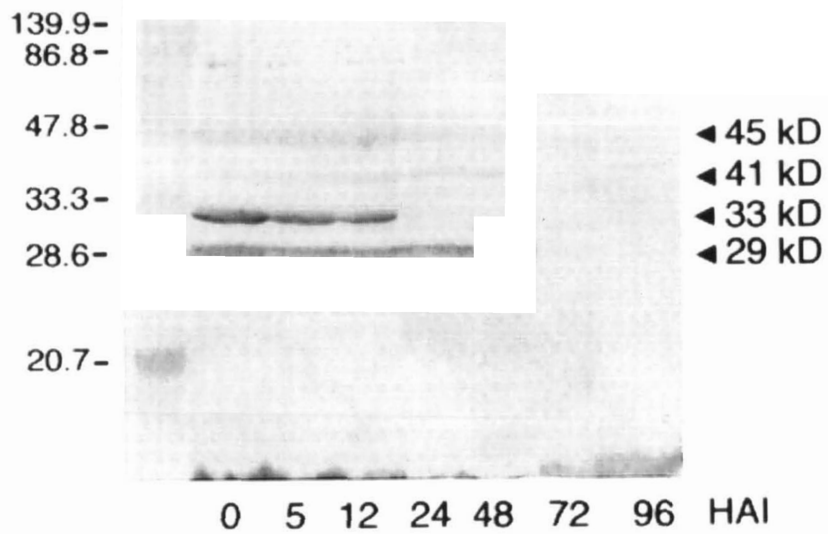
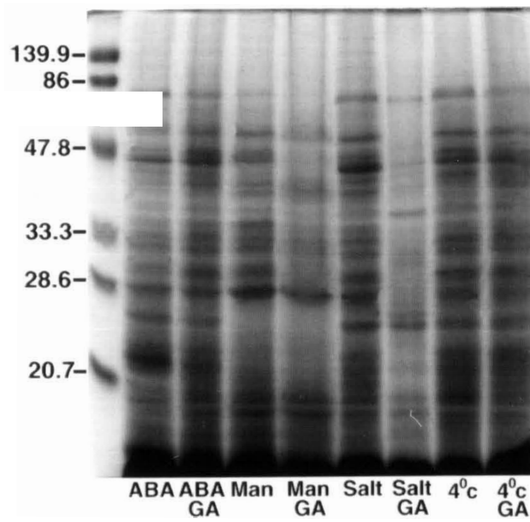
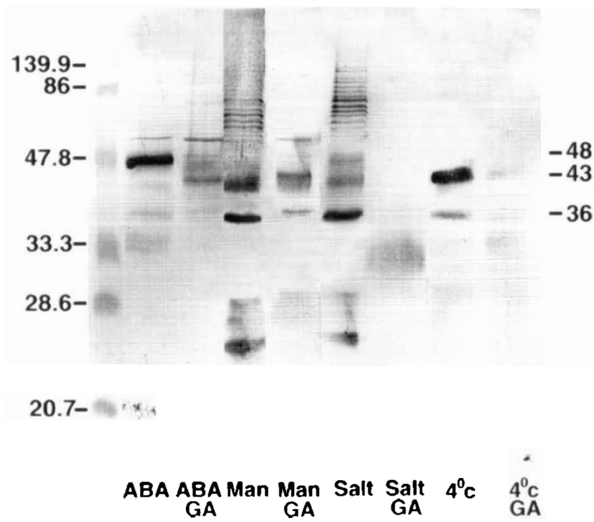


Figure 3.8 Effect of GA3 on production of dehydrin-related proteins in the endosperm of castor bean seedlings in response to ABA treatment and water-deficit-related treatments.

Upper panel is a Coomassie blue-stained SDS-polyacrylamide gel of heat stable proteins extracted from the endosperm of seedlings exposed to ABA or the stress treatment alone or from the endosperm of seedlings simultaneously exposed to GA3 (10^{-4} M) and either ABA or the water-deficit-related stress (cold, high salt or mannitol). Lower panels are Western blots of heat-stable proteins using a rabbit antibody to detect dehydrin-related polypeptides. Samples were loaded with equal amount of protein as in Fig. 3.3. Numbers on the right indicate the approximate molecular weights of the dehydrin-related polypeptides; only those polypeptides not found on control Western blots using pre-immune serum are indicated. ABA, abscisic acid (10^{-5} M); Man, mannitol; GA, gibberellin (10^{-4} M).

ENDOSPERM





simultaneously to GA_3, and either ABA, high salt, high mannitol or low temperature. In the endosperm, the production of the 48-KD dehydrins elicited following exposure of seedlings to exogenous ABA, water stress, and desiccation (Fig. 3.4), was completely suppressed when seedlings were exposed simultaneously to ABA and GA_3 (Fig. 3.8). Salt and low temperature induced dehydrin-related proteins of 36- and 43-KD in relatively high amounts in the endosperm (Figs 3.4 and 3.8); production of both these proteins was effectively suppressed when GA was applied simultaneously with the stress treatment (Fig. 3.8). An exception occurred in seedlings treated with mannitol, in which GA was relatively ineffective in preventing the production of the 36- and 43-KD proteins.

3.4. Discussion

In the present study, we examined the stress inducibility of dehydrin protein production in immature seeds and seedlings of castor bean by subjecting them to ABA and various water-deficit-related treatments including desiccation, water stress, high salt, high osmolarity and low temperature. In castor bean, production of dehydrin-related polypeptides was organ-specific, dependent upon the physiological stage of the seed and exhibited some qualitative and quantitative differences in response to different water-deficit-related stresses. In several other species, dehydrin production exhibits tissue/organ-specificity. For example, in tomato and wheat plants, dehydration leads to the production of dehydrin/LEA proteins exclusively within the shoot (Reid and Walker-Simmons, 1993), with no synthesis occurring in the roots. However, in other plants or seeds, dehydrin/LEA proteins are present in all tissue types (e.g., in wheat seedlings and in embryos of maize and cotton)

(Danyluk *et al.*, 1994; Asghar *et al.*, 1994; Roberts *et al.*, 1993). Several researchers have restricted their analyses of dehydrin gene expression to specific tissue types and to certain stress conditions and it is not yet clear whether dehydrin genes which are expressed in cells of different tissues respond equally to ABA and water-deficit-related stresses.

Stress-specific differences in dehydrin synthesis were found in different organs/tissues of castor bean seedlings; however, dehydrins inducible by exogenous ABA were consistently produced in the cotyledons and radicle of stressed seedlings. On the other hand, two distinct patterns of dehydrin production were elicited in the endosperms of castor bean seedlings subjected to the different stresses -- one in response to ABA, desiccation and water stress; another was elicited by the high osmoticum (mannitol), high salt, and cold temperature treatments. Although we can not make any conclusions about the differential induction of specific dehydrin genes in castor bean, others have found that ABA and water-deficit-related stresses affect plant molecular processes through different pathways and induce different dehydrin genes. In *Arabidopsis thaliana*, two dehydration responsive genes (*rd29A* and *rd29B*) respond differently to ABA, drought, cold and salt (Yamaguchi-Shinozaki and Shinozaki, 1994). The authors propose the existence of at least two independent signal transduction pathways (one which is ABA-independent and the other, ABA-responsive), between the environmental stress and the expression of the two genes. Both pathways are involved in the expression of one dehydration responsive gene (*rd29A*); the other (*rd29B*) requires only the ABA responsive pathway for induction. For example, the *rd29A* gene has at least two *cis*-acting elements. One appears to be involved in an ABA-associated slow response to dehydration (ABRE), and the other may function in ABA-independent rapid induction (DRE). The stresses also function differently; salt or drought may

affect gene expression by the ABA-dependent pathway, while cold or drought stimulate gene expression by the ABA-independent pathway. Further support for two pathways in *A. thaliana* comes from studies of ABA-deficient and ABA-insensitive mutants in relation to freezing tolerance (Mäntylä *et al.*, 1995).

More work is needed to clarify whether there are dehydrin genes in castor bean that respond differently to specific stress inducers or whether different inducers stimulate dehydrin synthesis by different pathways. Dehydrin synthesis is often tissue-specific and the signal transduction pathways may also be tissue-specific.

Dehydrin production was also dependent upon the duration of the stress treatment and the rate of water loss may be important to this physiological process. For example, drying of castor bean shoots, led to a FW (fresh weight) decline of 88% within 40 min and dehydrins were detected within a short period of time (6 h after the treatment). However, in castor bean seedlings subjected to water stress, dehydrins were undetectable at 2 days of the treatment. A lack of dehydrin induction may be due to the very gradual rate of water loss effected by the treatment (FW loss was less than 12% after 2 d). Only after 4 days of water stress, were dehydrins induced. Furthermore, different developmental stages of the plant (vegetative shoots vs. seedlings) may also exhibit different rates of dehydrin production.

Chapter 4.

Production of dehydrins in Seeds and Seedlings of a recalcitrant species (*Castanospermum australe*) in Response to ABA and Water-Deficit-Related Stresses

4.1 Introduction

Unlike the seeds of most angiosperm species, which pass through a period of natural dehydration on the plant before shedding, there is a group of species which shed their seeds at high moisture contents. Although desiccation tolerance increases throughout development, mature seeds of these species do not survive drying (Finch-Savage 1992, Hong and Ellis, 1990) and they have been termed "recalcitrant" because of their limited storage potential (Roberts 1973). The cause of their desiccation sensitivity, and indeed the mechanism of desiccation tolerance in seeds of orthodox species, is not well understood. It has been suggested that the increasing desiccation sensitivity in recalcitrant seeds during storage may be due to the initiation of germination associated events and may, therefore, be analogous to the sensitivity of germinating seeds of orthodox species (Berjak *et al.*, 1984).

The question arises as to whether the desiccation sensitivity of recalcitrant seed is at least partially the result of an insufficient accumulation of dehydrins or whether other factors are more important, including a lack of protective sugars, an inability to rapidly effect mechanisms that serve to repair desiccation-induced damage upon subsequent rehydration and the proportion and distribution of freezable and non- freezable (bound) water within the seed (Berjak et al., 1992; reviewed in Bewley and Oliver, 1992; Vertucci and Farrant, 1995). Dehydrins have been reported to be absent during the late stages of development of recalcitrant mangrove (*Avicennia marina*) seeds, a finding suggested to account for the lack of desiccation tolerance of this species (Farrant *et al.*, 1992). However, only heat-stable proteins were examined and use of the dehydrin antibody will be necessary to confirm these results. Work with *Zizania palustris*, a minimally recalcitrant seed type, indicates that dehydrins

can be detected by the maize dehydrin antiserum in both embryos and seedlings (Bradford and Chandler, 1992). The *Z. palustris* embryos and seedlings are also capable of ABA accumulation during limited dehydration. The intolerance of *Z. palustris* seeds to dehydration at low temperature does not seem to be due to an absence of dehydrins or an inability to accumulate ABA (Bradford and Chandler, 1992). This indicates that the presence of dehydrins alone is not sufficient to prevent desiccation injury (Blackman *et al.*, 1991, Bradford and Chandler 1992). Finch-Savage *et al.* (1994) detected dehydrins in mature seeds of many desiccation sensitive (recalcitrant) trees, and *Lea* mRNA can also be induced in stored recalcitrant seeds of *Quercus robur* L. (English oak) by exogenous ABA and limited drying treatments.

Although all recalcitrant seeds are considered to be desiccation sensitive, the degree of water loss tolerated varies with the species; hence, dehydrin gene expression needs to be investigated in a wider range of recalcitrant seed types. Further, temporal studies to examine changes in dehydrin production during development and following germination are necessary. This is especially important since there is often no clear-cut event delineating the end of seed development and the start of germination in recalcitrant species. Moreover, even though seeds of recalcitrant species may be capable of dehydrin synthesis during their development, a decline in the synthesis of dehydrins (or an inability to produce specific dehydrins) following the transition to germination/seedling growth may be an important factor contributing to losses in viability after seed shedding or during storage.

Castanospermum australe is grown as a street tree in Pietemariztbury, South Africa. *C. australe* is a recalcitrant species; its seeds are large and do not undergo substantial maturation drying during development. The seeds are shed at high water content and are desiccation sensitive. The responses of

seeds to desiccation are commonly assessed by electrolyte leakage and the rate of electrolyte leakage is an indicator of the degree of tissue damage induced by drying. Following rapid drying of *C. australe* excised axes, the electrolyte leakage rate is 1.0 g/g DW.

In this chapter, a dehydrin antibody, provided by T. Close (Close *et al.*, 1993a), was used to determine if dehydrins are present in immature and mature seeds of *Castanospermum australe*. Further, dehydrin accumulation in seeds and seedlings of *C. australe* in response to ABA and various water-deficit-related stresses was assessed. In order to determine whether dehydrins were present in other recalcitrant species, seeds of *Artocarpus heterophyllus* were also analyzed.

4.2. Materials and Methods

4.2.1. Plant material

Seeds of *C. australe* were collected at mid-maturation and at maturity. Axes and cotyledons were obtained from immature seeds at mid-maturation, from mature seeds and from 5-d-old seedlings (obtained by germinating mature seed). Some of the immature seeds and seedlings were used in the fresh state; other were first subjected to the various treatments outlined below.

A. heterophyllus is grown in private gardens in Durban. Seeds of this species were collected at mid-maturation and at maturity.

4.2.2. Seed germination

Mature seeds were surface-sterilized in a solution of 1% hypochlorite,

0.01% SDS for 10 min, and then rinsed three times in sterilized water. The seed coats were then removed under asceptic conditions in a flow hood. Seeds were placed in Petri dishes containing sterile filter paper moistened with 10 ml of sterile water and allowed to germinate at 26°C in the dark. After 5 d, seedlings were used for the various treatments.

4.2.3. ABA treatments

Five-d-old seedlings were incubated for up to 7 d in different concentrations of ABA (10^{-3} M, 10^{-4} M, 10^{-5} M, and 10^{-6} M). For these treatments, the seedlings were placed in Petri dishes containing sterile filter paper and 10 ml of the different ABA solutions (sterilized by passing through a 0.22 µm filter). The 7-d treatment was carried out under ambient laboratory conditions.

4.2.4. Water stress treatment

Immature seeds and 5-d-old seedlings were subjected to water stress by equilibrating them in a desiccator over 25% glycerol. The osmotic potential of the glycerol solution was determined to be -12.6 MPa, which produces a relative humidity in the chamber of approximately 87%. The treatment was carried out for up to 7 d under ambient laboratory conditions.

4.2.5. Desiccation treatment

Immature seeds and 5-d-old seedlings were dried slowly to known percentage water contents by placing them in a desiccator over stirred saturated

salt solutions. The drying regime used for 30 DAP seeds and for 5-d-old seedlings was as described in Kermode and Bewley (1985) (see 30 DAP seed treatment).

4.2.6. High salt and high osmolarity treatments

Immature seeds and 5-d-old seedlings were placed in Petri dishes containing sterile filter paper moistened with either 10 ml of 0.2 M NaCl (high salt treatment) or 10 ml of 0.3 M mannitol (high osmolarity treatment). Both solutions were previously sterilized by passing through a 0.22 μm filter. The treatment was carried out for up to 7 d under ambient laboratory conditions.

4.2.7. Cold treatment

Immature seeds and 5-d-old seedlings were placed in Petri dishes containing sterile filter paper moistened with 10 ml of sterile water, and then maintained at 4°C for up to 7 d.

4.2.8. Extraction of heat stable proteins and Western blot analysis

Heat stable proteins were extracted from endosperms isolated from immature seeds and seedlings, and from cotyledons and radicles isolated from seedlings as outlined in Close *et al.* (1993a). Briefly, total soluble proteins were extracted by grinding tissues in 30 mM TES buffer pH 8.0, containing 20 mM NaCl and 1 mM PMSF using a glass ground homogenizer. This was followed by centrifugation at top speed in a microcentrifuge for 15 min at 4°C. The supernatants were boiled at 100°C for 10 min, kept on ice and then centrifuged

as before. Protein concentrations were determined by the Bio-Rad protein assay (Bradford, 1976) using bovine serum albumin as a standard. Aliquots containing equal amounts of heat stable proteins were precipitated with four volumes of acetone and then resuspended in SDS-PAGE sample buffer (0.125 M Tris-HCl, pH 6.7, 2% SDS, 10% Glycerol, 0.0003% bromophenol blue and 5% ß-mercaptoethanol). The mixture was heated for 10 min at 95°C, cooled and then fractionated by SDS-PAGE on 12% gels using a Hoefer Model SE 280 apparatus, according to the method of Laemmli (1970). After electroblotting onto nitrocellulose (using a BioRad Trans-Blot Semi-Dry Transfer Cell), dehydrin proteins were detected with rabbit anti-dehydrin serum (diluted 1/6000 before use), followed by goat anti-rabbit IgG (Promega, Madison, WI) to which was conjugated alkaline phosphatase. The anti-dehydrin serum was produced from a synthetic polypeptide containing a highly conserved sequence (KIKEKLPG) (kindly provided by T.J. Close) (Close *et al.*., 1993a, 1993b). The pre-immune serum was utilized as a control.

4.3 Results

4.3.1. Dehydrin proteins accumulate during development of *C. australe*

Similar to orthodox species (e.g. castor bean), new dehydrins were synthesized as seeds of *C. australe* matured. Figure 4.1 shows dehydrins in axes and cotyledons of mature and immature seeds of *C. australe* determined by Western blotting. In immature seeds, 31-KD and 85-KD dehydrins were detected in axes (Fig. 4.1, lane 3); both dehydrins were not present in mature axes, in which polypeptides of 37 and 40 KD accumulated (Compare Lane 3 with Lane 1). In immature cotyledons, only the 31-KD polypeptide was

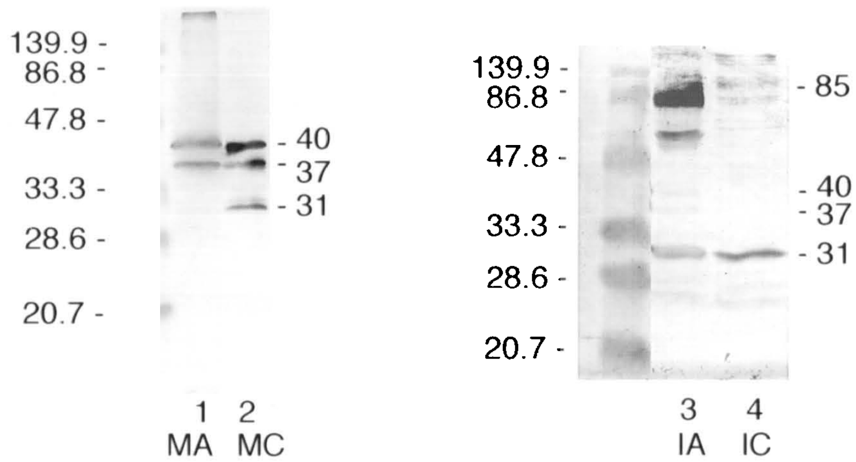
Figure 4.1. Western blot analysis of dehydrins in C. *australe* seeds at mid-maturation and maturity.

Lane 1: mature axes; Lane 2: mature cotyledons; Lane 3: immature axes; Lane 4: immature cotyledons. Dehydrins were detected by a dehydrin antiserum and blots using the pre-immune serum served as controls. Heat-stable protein (64 μg in 32 μl sample buffer) was loaded onto each lane. Numbers on the right indicate the approximate molecular weights (KD) of the dehydrins; only those proteins not found on control Western blot are indicated.

Castanospermum australe

- mature and immature seeds

immune



pre-immune

predominant, but at maturity polypeptides of 31-, 37- and 40-KD were prominent (Compare Lane 4 with Lane 2). Thus, the nature of the dehydrins accumulated in the cotyledons and axes changes as seeds matured.

4.3.2. New Dehydrins are induced in immature seeds by ABA and moderate stresses but not in response to full desiccation

Figure 4.2 shows dehydrins detected by Western blotting in axes and cotyledons of immature C. *australe* seeds following treatment of seeds with ABA or various water-deficit-related stresses. Figure 4.2(A) shows that a 40 KD dehydrin was induced in the axis by ABA, water stress, high osmolarity, high salt and cold, while the 31- and 85-KD proteins present in untreated axes declined. A desiccation treatment that resulted in more extreme water loss, did not elicit dehydrin synthesis. In cotyledons, 40-KD dehydrins were induced by the above treatments (possibly due to seed detachment), and similar to the situation in axes, no dehydrins were present following the desiccation treatment (Fig. 4.2B). Thus it appears that dehydrins fail to accumulate under conditions of extreme water loss. However, the possibility can not be ruled out that the seeds do not survive the treatment and therefore are incapable of protein synthesis. Dehydrins were present in cotyledons of seeds subjected to a control water treatment but not in the axes of these control seeds. As in castor bean (Chapter 3), the detachment of immature seeds from the parent plant can induce dehydrin production. There was no 31-KD dehydrin in ABA-treated cotyledons of seeds; instead a 37-KD protein was detected (Fig. 4.2B). The 37-KD protein was also detected in ABA-treated axes (Fig. 4.2A). All concentrations of ABA (10^{-3} to 10^{-6} M) induced 40-KD and 37-KD dehydrin proteins in whole seeds (axis and cotyledons) (Fig. 4.2C).

Figure 4.2. Western blot analysis of changes in dehydrins in the axes and cotyledons of immature *C. australe* seeds subjected to ABA and various water-deficit-related stresses.

(A): Axis of immature *C. australe* seeds subjected to various water-deficit-related stresses. (B): Cotyledons of immature *C. australe* seeds subjected to various water-deficit-related stresses. (C): Whole seeds of immature *C. australe* seeds subjected to different concentrations of ABA. Dehydrins were detected by a dehydrin antiserum and blots using the pre-immune serum served as controls. Heat-stable protein (64 μg in 32 μl sample buffer) was loaded onto each lane. Numbers on the right indicate the approximate molecular weights (KD) of the dehydrins; only those proteins not found on control Western blot are indicated. ABA, abscisic acid (10^{-5} M); D, desiccation; WS, water stress; Man, mannitol.

Castanospermum australe
-Immature seeds

(A) axis

immune

139.9-
86.8-
47.8-
33.3-
28.6-
20.7-

◄40
◄37

ABA D WS Man Nacl 4°C H_2O

pre-immune

ABA D WS Man Nacl 4°C H_2O

(B) coty

immune

139.9-
86.8-
47.8-
33.3-
28.6-
20.7-

◄40
◄37
◄31

ABA D WS Man Nacl 4°C H^2O

pre-immune

ABA D WS Man Nacl 4°C H^2O

(C) whole seed

139.9-
86.8-
47.8-
33.3-
28.6-
20.7-

◄40
◄37

10^{-3} 10^{-4} 10^{-5} 10^{-6} M ABA

4.3.3. Dehydrins are detectable following ABA and various water-deficit-related treatments in the cotyledons of *C. australe* seedlings, but are undetectable in the roots of the seedlings

Figure 4.3 shows dehydrins detected by Western blot analysis in cotyledons and roots of *C. australe* seedlings following treatments of seedlings with different water-deficit-related stresses. In all the experiments, 5-d-old seedlings were subjected to 7-d treatments. After germination for 5 days, the dehydrins present in cotyledons of mature seeds persisted (Compare Fig. 4.1 mature cotyledons, Lane 2, with Fig. 4.3, Con). Following the 7-d treatments, in the cotyledons of the seedlings , all dehydrins became less abundant with the exception of a 40-KD protein in cold treated-seedlings (4°C). Even in the water-treated seedlings, some of the dehydrins persisted (40 and 37 KD), although the 31-KD protein declined. Dehydrins were not detectable in cotyledons of desiccation-treated seedlings, which was similar to the situation of the desiccation-treated immature seeds (Fig. 4.2, B).

Interestingly, no dehydrins were detectable in the roots of *C. australe* seedlings either after 5 days of germination or following the different water-deficit-related treatments (Fig. 4.3). A lack of dehydrin synthesis in the roots may contribute to desiccation sensitivity in *C. australe.*

Figure 4.4 shows dehydrins detected by Western blot analysis in cotyledons and roots of *C. australe* seedlings following ABA treatments of the seedlings. As in Figure 4.3, seedlings were treated with ABA for 7 days after 5 days of germination. In the cotyledons of seedlings, following 7 days of treatment with 10^{-5} and 10^{-6} M ABA, all three dehydrin bands (40 KD, 37 KD and 31 KD) persisted (Compare Fig. 4.4 Coty, 10^{-5} M ABA and 10^{-6} M ABA with Fig. 4.3 Coty, Con). In the 10^{-4} M ABA treated seedlings, the 31-KD dehydrin

Figure 4.3. Western blot analysis of changes in dehydrins in cotyledons and roots of C. *australe* seedlings subjected to various water-deficit-related stresses. Untreated seedlings (5-d-seedlings: Con) and seedlings maintained on water (H_2O) served as controls.

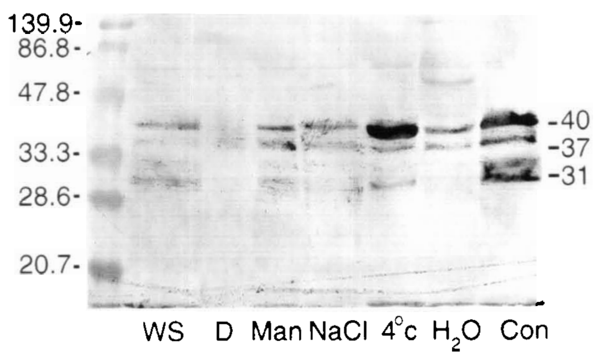
Dehydrins were detected by a dehydrin antiserum and blots using the pre-immune serum served as controls. Heat-stable proteins (64 μg in 32 μl sample buffer) were loaded onto each lane. Numbers on the right indicate the approximate molecular weights (KD) of the dehydrins; only those proteins not found on control Western blot are indicated.

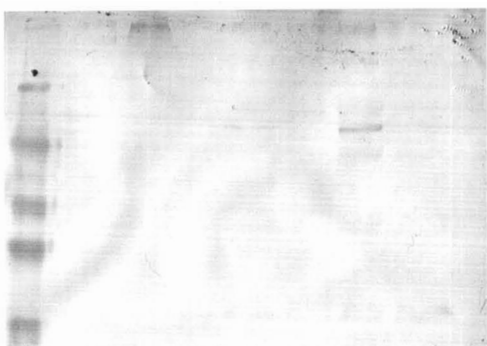
Castanospermum australe
-seedlings

immune

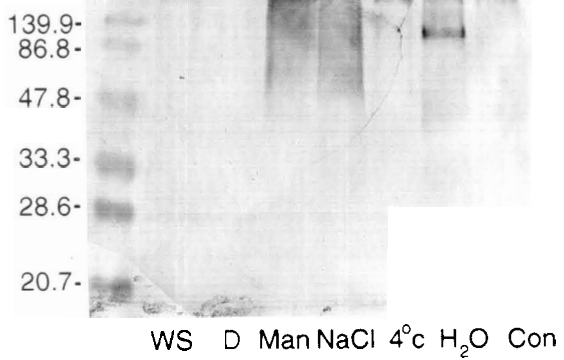
pre-immune

Coty





Root



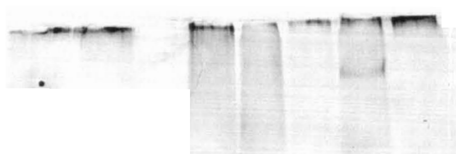
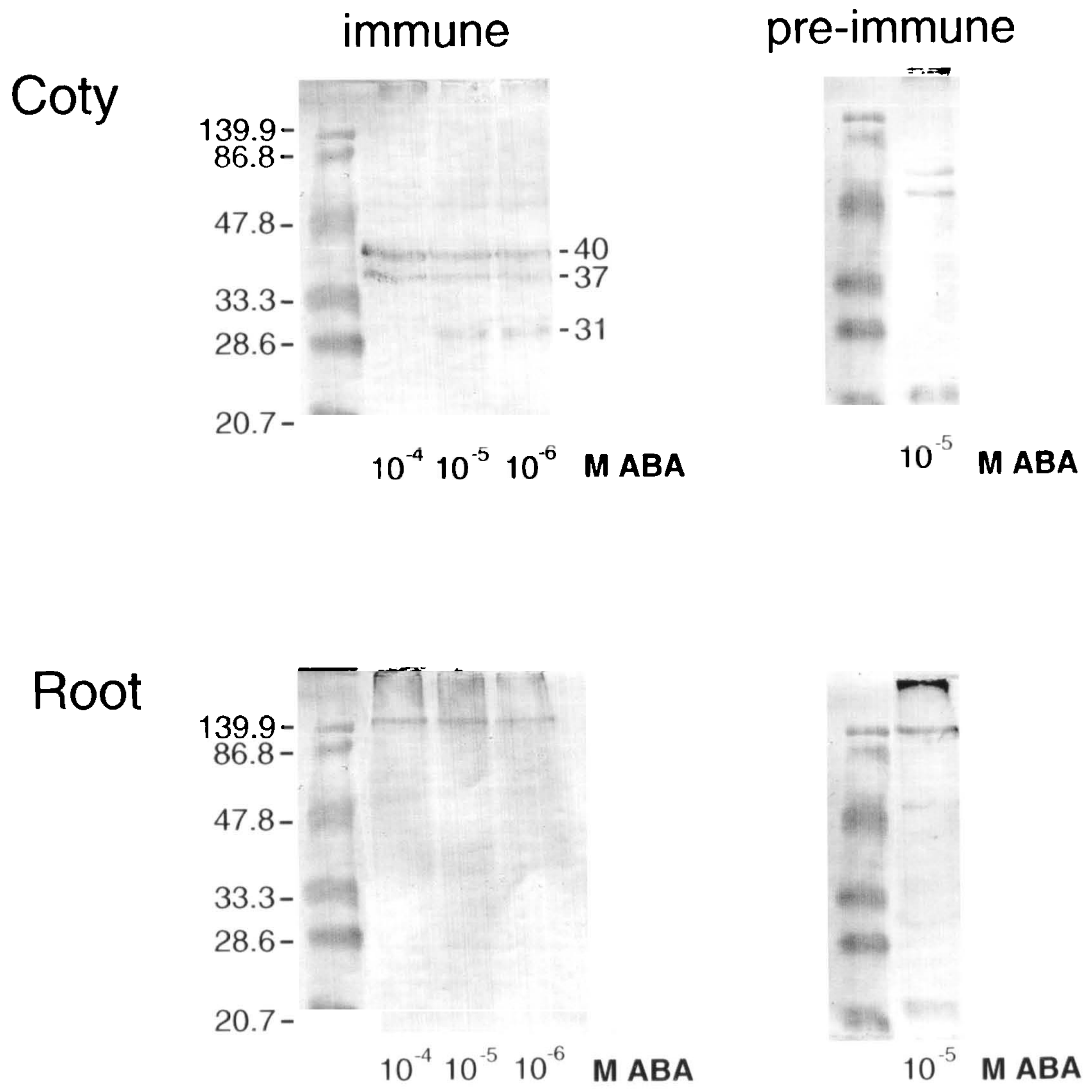


Figure 4.4. Western blot analysis of the changes in dehydrins in cotyledons and roots of *C. australe* seedlings subjected to different concentrations of ABA.

Dehydrins were detected by a dehydrin antiserum and blots using the pre-immune serum served as controls. Heat-stable protein (64 μg in 32 μl sample buffer) was loaded onto each lane. Numbers on the right indicate the approximate molecular weights (KD) of the dehydrins; only those proteins not found on control Western blot are indicated.

Castanospermum australe
-seedlings



was undetectable in cotyledons, but 40-KD and 37-KD dehydrins were obvious. Consistent with the response to the stresses, in the roots, no dehydrins were detected when seedlings were subjected to all three different concentrations of ABA (Fig. 4.4 Root).

4.3.4. Dehydrin proteins accumulate during development of *A. heterophyllus*

Figure 4.5 shows dehydrins in the axes and cotyledons of mature and immature seeds of *A. heterophyllus* determined by Western blotting. In the axes of the mature seed, 50-KD and 52-KD dehydrins were detected (Fig. 4.5, lane 1). Only the 52-KD dehydrin appeared in trace amounts in the immature axes (Fig. 4.5, lane 2). In the cotyledons of mature seeds, no dehydrins were present in detectable amount (Fig. 4.5, lane 3).

4.4. Discussion

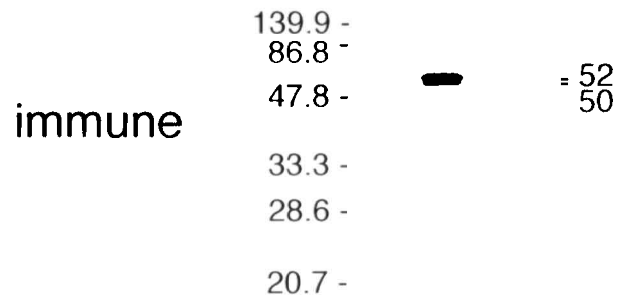
There are several hypotheses to explain how certain plant cells are able to cope with water stress or full desiccation (Bray, 1993). Although an essential component of desiccation tolerance may well involve the ability to effect repair upon subsequent rehydration, it is likely that desiccation tolerant tissues accumulate protective substances that limit the amount of damage that otherwise would be induced by water loss. Sugars (disaccharides such as sucrose and oligosaccharides such as raffinose and stachyose) may play a key protective role accumulating under water deficit conditions and functioning to replace water and thus stabilize membranes and other sensitive systems (Crowe *et al.*, 1992). Another protective mechanism may involve the group of proteins induced in seeds and plant vegetative tissues undergoing mild

Figure 4.5. Western blot analysis of dehydrins in *A. heterophyllus* seeds at mid-maturation and maturity.

Lane 1: mature axes; Lane 2: immature axes; Lane 3: mature cotyledons. Dehydrins were detected by a dehydrin antiserum and blots using the pre-immune serum served as controls. Heat-stable protein (64 μg in 32 μl sample buffer) was loaded onto each lane. Numbers on the right indicate the approximate molecular weights (KD) of the dehydrins; only those proteins not found on control Western blot are indicated.

Artocarpus heterophyllus

- mature and immature seeds



1 2 3
M I M
A A C

pre-immune

1 2 3
M M M
A A C

water loss or full desiccation, namely dehydrins.

Desiccation tolerance is acquired during development of orthodox seeds; tolerance to full desiccation is generally lost after germination. Recalcitrant seeds, unlike orthodox seeds, are sensitive to desiccation when shed from the parent plant, and thus, provide a system to study temporal and stress-induced changes in dehydrin synthesis.

In this study, dehydrin-related polypeptides were identified in recalcitrant seeds of two tropical species, including *Castanospermum australe, Artocarpus heterophyllus* (jackfruit). Notably, dehydrins were absent from the cotyledons of *A. heterophyllus*, although the axes of the seed contained two prominent dehydrin-related polypeptides (Mr ~ 50 KD). *C. australe* exhibited dehydrins in both the axis and cotyledons of the seed.

There were striking changes in the nature of the dehydrins synthesized during development of *C. australe* seeds. A distinct set of dehydrin-related polypeptides were present in the axes and cotyledons of immature seeds at mid-maturation; new dehydrins were synthesized when seeds reached maturity. Immature *C. australe* seeds subjected to ABA or various water-deficit-related treatments synthesized some of the dehydrins associated with late development, but some of these were also synthesized in detached hydrated seeds, at least in the cotyledons. The general characteristics of dehydrin production in the recalcitrant *C. australe* seeds during development (i.e., new polypeptides induced during late development and following mild stress or detachment of immature seeds) are very similar to those exhibited by seeds of an orthodox species that we have been studying, namely castor bean (*Ricinus communis* L.) (Han et al., 1995, 1996, Han and Kermode, 1996).

In relation to dehydrin production, the major differences between seeds of *C. australe* and castor bean appear to be exhibited following the transition to

germination and seedling growth. Similar to the castor bean endosperm, the dehydrin-related polypeptides persist in the cotyledons of *C. australe* seeds for at least 5 days following the start of imbibition. However, following ABA treatments or the imposition of all water-deficit-related stresses (except cold) these dehydrins became less abundant in the cotyledons of *C. australe* seedlings and no new polypeptides were detected. In contrast, castor bean seedlings exhibit a strong induction of dehydrin synthesis in the endosperm, cotyledons and axis as a consequence of ABA or stress imposition (Chapter 3; Han and Kermode, 1996). Furthermore, in the endosperm of the castor bean seedlings the stress-inducible dehydrins are distinct from those that are induced during late seed development and which persist following germination/growth of mature seeds.

Perhaps the most significant finding is the lack of dehydrins in the axes of *C. australe* following seed germination and the inability of high concentrations of ABA or stress imposition to induce their production. It seems that the axes are very sensitive even to mild water-deficit-related stresses and are not capable of dehydrin synthesis, despite having this capacity during seed development.

A decline in the ability of recalcitrant seeds to produce dehydrins following the transition to seedling growth is likely more important to the viability of shed seed than a previous capacity for synthesis during seed development. It may be significant that the changes that occur on dehydration of recalcitrant seeds of the mangrove, *Avicennia marina,* are very similar to changes brought about by desiccation of orthodox seeds during the intolerant stage following germination. In fact, recalcitrant seeds may be desiccation intolerant because germination is initiated upon abscission, and attempting to store these seeds is akin to air-dry storage of germinated, orthodox seeds

(Farrant et al., 1986). There is no clear-cut event delineating the end of seed development and the start of germination; thus it is difficult to separate these two phases. At all stages recalcitrant seeds appear to remain metabolically active, although the axes may undergo a very brief period when they are quiescent. If indeed the shed recalcitrant seed responds in a manner similar to germinated orthodox seeds, it is also important to distinguish between dehydrin production in storage tissues (cotyledons or endosperm) and that occurring in the axis. For example, the axes of orthodox seeds such as soybean rapidly lose their tolerance to a full desiccation event (brought about by air-drying or drying over silica gel) during the course of germination, while the cotyledons remain tolerant for a considerably longer period (Senaratna and McKersie, 1983).

Our results differ somewhat from those reported by Finch-Savage *et al.* (1994) who showed an induction of *Lea* mRNA in axes of stored recalcitrant seeds of *Quercus robur* L. (English oak) following treatment with ABA or limited desiccation. However, at the protein level, there was no true induction nor even enhanced synthesis of dehydrin-related polypeptides in response to ABA or limited desiccation.

Our results support the contention that desiccation-sensitivity of recalcitrant seeds may be due in part to an inability to accumulate sufficient dehydrins and/or to the absence of specific LEA or dehydrin proteins following the transition to a germination/growth program -- especially under stress conditions.

Further studies are needed to determine whether there is a direct relationship between a loss of viability during storage and a decline in the ability of the axes of recalcitrant species to synthesize/maintain dehydrins. It is likely that the underlying basis of desiccation tolerance is diverse and is not

simply restricted to the synthesis of specific proteins.

Conclusions

The accumulation pattern of dehydrins during seed development and following germination of an orthodox species (*Ricinus communis* L.) was studied. In addition, the effects of ABA and water-deficit-related stresses imposed during seed development and following germination were examined. To compare the responses of orthodox vs. recalcitrant species, I further analyzed inducibility of dehydrin synthesis in a recalcitrant species (*Castanospermum australe*). General conclusions that can be drawn from the above studies are summarized as follows:

(1). The nature of the dehydrins accumulated in seeds changed during the late stages of seed development in *R. communis* L. These dehydrins did not undergo degradation following imbibition up to 96 h.

(2). A premature drying treatment induced maturation-related dehydrin synthesis in *R. communis* L. seeds when imposed during mid-maturation. These dehydrins declined much faster following rehydration.

(3). Exogenous ABA and various water-deficit-related treatments induced maturation-related dehydrins in immature *R. communis* seeds. However, the dehydrin production may simply be a result of detachment from the parent plant.

(4). Dehydrins accumulated in all three organs of *R. communis* L. seedlings when the seedlings were treated with ABA and various water-deficit-related stresses. The production of dehydrins was organ-specific and appeared

to be differentially regulated by different stress treatments.

(5). A high concentration of the plant hormone ABA (10^{-3} M) did not suppress dehydrin production in *R. communis* L. seedlings. An antagonistic plant hormone (GA_3) suppressed dehydrins induced by ABA and stress when applied simultaneously to seedlings.

(6). The recalcitrant plant (*C. australe*) accumulated dehydrins in seeds during development. Like the orthodox species, the nature of the dehydrins accumulated changed as development progressed. The dehydrins present in mature seeds persisted in the cotyledons after 5 days of germination but were not detectable in axes.

(7). ABA and mild water-deficit-related stresses imposed on immature seeds of *C. australe* induced some maturation-related dehydrins. But a more extreme desiccation treatment resulted in no detectable dehydrins perhaps due to seed death.

(8). ABA and mild water-deficit-related stresses did not induce new dehydrin synthesis in cotyledons of *C. australe* seedlings; in the axes dehydrins were absent.

Desiccation-sensitivity in recalcitrant seeds is thought to be caused in part because seeds start germination on the parent plant during the late stages of seed development. Even though dehydrins are found in many species of recalcitrant seeds during development, the dehydrins may decline to very low amounts (especially in the axis) before seeds are shed from the parent plant. I

have found that dehydrins previously present in axes of mature C. *australe* seeds are not detectable after 5 days of germination.

The results from my studies showing an absence of dehydrins in the axes of recalcitrant species when seedlings are subjected to stress provides some tantalizing evidence that dehydrins may function as protectants in plant cells during water-deficit-related stresses.

Further studies are needed to determine whether there is a direct relationship between a loss of viability during storage and a decline in the ability of the axes of recalcitrant species to synthesize/maintain dehydrins.

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